UNIVERSITAT DE BARCELONA

UNIVERSITÉ LOUIS PASTEUR STRASBOURG

THESE en-cotutelle entre:

L'UNIVERSITE LOUIS PASTEUR DE STRASBOURG

et

L'UNIVERSITAT DE BARCELONA

présentée pour l'obtention du grade de

DOCTEUR DE L'UNIVERSITE LOUIS PASTEUR DE STRASBOURG

Discipline Chimie

par

Stéphanie BOUSSERT

Structural studies of proteins and protein complexes by mass spectrometry and atomic force microscopy

Soutenue le 25 juin 2008 devant la commission d'examen:

Dr. Alain VAN DORSSELAER	Co-directeur de thèse
Dr. Ernest GIRALT	Co-directeur de these
Prof. Laurence SABATIER	Rapporteur interne
Prof. Christine ENJALBAL	Rapporteur externe
Prof. Fausto SANZ CARASCO	Rapporteur externe
Prof. Anna GRANDAS SEGARRA	Examinateur
Dr. François BESSUEILLE	Examinateur
Dr. Félix ELORTZA	Examinateur

Acknowledgements

I would like to thank my three supervisors. First of all, Eliandre de Oliveira, because she believed in me. Eliandre was a model of patience, kindness, availability and a great source of ideas and advices. She will definitely remain for me an example to follow. Alain Van Dorsselaer because he introduced me to the world of mass spectrometry. His long distance help was really appreciated. Ernest Giralt because he gave me the opportunity to follow many research lines. Ernest transmitted to me the virus of scientific curiosity and enthusiasm. Eliandre, Ernest and Alain are the main catalysts of the work presented here.

I thank the members of my tribunal for the time they spent for the evaluation of my thesis: Pr. Anna Grandas, Pr. Christine Enjalbal, Pr. Laurence Sabatier, Pr. Fausto Sanz, Dr. Francois Bessueille and Dr. Felix Elortza.

I woud like to warmly thank the iniators of my project in Barcelona: Eliandre de Oliveira, Marcelo Kogan and Fernando Albericio, who make this happens. I also thank Lluis Ribas de Pouplana for his help to open me the right doors.

I would like to thank the people I have collaborated with during these years: Anass Jawhari and Arnaud Potzerman, Nathalie Zorn and Emmanuel Bisse, Ismael Diez and Federico Rua, and Nacho Pons.

I would like to thank all the people who have contributed directly to the achievement of my thesis: Antonia (known also as Janet), David and Alex for the proteomic team of Barcelona. I had great time with you: our casual fridays, our " no tocar David" (I have greatly improved my skills to drawn girly-skulls on your side), our week-ends in the mountains (specially the "carros de foc" , "qu'estem fent David? quina m...de pedres!!"). Many thanks also to my little summer slave Maria, her help during my last summer of investigation was greatly appreciated. And of course, Alberto for his great help on solid phase synthesis, for teaching me catalan, and for so much oher things.

Thanks to my other MS-friends: Helene, Karine, Sophie and Nathalie for pour happy moments and also for your kind support in difficult times, I have not exported the "bolino-vache-qui-rit" in Barcelona, you were the only lucky beneficiaries, I hope you have enjoyed it. Thank's

also to Daniele, Noelle, Emmanuelle, Sarah, Virginie, Chriselle, Jean-Marc, Fabrice, they all have been a great help during my stay in Strasbourg.

Special thanks to Eva and Veronique for their ability to solve any administrative problems, and personal ones too.

Life in the labs would not have been the same without all the friends I met during these years. They made my work a lot easier, I thank them all for the support and happiness they provided me. Many thanks to: Laia and our tea time at 5pm, Nessim for trying to teach me castilian..sorry it didnt work, Eli for her catalan skills...among others, Yesi for trying to eat faster, Nano-Marta for understanding that I will not go out all night (yes I need some rest at my age), Marta qui "molt poc cops guanya la cursa del migdia", Esther for finding me anything anywhere in the lab (and most of the time at the place it should be), Silvia for our cultural asiatic movies, Tere for her help and advices in biology, Muriel well known as Soclamu for her help in the nanoworld, Sergi for his help in LateX, Natxo for the climbing times, Txell for her help and advices in chemistry, Allbert Susana Fayna and Javi for their help in the administrative jungle of the university, Irene for speaking with me french-swiss, Morteza for being so kind and calm, Giovanna for the bollywood parties, Martina for our cultural asiatic movies, Pau for his orxata de Valencia, Birgit for the establishment of the beautiful-fridays, Roger for his very good taste regarding my hair-style, Carles for speaking french with me, Miriam for also trying to eat faster, Eduard for his help in catalan (level K, no comment), Rosa for sharing taste of french movies, and so many other: Lorena, Leticia, Natalia, Gemma, Judith, Dolors, Laura, Ramon, Xavi, Oscar, Jan, Tomasso, Gerardo, Josep, I am sure I have forgotten names...

Thank's to the AFM team: Carolina, Jordi, Gerard, Aleix, Felipe, Juan-ma and Pau for all their advices. Thank's to Ismael and Federico for sharing yerba-mate, time was shorter and funnier when imaging with you.

I thank my dear friends out of the lab, so far and so close at the same time, Pascale, Geraldine, Nathalie, Helene, Christel, Celine, Sophie, Anne-Lise, Greg.

And last but not least, my family for its support, and Renaud. This thesis would have not been possible without them.

Glossary

AA, amino-acid;

AAA, amino-acid analysis;

Aβ, beta-amyloid peptide;

Aβ40, beta-amyloid 1-40;

AFM, atomic force microscopy;

Boc-AA, N-alpha-tert-butyloxycarbonylamino acid;

DCM, dichloromethane;

DIEA, diisopropylethyl amine

DMAP, 4-(N,N-dimethylamino) pyridine;

DMF, dimethylformamide;

DIPCDI, 1,3-diisopropylcarbodiimide;

EDT, ethane-1,2-dithiol;

EG, ethylene glycol;

ESI-MS, electrospray ionization mas spectrometry;

Fmoc-AA, N-fluorenylmethyloxycarbonylamino acid;

HOBT, N-hydroxybenzotriazole;

HPLC, high-pressure liquid chromatography;

IgG, immunoglobulin G;

LC, liquid chromatography;

Lys, lysine-amino-acid;

MeCN, acetonitrile;

MALDI-MS, matrix-assisted laser desorption ionization mass spectrometry;

MS, mass spectrometry;

MS-MS, tandem mass spectrometry;

μCP, micro-contact printing;

PBS, phosphate buffered saline;

PDMS, poly-dimethyl-siloxane;

PEG, poly-ethylene-glyol;

TBTU, 1-[bis(dimethylamino)methylene]-1H-benzotriazolium hexafluorophosphate 3 oxide;

TOF, time-of-flight;

RP, reverse phase high-pressure liquid chromatography;

SAM, self-assembled monolayer;

SAMDI-MS, self-assembled for matrix assisted laser desorption/ionization mass spectrometry;

TFA, triflouroacetic acid;

TIS, triisopropylsilane;

Contents

General Introduction

Chapter I

Mass Spectrometry

1 A brief Introduction to Mass Spectrometry

The basic principle of mass spectrometry (MS) consists on generating ions from either inorganic or organic compounds by any suitable method. The ions are then separated based on their mass-to-charge ratio (m/z) and are finally detected by their respective m/z and abundance. Mass spectrometer is composed of three parts: an ionization source, a mass filter/analyzer and an ion detector (see Fig.I.1).

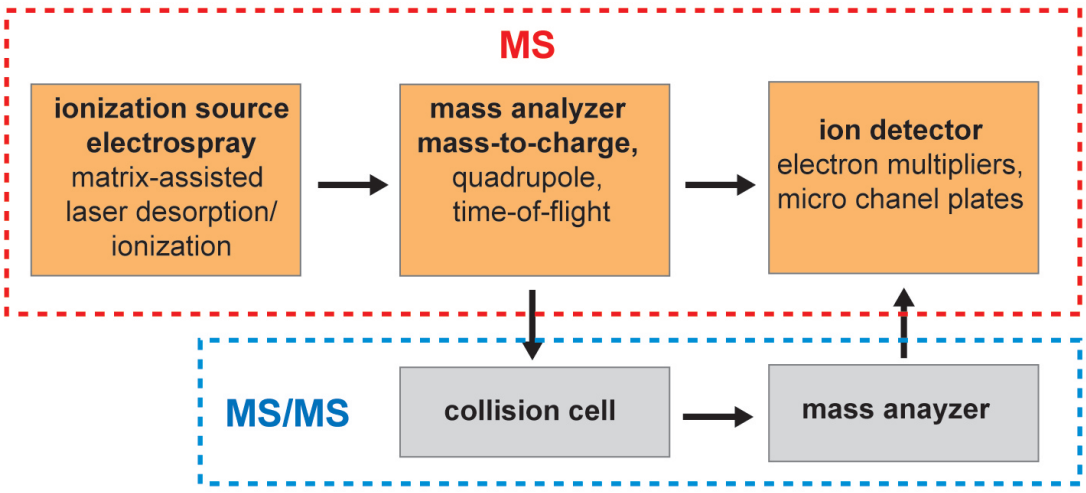


Figure I.1: Schematic representation of a mass spectrometer

MS started in 1897 with the physicist John Thomson and his discovery of the electron and the measure of its m/z ratio. Thomson built the first mass spectrometer (called "parabola spectrograph") at the beginning of the XXe century and finally received the Nobel Prize in Physics in 1906.

In the 60's, one of the first analysis of peptide was done by EI (Electron Impact) ionization [1]. But it was only at the beginning of the 80's that MS studies of biological compounds really start

[1] M Barber, P Jolles, E Vilkas, and E Lederer. *Biochem Biophys Res Commun*, 18:469–473, 1965.

with the discovery of new ionization sources: PDMS (Plasma Desorption Mass Spectrometry) [2], FAB (Fast Atom Bombardment), and SIMS (Secondary Ion Mass Spectrometry) and LSIMS (Liquid Secondary Ion Mass Spectrometry) [3] [4].

In the latter part of the 20th century two new ionization methods were described for biological macromolecules analysis: the ESI (ElectroSpray Ionization) [5] [6] and the MALDI (Matrix-Assisted Laser Desorption/Ionization) [7] [8].

In both methods, peptides are converted to ions by the addition or loss of one or more protons. ESI and MALDI are soft ionization methods that allow the formation of ions without significant loss of sample integrity. This is important because it enables achievement of accurate mass information of proteins in their native states. These two techniques have opened up the world of biological chemistry to mass spectrometry.

1.1 Ionizations methods:

1.1.1 ESI source

The ESI source consists of a very fine needle and a series of skimmers that generates ions directly from solution (usually an aqueous or aqueous/organic solvent system) by creating a fine spray of highly charged droplets in the presence of an electric field. The solution has to provide some electrical conductivity that may be obtained by use of ionic analytes, ionic additives such as buffers or by some degrees of electrolytic dissociation of the solvent. The spray is affected by the charge separation at the liquid surface, making a deformation of the emerging drop (Taylor cone), which finally disintegrates to yield thousands of micrometers, sized droplets. As the droplets carry an excess of ions of one charge state they drift towards the counter electrode. On their way, the droplets are rapidly shrinking by evaporation of solvent, increasing charge density on their surface. What happens exactly afterwards is not clear, but one of the hypotheses is that the droplets may repetitively undergo coulomb explosions yielding smaller and smaller droplets, that finally contain only one charged molecule and perhaps still a few solvent molecules (charged residue model [9]). Anyway, the process ends up with (often highly) charged molecules sometimes still carrying solvent molecules. Emerging ions are directed trough electrostatic lenses to the mass analyzer. (See Fig.I.2 and Fig.I.3).

Because the mass spectrometer measures the mass-to-charge ratio (m/z), multiple charg-

[2] D F Torgerson, R P Skowronski, and R D Macfarlane. *Biochem Biophys Res Commun*, 60(2):616–621, 1974.
[3] A Benninghoven and et al. *Appli Phys*, 11(35–39), 1976.
[4] A Dell and H R Morris. *Biochem Biophys Res Commun*, 106(4):1456–1462, 1982.
[5] Fenn JB. *American Society of mass Spectrometry Conference, San Diego*, 1988.
[6] J B Fenn, M Mann, C K Meng, S F Wong, and C M Whitehouse. *Science*, 246(4926):64–71, 1989.
[7] F Hillenkamp. *International Mass Spectrometry Conference, Bordeaux (France)*, 1988.
[8] M Karas and F Hillenkamp. *Anal Chem*, 60(20):2299–2301, 1988.
[9] Dole M and et al. *J Chem Phys*, 49:2240, 1968.

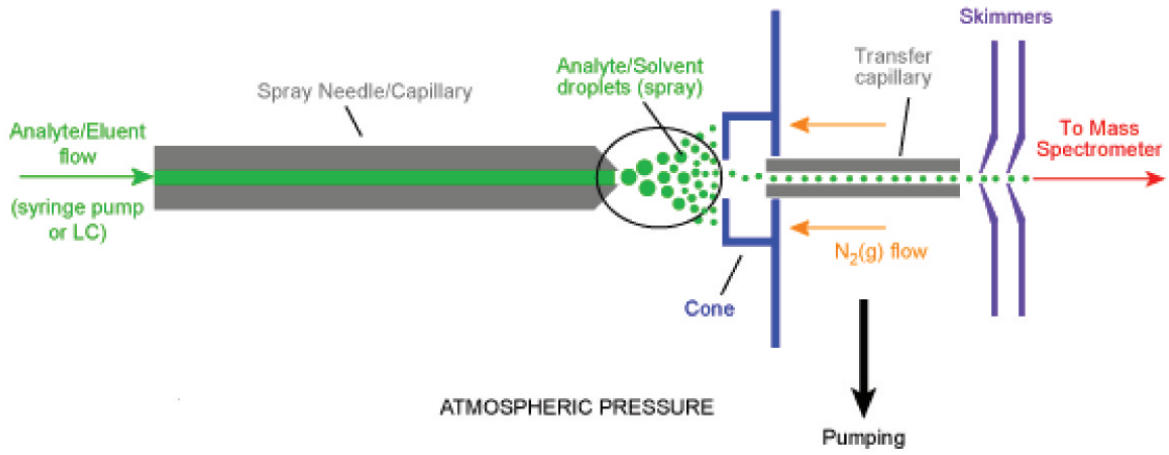


Figure I.2: Schematic representation of the electrospray source interface (Fig. from: http://www.bris.ac.uk/nerclsmsf/techniques/hplcms.html).

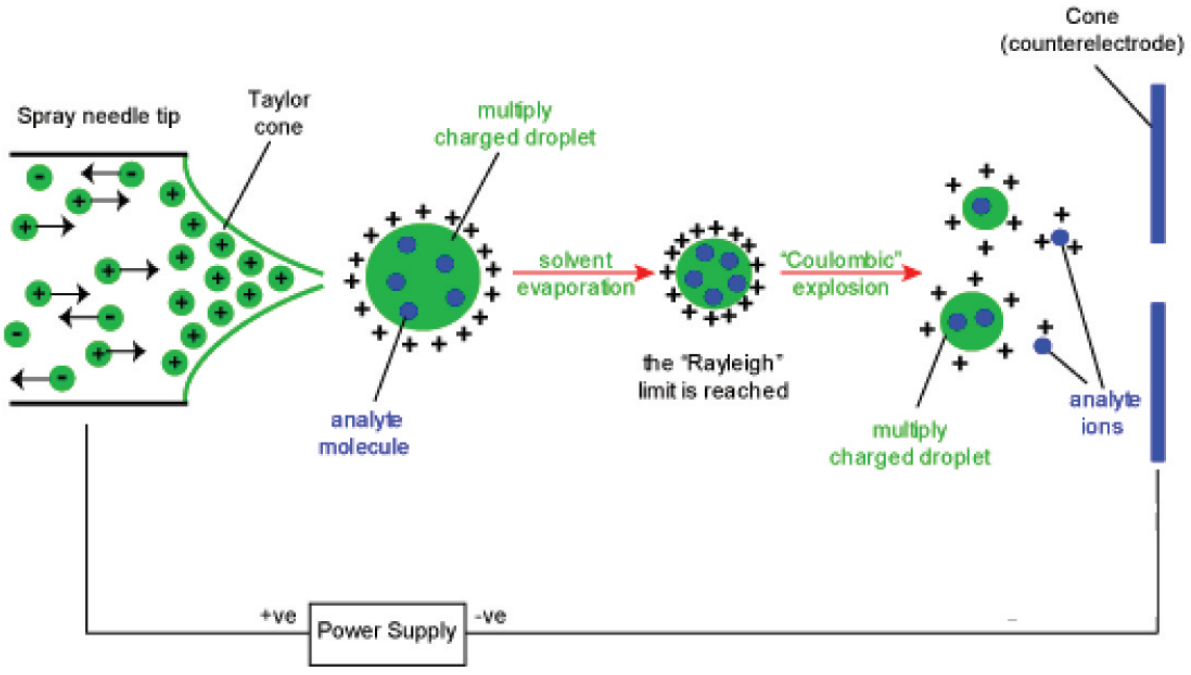


Figure I.3: A schematic representation of the mechanism of ion formation (Fig. from: http://www.bris.ac.uk/nerclsmsf/techniques/hplcms.html).

ing state makes possible to detect very large molecules with an instrument having a relatively small mass range.

The continuous introduction of solution enables coupling of chromatography liquid (LC) to the mass spectrometer (LC-ESI-MS) and separation of a mixture of compounds. Low flow electrospray, originally described by Wilm and Mann [10], has been called nano-electrospray and micro-electrospray. In nanospray ionization, the microcapillary tube has a spraying orifice of 1 to 2 um and flow rates as low as 5 to 10 nL/min. The end result of this rather simple adjustment is increased efficiency, which includes a reduction in the amount of sample needed, increasement of sensitivity and a reduction in time analysis.

1.1.2 MALDI source

In MALDI technique, the sample is co-crystallized with a large excess of small UV-absorbing organic molecules (matrix) on a metal plate (MALDI plate) (see Fig.I.5). Both, analyte and matrix are usually dissolved in a solution of acetonitrile (or other volatile solvent) and water-acid. The analyte is spotted along with the matrix and allowed to evaporate, resulting in the formation of crystals. The sample plate is inserted into a vacuum lock and transferred into a vacuum chamber (ion source) where the MALDI takes place. A pulsed laser beam is fired, resulting in the desorption of matrix and co-crystallized analyte molecules into the gas phase. The matrix serves three functions: the isolation of the biomolecules from each other, the absorption of the energy from the laser light to transfer it into excitation energy, and finally the ionization of the biomolecules (see FigI.4).

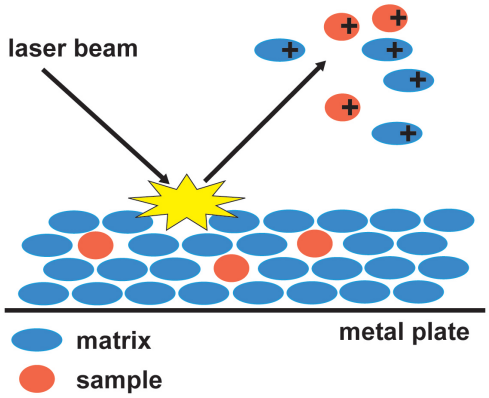


Figure I.4: Schematic representation of MALDI process

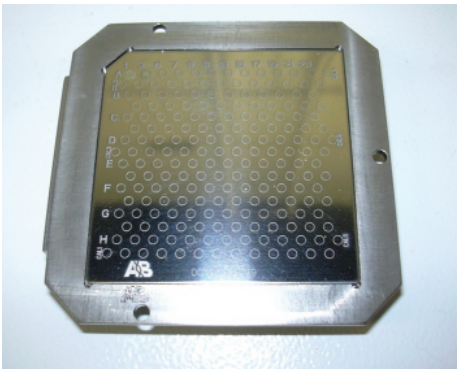


Figure I.5: Example of a 192 sample wells MALDI-plate

The exact desorption/ionization mechanism is not known, and several theories have been developed to explain MALDI ion formation. Gluckmann and Karas have discussed the relevance

[10] M Wilm and M Mann. *Anal Chem*, 68(1):1–8, 1996.

of cluster ejection and decay with respect to the ionization of analyte molecules in MALDI in a phenomenological lucky survivor model [11], while Zenobi and co-workers state that ion formation can be divided up into formation of primary ions during the laser pulse, followed by secondary ion-molecule reactions in the expanding plume that lead to the observed ions on the detector (two-step model Zenobi [12]). Once in the gas phase, the desorbed charged molecules are then directed electrostatically from the MALDI ionization source into the mass analyzer. MALDI is also a soft ionization method and produces mainly singly-charge ions.

1.2 Analyzers:

Once the ions have been produced, they need to be separated according to masses, which must be determined. The three main characteristics of an analyzer are the mass limit, the transmission and the resolution. One of the most common mass analyzers is the quadripole analyzer (Q), where ions are separated by an electric field created by an array of four parallel metal rods[13] [14].

The time-of-flight (TOF) mass analyzer measures the m/z ratio of an ion by determining the time required to traverse the length of a flight tube. TOF mass analyzers can work in linear mode or reflectron mode [15]. The reflectron mode consists on increasing the length of the tube via an ion mirror (electrostatic reflector), which contributes to correct small energy differences among ions and increases mass resolution[13].

The ion trap (IT) mass analyzers function is to trap molecular ions in a 3-D electric field. The main advantage of an IT mass analyzer is its ability to store ions and selectively eject them from the IT, increasing sensitivity [16]. In a Fourier transform (FT) analyzer, or Fourier-transform ion-cyclotron resonance (FTI-CR) mass analyzer, ions move in circular orbits in a magnetic field. The orbits vary by subjecting the ions to a resonant alternative electric field where ions oscillate around the magnetic field at frequencies that are related to their m/z ratio. As the ions oscillate near the top and bottom metal plate of the cubic trapping cell, they induce an alternating current that can be measured and related to their m/z ratio.

Different types of mass analyzers can be coupled together in tandem to take advantage of the strength of each. In that case they are called hybrid instruments, and they were developed to perform tandem mass spectrometry (MS/MS). Within the variety of hybrid instruments that were developed in the 1980s and the 1990s, two of them, the quadrupole/time-of-flight

[11] M Karas, M Gluckmann, and J Schafer. *J Mass Spectrom*, 35(1):1–12, 2000.
[12] R Zenobi and R Knochenmuss. *Mass Spec Rev*, 17(337-366), 1998.
[13] J R 3rd Yates. *J Mass Spectrom*, 33(1):1–19, 1998.
[14] A L Burlingame, R K Boyd, and S J Gaskell. *Anal Chem*, 70(16):647R–716R, 1998.
[15] F Sobott, H Hernandez, M G McCammon, M A Tito, and C V Robinson. *Anal Chem*, 74(6):1402–1407, 2002.
[16] R G Cooks, S A glish, S A McLuckey, and R E Kaiser. *Chem Eng Newsl*, 25:26–41, 1991.

(Q/TOF)[17] and the tandem time-of-flight (TOF/TOF)[18], have evolved into a mainstay of modern MS/MS instrument.

MS/MS enables structural information of the molecule analyzed in two stages of MS. In the first stage, ions of a desired m/z ratio are isolated from a mixture of ions. These selected ions (called parent ions or precursor ions) are then passed into a collision chamber. Inside the collision chamber, molecules are fragmented by interaction with an inert gas (usually nitrogen or argon) by a process called collision-induced dissociation (CID) [19]. The resulting ions, termed product or fragment ions, are analyzed in the second stage of the MS/MS. In peptides, fragmentations usually take place along the peptide backbone, but can also occur along amino acid side chain [20] [21] [13]. The resulting fragment ions can be used to identify the amino acid sequence of the peptide being analyzed. Moreover MS/MS enables characterization of protein modifications such as phosphorylation, methylation, nitration [14]. This technique also allows determination of modified sites in proteins.

1.3 Detectors:

Once the ion beam passes through the mass analyzer, it is detected and transformed into usable signal by a detector. One of the most used detector employed nowadays is the electron multipliers detector. Some instruments, such as the MALDI-TOF and the Q-TOF, use a modern electron multiplier detector called micro channel plates (MCPs) detector. MCPs can multiply the number of electrons by 10^5 via a cascade of electrons that finally results in a measurable current at the end of the electron multiplier.

1.4 MS and proteomics

With the development of ESI and MALDI techniques, MS has become an emerging method for the characterization of biomolecules and proteomic studies [22]. The term proteome was first coined to describe the set of proteins encoded by the genome. The study of the proteome evokes not only all the proteins in any given cell, but also the set of all proteins isoforms and modifications, the interaction between them, the structural description of proteins and their

[17] G L Glish and D E Goeringer. *Anal Chem*, 56(2291–2295), 1984.

[18] T J Cornish and R J Cotter. *Anal Chem*, 65(8):1043–1047, 1993.

[19] J M Wells and S A McLuckey. *Methods Enzymol*, 402:148–185, 2005.

[20] D F Hunt, A M Buko, J M Ballard, J Shabanowitz, and A B Giordani. *Biomed Mass Spectrom*, 8(9):397–408, 1981.

[21] D F Hunt, J R 3rd Yates, J Shabanowitz, S Winston, and C R Hauer. *Proc Natl Acad Sci U S A*, 83(17):6233–6237, 1986.

[13] J R 3rd Yates. *J Mass Spectrom*, 33(1):1–19, 1998.

[14] A L Burlingame, R K Boyd, and S J Gaskell. *Anal Chem*, 70(16):647R–716R, 1998.

[22] M Mann, R C Hendrickson, and A Pandey. *Annu Rev Biochem*, 70:437–473, 2001.

higher-order complexes, and for that matter almost everything 'post genomic' [23] [24]. Proteomics means protein biochemistry on an unprecedented, high-throughput scale.

MS-based proteomics is typically carried out by enzymatic digestion of proteins into smaller peptides using proteases such as trypsin, chymotrypsin or pepsin, either in-solution or in-gel [25] after electrophoretic separation. The collection of peptide products is then introduced into the mass spectrometer. When the characteristic pattern of peptides is used for the identification of the protein the method is called peptide mass fingerprint (PMF) [26] [27]. When the protein identification is performed using the sequence data determined in tandem MS analysis, it is called MS/MS protein identification [28] (see Fig. I.6).

These procedures of protein analysis are also referred to as the "bottom-up" approach and they use protein and/or genomic databases to compare experimental data against theoretical data set (databases). When the protein being analyzed is not present in the database, MS-MS data is analyzed to reach an amino acid peptide sequence in order to identify the protein by homology with other species. This kind of analysis is named "de novo" sequencing [29].

More recently, the strategy of "top-down" analysis has been developed [30] [31]. Intact proteins are ionized by either one of the two techniques described above, and then introduced to a mass analyzer and subjected to gas-phase fragmentation. In recent years, highly efficient fragmentation methods have been developed such as electron capture dissociation (ECD)[32] and electron transfer dissociation (ETD) [33]. The mass range of top-down has been extended to proteins as large as 229 kDa [34], and increasingly large numbers of intact proteins can be detected in a single analysis.

A melting of the two strategies is already in progress with the emergence of "middle-down" proteomics [35]. In this approach, large proteins are subjected to limited proteolysis by enzymes such as LysC to yield products in the 5-20 kDa range. These more manageable polypeptides are then sequenced using top-down methodologies.

---

[23] J S Andersen and M Mann. *FEBS Lett*, 480(1):25–31, 2000.

[24] A Pandey and M Mann. *Nature*, 405(6788):837–846, 2000.

[25] M Wilm, A Shevchenko, T Houthaeve, S Breit, L Schweigerer, T Fotsis, and M Mann. *Nature*, 379(6564):466–469, 1996.

[26] P James, M Quadroni, E Carafoli, and G Gonnet. *Biochem Biophys Res Commun*, 195(1):58–64, 1993.

[27] O N Jensen, A V Podtelejnikov, and M Mann. *Anal Chem*, 69(23):4741–4750, 1997.

[28] J R 3rd Yates, J K Eng, A L McCormack, and D Schieltz. *Anal Chem*, 67(8):1426–1436, 1995.

[29] R S Johnson and J A Taylor. *Methods Mol Biol*, 146:41–61, 2000.

[30] B Bogdanov and R D Smith. *Mass Spectrom Rev*, 24(2):168–200, 2005.

[31] N L Kelleher. *Anal Chem*, 76(11):197A–203A, 2004.

[32] R W Giese. *J Chromatogr A*, 892(1-2):329–346, 2000.

[33] S A McLuckey and J L Jr Stephenson. *Mass Spectrom Rev*, 17(6):369–407, 1998.

[34] X Han, M Jin, K Breuker, and F W McLafferty. *Science*, 314(5796):109–112, 2006.

[35] P M Thomas, C D Wenger, B A Parks, D E Robinson, R T Fellers, Y Kim, R D Zamborg, R D LeDuc, A J Forbes, and Kelleher N L. *54th ASMS Conf Mass Spectrometry and Alllied Topic, Seattle, WA*, 2006.

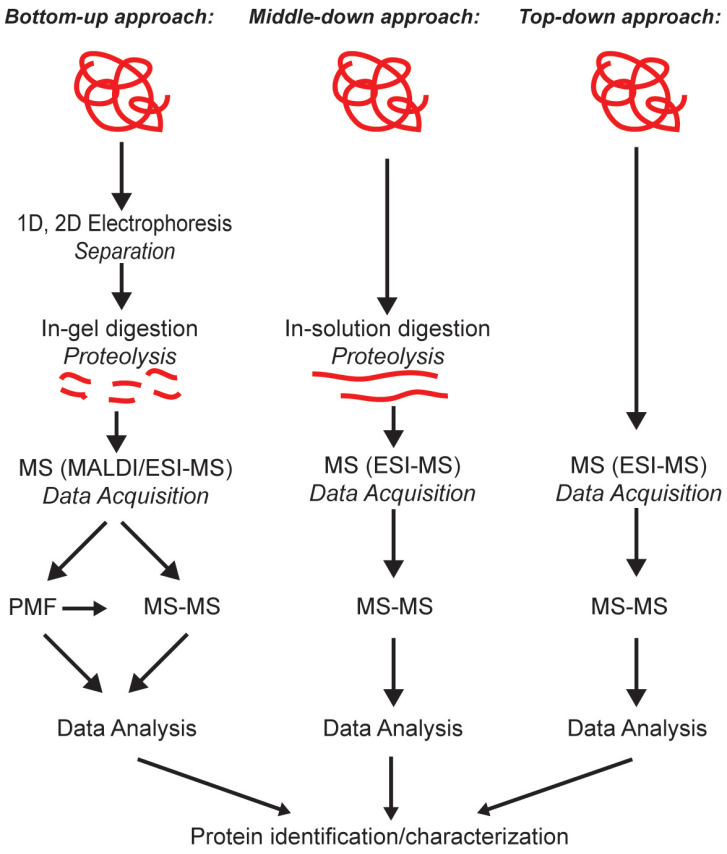


Figure I.6: MS based methods for proteomic studies

2 Study of protein interactions by MS

Establishment of the role and behavior of new identified proteins is a further goal and challenge of proteomics. The biological function of proteins invariably depends on their direct physical interaction with other molecules. All proteins in a given cell are connected through an extensive network, where non-covalent interactions are continuously forming and dissociating. The forces responsible for such interactions include electrostatic forces, hydrogen bonds, Van der Waals forces and hydrophobic effects.

Until recently, strategies allowing the understanding and identification of complex interaction networks have been lacking. However, data related to protein complex formation are now steadily growing in databases thanks to the development of technologies to study protein interactions (X-ray crystallography [36], nuclear magnetic resonance spectroscopy (NMR), electron crystallography [37], electron tomography [38], immuno-electron microscopy [39], fluorescence

[36] N Ban, P Nissen, J Hansen, P B Moore, and T A Steitz. *Science*, 289(5481):905–920, 2000.

[37] N Volkmann, K J Amann, S Stoilova-McPhie, C Egile, D C Winter, L Hazelwood, J E Heuser, R Li, T D Pollard, and D Hanein. *Science*, 293(5539):2456–2459, 2001.

[38] W Baumeister. *Curr Opin Struct Biol*, 12(5):679–684, 2002.

[39] M P Rout, J D Aitchison, A Suprapto, K Hjertaas, Y Zhao, and B T Chait. *J Cell Biol*, 148(4):635–651, 2000.

energy transfer (FRET)[40], site directed mutagenesis [41], yeast two-hybrid system [42],).

The word ”interactome” defines the entire pool of protein interactions in a given cell or tissue. There are two classes of complexes: one involves ”domain-domain” interactions, in which both components contain prefolded structural units, and the other one involves ”domain-peptide” interactions, in which one component is a short motif that is, usually, unstructured in the absence of its binding partner.

MS technology has emerged as the method of choice to characterize the interactome. This method presents several advantages over the conventional methods. For instance, MS allows the use of small quantities of sample (microgram to nanogram instead of multi-milligram typically required for NMR and crystallography-based methods) and the samples do not need to be labeled or chemically modified. MS analysis is fast and can be automated (from sample preparation to spectra interpretation), and in some cases the purity of the samples is not a strict requirement as the interaction would be differentiated by molecular mass.

When studying protein interactions, MS allows the identification of the partners involved in the protein interaction, and also the identification of interaction interfaces (contact residues).

We present here the main MS methods used to identify partners, and contact residues of domain-peptide and domain-domain complexes (see Fig.I.7).

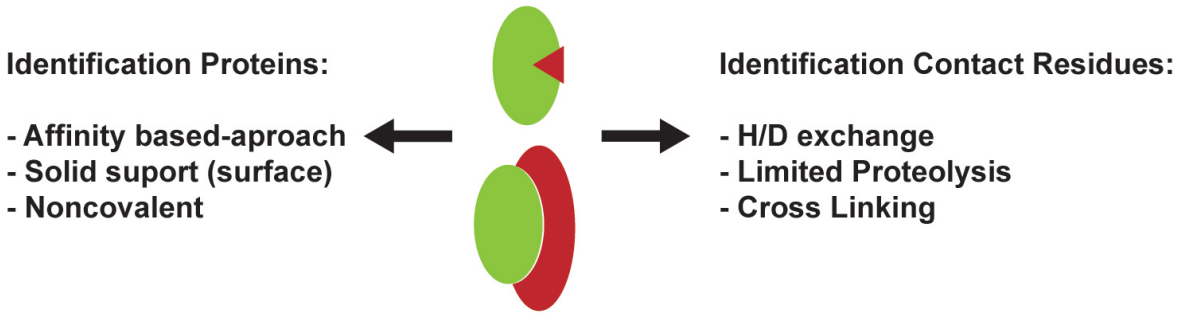


Figure I.7: MS based methods for protein interaction studies

[40] R M Siegel, J K Frederiksen, D A Zacharias, F K Chan, M Johnson, D Lynch, R Y Tsien, and M J Lenardo. *Science*, 288(5475):2354–2357, 2000.

[41] I Miras, F Schaeffer, P Beguin, and P M Alzari. *Biochemistry*, 41(7):2115–2119, 2002.

[42] S Fields and O Song. *Nature*, 340(6230):245–246, 1989.

2.1 Identification of partners by MS

2.1.1 Affinity Capture MS methods

Protein interaction can be studied by a range of affinity capture methods. These include co-immunoprecipitation[43] and affinity chromatography [44]. Following affinity purification, proteins are separated from one another either by gel electrophoresis or LC, afterwards enzymatically digested to be analyzed by MS and/or MS/MS and finally identified using a database.

2.1.2 Affinity Capture On-chip MS methods

Affinity Capture on-chip technology (AC on-chip) consists of a set of ligands or proteins covalently bound onto an array. The surface is incubated with a mixture of proteins liable to contain the target protein. The immobilized ligands/proteins interact with the target proteins and a complex is formed on the surface. After washing steps to remove unspecifically bound proteins, there are several possibilities to analyze the surface. The MS analysis can be directly done on the surface by simply adding matrix reagent, in that case the entire protein will be detected by direct on-chip MS analysis. The protein complex present on the surface can be digested on the surface (on-chip digestion) and then analyzed by MS (off-chip MS analysis). A third possibility is to recover the complex from the surface by elution and analyze it by MS (off-chip MS analysis) (see Fig.I.8).

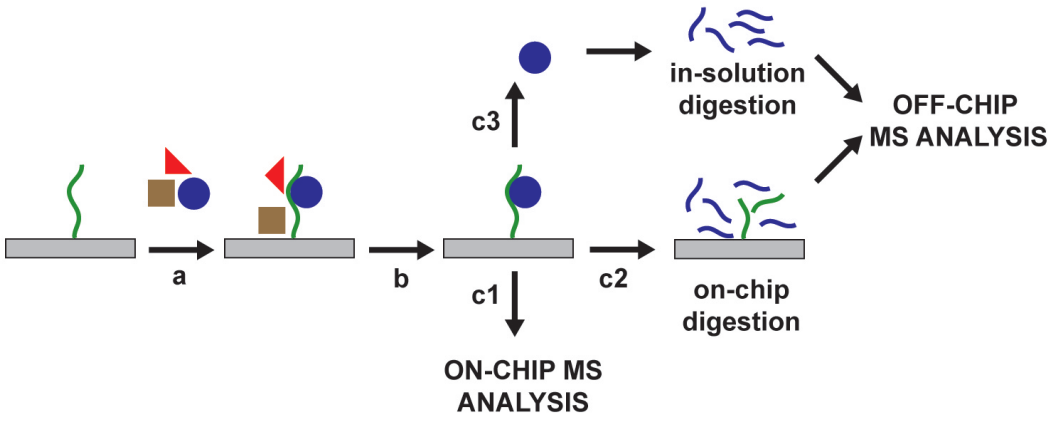


Figure I.8: Schematics of affinity capture on-chip to study protein interactions by MS. (a) incubation with a protein mixture; (b) washing steps; (c1) direct on-chip MS analysis, (c2) on-chip digestion, (c3) elution and in-solution digestion.

Direct on-chip MS analysis and on-chip digestion do not allow re-use of the chip for further

[43] P Licciardo, S Amente, L Ruggiero, M Monti, P Pucci, L Lania, and B Majello. *Nucleic Acids Res*, 31(3):999–1005, 2003.

[44] A Bauer and B Kuster. *Eur J Biochem*, 270(4):570–578, 2003.

fishing experiments, while elution method enables multiple capture/recovery cycles to increase the overall yield for MS analysis [45].

Depending on the application and the experimental conditions, preparation of peptide chips or protein chips requires a suitable choice of support and a suitable method to immobilize peptide or protein onto the surface in a way that preserves their specific activity [46].

2.1.2.a SAMDI MS One of the applications of on-chip MS based method is the strategy that combines MS and self-assembled-monolayers (SAMs), named SAMDI-TOF MS. SAMs are defined as ordered molecular assemblies formed by the adsorption of an active surfactant on a solid surface. Alkanethiols are typically used to functionalize gold surface. Alkanethiols are formed by a thiol head-groups that interact with a gold surface and by an alkyl chain that helps to compact the monolayer. For biological interaction studies, alkanethiols can be modified with oligoethylene or polyethylene glycol (PEG) groups, which are widely used to generate inert surfaces, since they confer surface resistance to non-specific adsorption of biomolecules or cells [47] [48].

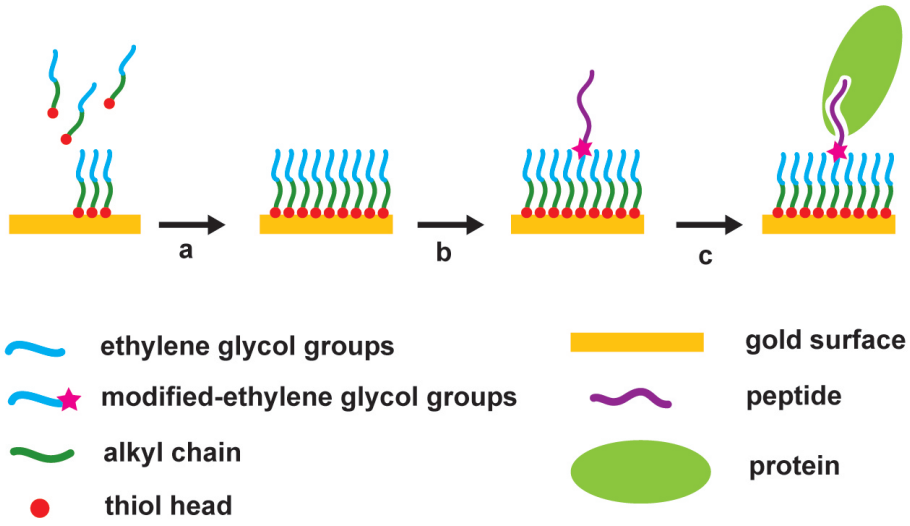


Figure I.9: Self-assembled monolayer formation for SAMDI analysis in protein interaction studies. (a) formation of the SAM with ethylene-glycol-groups-terminated alkanethiols and modified ethylene-glycol-groups terminated alkanethiols; (b) peptide immobilization; (c) the surface is ready for protein interaction studies

By some chemical modification of PEG-alkanethiols prior to SAM, it is possible to achieve specific attachment of a peptide or protein on the surface. This can be done via a bi-functional

[45] J Kikuchi, Y Furukawa, and N Hayashi. *Mol Biotechnol*, 23(3):203–212, 2003.
[46] D H Min, J Su, and M Mrksich. *Angew Chem Int Ed Engl*, 43(44):5973–5977, 2004.
[47] K L Prime and G M Whitesides. *Science*, 252(5010):1164–1167, 1991.
[48] R R Siegel, P Harder, R Dahint, M Grunze, F Josse, M Mrksich, and G M Whitesides. *Anal Chem*, 69(16):3321–3328, 1997.

maleimide group that allows specific binding with amine and thiol groups (see Results, IV, page 77).

Once the SAM is formed and functionalized, the surface can be characterized by adding matrix and performing MALDI-MS directly on the surface.

The functionalized surface can be used for further protein interaction studies such as screening applications [49] [46] [50].

2.1.2.b SELDI-MS Surface-Enhanced Laser Desorption/Ionization (SELDI) is based on the ability of preactivated surfaces to bind different kind of molecules, especially peptides and proteins. These preactivated surfaces can be divided in two groups: the first one involves chemical surfaces, including hydrophobic, hydrophilic, cationic, anionic or immobilized metal-ion affinity surfaces which can capture target proteins nonspecifically. The second group involves more biological surfaces, including antibody, DNA, enzyme, receptor or drug surfaces, which can recognize specifically target proteins.

SELDI-TOF MS has been applied to the screening of tumor biomarkers [51] [52] and biomolecular interaction [53]. Although SELDI has many advantages including speed, sensitivity and abundant information, it still faces several challenges such as the identification and verification of biomarkers, and the proper interpretation of sophisticated SELDI-MS data. In addition, for the studies of biomarkers in serum or plasma, treatmenst of pre-enrichement [54] are required since most of the proteins in serum are present in very low concentrations, and can not be detected by the SELDI technique.

2.1.2.c SPR-MS Surface Plasmon Resonance (SPR) technique is an optical method for measuring the refractive index of very thin layers of material adsorbed on a metal. In case of protein-adsorption, the difference between the refractive index of the buffer and the one of the adsorbate can be easily converted into mass and thickness of the adsorbate, as all proteins have almost identical refractive indices [55]. This technology enables the detection and quantification of molecular interaction in real time and without the use of label.

---

[49] D H Min, W J Tang, and M Mrksich. *Nat Biotechnol*, 22(6):717–723, 2004.

[46] D H Min, J Su, and M Mrksich. *Angew Chem Int Ed Engl*, 43(44):5973–5977, 2004.

[50] W S Yeo and M Mrksich. *Advanced Materials*, 16:1352–1356, 2004.

[51] G Malik, M D Ward, S K Gupta, M W Trosset, W E Grizzle, B L Adam, J I Diaz, and O J Semmes. *Clin Cancer Res*, 11(3):1073–1085, 2005.

[52] J Li, R Orlandi, C N White, J Rosenzweig, J Zhao, E Seregni, D Morelli, Y Yu, X Y Meng, Z Zhang, N E Davidson, E T Fung, and D W Chan. *Clin Chem*, 51(12):2229–2235, 2005.

[53] Y G Amaar, G R Thompson, T A Linkhart, S T Chen, D J Baylink, and S Mohan. *J Biol Chem*, 277(14):12053–12060, 2002.

[54] R Lehmann, C Melle, N Escher, and F von Eggeling. *J Proteome Res*, 4(5):1717–1721, 2005.

[55] A Szabo, L Stolz, and R Granzow. *Curr Opin Struct Biol*, 5(5):699–705, 1995.

The first coupling SPR-MS was described in 1997 [56] [57] : after an affinity-capture step of proteins on the surface, a matrix solution was applied onto the surface and on-chip detection of proteins was done by MS. Coupling SPR with MS has increased limits of detection, multi-protein analysis and protein-complex delineation.

Currently, there are two ways to perform SPR-MS analysis after affinity-capture on the SPR surface: or the surface can be analyzed directly by MS [58] [59] or the target proteins can be eluted and then analyzed by MS and MS-MS [60] [61].

2.1.2.d Microfluidics Microfluidics is based on the manipulation of continuous liquid flow through microchannels on a surface[62]. It is more and more used in chemical biology and has become a powerful technology in biology studies [63].

By combining microfluidics technique, functionalized surfaces and MS it is possible to affinity-capture on-chip a mixture of proteins, avoid evaporation of the samples, use small volumes and small quantities of sample (microliter to nanoliter) and perform highly sensitive MS analysis [64]. For instance, peptide array multi enzyme assays were performed by combining microfluidic network and SAMDI [65].

2.1.2.e Free matrix surfaces Various materials have been investigated with the purpose of enhancing the laser desorption without addition of matrix solution. Unlike matrix-assisted MALDI TOF-MS, matrix-free surfaces are convenient for the characterization of low molecular weight compounds as they allow analysis of small molecules below m/z 300. Thus, these surfaces can be useful to analyze very small ligands after their elution from an affinity surface.

In particular, carbon and porous silicon surfaces have been introduced for the study of polar analytes of low molecular weight.

- This matrix-free approach was first introduced by Wei *et al.* in 1999 [66]. Desorption/ionization on silicon (DIOS) - time-of-flight (TOF) is a matrix free technique where analyte molecules are trapped within a porous silicon surface which are laser-desorbed and ionized [67].

[56] J R Krone, R W Nelson, D Dogruel, P Williams, and R Granzow. *Anal Biochem*, 244(1):124–132, 1997.
[57] R W Nelson, J R Krone, and O Jansson. *Anal Chem*, 69(21):4363–4368, 1997.
[58] C P Sonksen, E Nordhoff, O Jansson, M Malmqvist, and P Roepstorff. *Anal Chem*, 70(13):2731–2736, 1998.
[59] R W Nelson, J W Jarvik, B E Taillon, and K A Tubbs. *Anal Chem*, 71(14):2858–2865, 1999.
[60] D Nedelkov and R W Nelson. *J Mol Recognit*, 13(3):140–145, 2000.
[61] R W Nelson, D Nedelkov, and K A Tubbs. *Anal Chem*, 72(11):404A–411A, 2000.
[62] G M Whitesides. *Nature*, 442(7101):368–373, 2006.
[63] D B Weibel and G M Whitesides. *Curr Opin Chem Biol*, 10(6):584–591, 2006.
[64] P Hoffmann, U Hausig, P Schulze, and D Belder. *Angew Chem Int Ed Engl*, 46(26):4913–4916, 2007.
[65] J Su, M R Bringer, R F Ismagilov, and M Mrksich. *J Am Chem Soc*, 127(20):7280–7281, 2005.
[66] J Wei, J M Buriak, and G Siuzdak. *Nature*, 399(6733):243–246, 1999.
[67] Eden P Go, Jessica E Prenni, Jing Wei, Arianna Jones, Steven C Hall, H Ewa Witkowska, Zhouxin Shen, and Gary Siuzdak. *Anal Chem*, 75(10):2504–2506, 2003.

DIOS-MS has been used for instance to study enzyme kinetics and inhibition reactions [68] [69].

- Surface Assisted Laser Desorption Ionization (SALDI) is a laser desorption method that use carbon powder to couple the laser UV energy into a liquid solution. This technique is well suited for the analysis of a wide range of polar and/or thermally labile bio-organic compounds like phospholipids, amino acids, as well as peptides and small proteins such as myoglobin and cytochrome C [70]. However, SALDI-MS only yield few desorbed ions at low m/z ratios compared to those produced by MALDI, and leads to ion source contamination due to carbon particles. For these reasons, another technique named graphite plate laser desorption ionization (GLPDI) has been developed [71]. GLPDI consists on a thin layer of carbon particles on a surface where an analyte solution is directly deposited. An electrical contact between the activated carbon layer and aluminum foils is used to improve peak resolution. This laser desorptio/ionization method presents a high sensitivity with good resolution and leads only to few background ions at low m/z. Bioorganic compounds were succesfully analyzed [71].

- The properties of SAM were used to develop an initial matrix layer on gold surface [72]. Different models were proposed such as SAMs which contain aromatic compounds. [73].

2.1.3 Identification of intact non-covalent complexes by MS

The advantage using MS in the analysis of protein-small molecules complexes, is its rapidity and the accuracy of the analysis. Nevertheless, the ionization and transfer of complexes from solution to the gas phase is still a challenge in the characterization of intact non-covalent complexes . The solvent free environment in the mass spectrometer may enhance some interactions based on charge, dipoles, and polarizability whereas hydrophobic interactions, which greatly contribute to form stable protein-protein complexes, may be reduced in the gas phase [74]. ESI has long been recognized as the method of choice for studying non-covalent complexes [75] [76]. MALDI has been much less frequently applied to this kind of studies. It is mainly because the non-covalent complex inclusion within a matrix crystal remains a difficult step, and

[68] J J Thomas, Z Shen, J E Crowell, M G Finn, and G Siuzdak. *Proc Natl Acad Sci U S A*, 98(9):4932–4937, 2001.

[69] D B Wall, J W Finch, and S A Cohen. *Rapid Commun Mass Spectrom*, 18(13):1482–1486, 2004.

[70] P Kraft, S Alimpiev, E Dratz, and J Sunner. *J Am Soc Mass Spectrom*, 9(9):912–924, 1998.

[71] M Han and J Sunner. *J Am Soc Mass Spectrom*, 11(7):644–649, 2000.

[72] C E Jordon, B L Frey, S Kornguth, and R M Corn. *Langmuir*, 10:3642–3648, 1994.

[73] S Mouradian, C M Nelson, and L W Smith. *J Am Chem Soc*, 118:8339–8345, 1996.

[74] J Loo. *Int J Mass Spectrom*, 200:175–186, 2000.

[75] J M Daniel, S D Friess, S Rajagopalan, S Wendt, and R Zenobi. *Int J Mass Spectrom*, 216:1–27, 2002.

[76] S A Hofstadler and R H Griffey. *Chem Rev*, 101(2):377–390, 2001.

many fundamental should be done to improve and preserve complex stability.

2.1.3.a Analysis of intact protein complexes by ESI-MS In 1991, Ganem et al reported two successive articles showing mass spectra of receptor-ligand and enzyme-inhibitor complexes ionized by ESI [77]. The ability of ESI-MS to show whether a ligand is or is not bound to a protein is now often used in the pharmaceutical field for screening potential drugs [78] [79] [80] [81].

ESI-MS analysis of peptide-protein is usually done by comparison of the molecular masses of species measured in the absence or the presence of ligands (see Fig.I.10 A1-A2) or by comparison of the molecular masses of species under denaturing and under non-denaturing conditions (See Fig.I.10 B1-B2). Some experimental parameters have to be carefully controlled to study non-covalent complex formation (source temperature, cone voltage, interfacial pressure, choice of the buffer) in order to allow the ionization and the transfer of fragile complexes from the solution to the gas phase [82] [83]. For example, proteins often require non-volatile salts, cofactors, additives, and neutral pH to maintain their native conformation and biological activity. These conditions are not ideal for ESI-MS detection where low pH and the presence of some organic solvents are usually preferred for efficient ionization. As a result, this approach is limited to proteins whose native state can be preserved at a relatively acid pH (pH 5.5 - 6.5) and in low concentration of volatile salts and buffers (10-50 mM ammonium acetate, Tris acetate or ammonium bicarbonate). Because its high mass accuracy for protein complexes, ESI-MS is ideal for the determination of stoichiometry of non-covalent complexes. ESI-MS can be used to probe cooperativity in the binding of a ligand or a metal to a protein, and can determine the complex stability [83]. Numerous reviews in this field have been published [84] [85] [86]. These have been focused on the application of ESI-MS to the identification and structural characterization of interaction partners [87] [88], the study of protein-ligand interactions, including small molecules, lipids, and nucleic acids [89] or viruses [85].

---

[77] Yu Tsyr Li Bruce Ganem and Jack D. Henion. *J Am Chem Soc*, 113:6294–6296, 1991.

[78] M T Cancilla, M D Leavell, J Chow, and J A Leary. *Proc Natl Acad Sci U S A*, 97(22):12008–12013, 2000.

[79] K F Geoghegan and M A Kelly. *Mass Spectrom Rev*, 24(3):347–366, 2005.

[80] M G McCammon, D J Scott, C A Keetch, L H Greene, H E Purkey, H M Petrassi, J W Kelly, and C V Robinson. *Structure*, 10(6):851–863, 2002.

[81] H M Wright, C B Clish, T Mikami, S Hauser, K Yanagi, R Hiramatsu, C N Serhan, and B M Spiegelman. *J Biol Chem*, 275(3):1873–1877, 2000.

[82] J L Benesch and Carol V Robinson. *Curr Opin Struct Biol*, 16(2):245–251, 2006.

[83] N Potier, H Rogniaux, G Chevreux, and A Van Dorsselaer. *Methods Enzymol*, 402:361–389, 2005.

[84] A E Ashcroft. *Nat Prod Rep*, 22(4):452–464, 2005.

[85] B Bothner and G Siuzdak. *Chembiochem*, 5(3):258–260, 2004.

[86] A J Heck and R H Van Den Heuvel. *Mass Spectrom Rev*, 23(5):368–389, 2004.

[87] J Borch, TJ Jorgensen, and P Roepstorff. *Curr Opin Chem Biol*, 9(5):509–516, 2005.

[88] R W Kriwacki and G Siuzdak. *Methods Mol Biol*, 146:223–238, 2000.

[89] M G McCammon and CV Robinson. *Curr Opin Chem Biol*, 8(1):60–65, 2004.

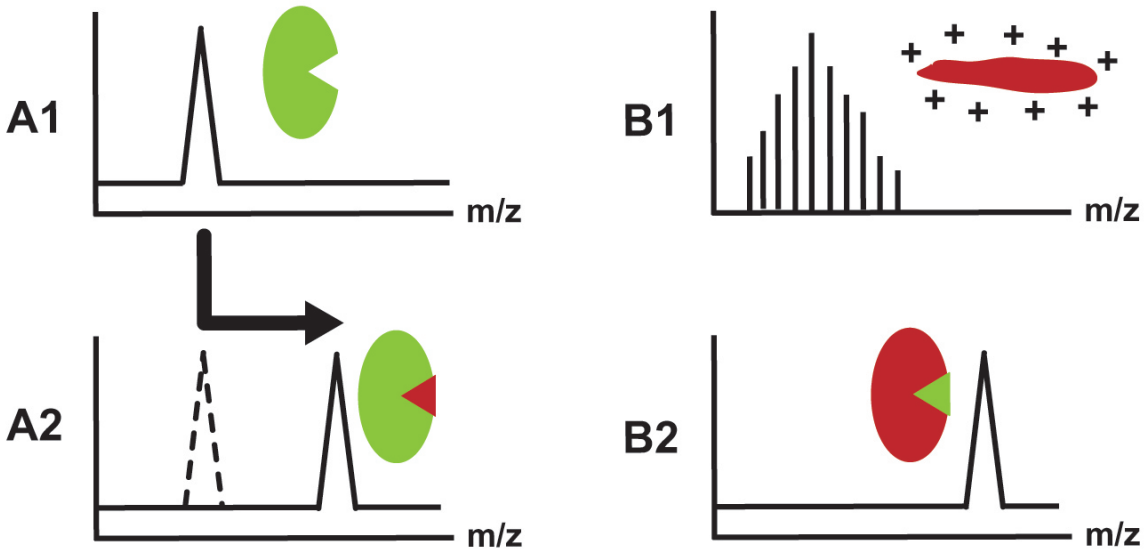


Figure I.10: Scheme of ESI-MS based methods for ligand-protein interaction studies. Mass spectra under non-denaturing conditions from a protein in absence of ligand (A1) and in presence of ligand (A2); the difference in the measured molecular mass corresponds to the molecular mass of the ligand. Mass spectra from a protein in presence of ligand under denaturing conditions (B1) and under non-denaturing conditions (B2); the difference in the measured molecular mass corresponds to the molecular mass of the ligand.

2.1.3.b Analysis of intact protein complexes by MALDI-MS There are only a limited number of reports describing the detection of molecular ions of intact non-covalent complexes by MALDI-MS (see for instance [90] [91] [92] [93]).

Considering that complexes are generally formed under under physiological conditions in solution phase, the dried sample mixed with organic matrix is not ideal for the observation of non-covalent complexes. In addition, it is difficult to differentiate the molecular ions of specific non-covalent complexes from those of non-specific associations. Consequently, application of MALDI-MS to the detection of molecular ions of non-covalent complexes is now far from being routine, while the literature on the ESI method for this purpose is now rapidly expanding.

2.2 Identification of contact residues by MS

H/D exchange, chemical cross linking and limited proteolysis are alternative and complementary methods to the traditional experimental methods of X-ray crystallography and NMR spectroscopy to study protein complexes. These methods enable contact residues information of

[90] J G Kiselar and K M Downard. *J Am Soc Mass Spectrom*, 11(8):746–750, 2000.
[91] A S Woods and M A Huestis. *J Am Soc Mass Spectrom*, 12(1):88–96, 2001.
[92] M Zehl and G Allmaier. *Anal Chem*, 77(1):103–110, 2005.
[93] B Tissot, F Gonnet, A Iborra, C Berthou, N Thielens, G J Arlaud, and R Daniel. *Biochemistry*, 44(7):2602–2609, 2005.

the complex.

2.2.1 Hydrogen exchange mass spectrometry

First described by Smith and Zhang in 1993 [94], hydrogen exchange mass spectrometry (HX-MS) involves the exchange of amide hydrogen atoms in a protein with a heavier isotope, typically deuterium, and MS analysis. The protein is incubated in a deuterium rich solvent whereupon hydrogen atoms may exchange with deuterium depending on the accessibility of the backbone to the bulk solvent (See Fig. I.11 A). The isotopic exchange rate is directly related to the secondary and tertiary structures of the studied protein. Rapid digestion of the protein at low pH and temperature (pH 2-3, 0 ℃), with proteolytic enzyme minimizes further back exchanges.

In a protein complex study, H/D exchange occurs only in the amide hydrogens that are not implicated in the interaction (see Fig. I.11 B). Proteolytic digestions of both the protein alone and the protein complex leads to two peptide sets that are analyzed by MS (typically LC-MS). H/D exchange rates are examined by determining the mass changes (Δ mass) from the two peptide sets. MS and MS-MS allow the identification of the peptides and amino acids residues involved in the protein complex interaction [95] [96].

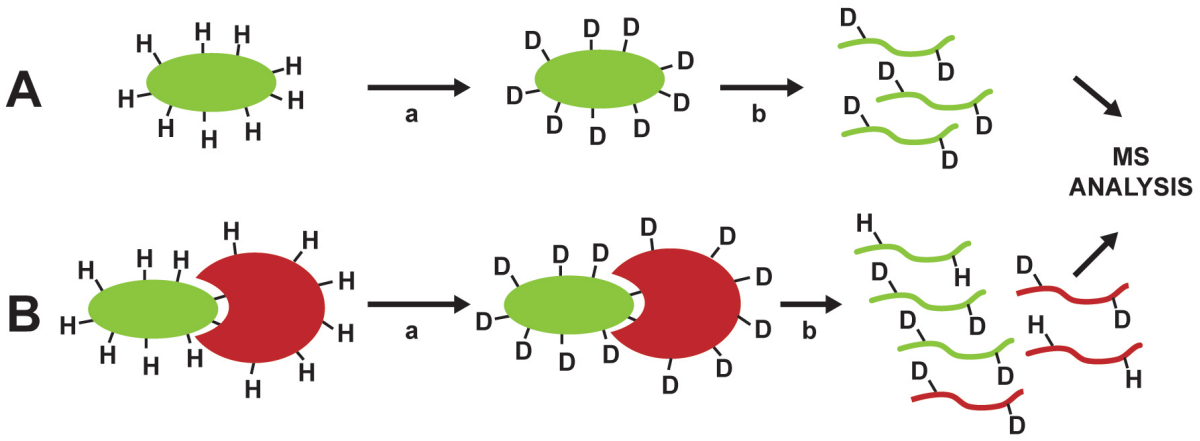


Figure I.11: H/D-MS based methods for the identification of contact residues in protein interaction studies. (A) H/D exchange of protein alone, proteolytic digestion and MS analysis of peptides; (B) H/D exchange of protein-protein complex, proteolytic digestion and MS analysis of peptides; Δ mass measured by MS analysis of two peptide sets allows identification of peptides involved in the interaction.

[94] Z Zhang and D L Smith. *Protein Sci*, 2(4):522–531, 1993.
[95] J R Engen. *Analyst*, 128(6):623–628, 2003.
[96] J R Engen and D L Smith. *Anal Chem*, 73(9):256A–265A, 2001.

2.2.2 Chemical cross-linking

Following the advance of MS, which enables rapid and accurate mass determination of modified proteins or peptides with a small amount of sample, the number of protein complexes whose interaction sites have been characterized by chemical cross-linking has significantly increased [97] [98] [99]. Cross-linking involves a chemical and irreversible modification between side chain groups engaged in the protein interaction. Depending on the length of the cross-linker, its functional chemical and/or photoactive group and its specificity to react with certain amino acid, two near- neighbor amino acids can be cross-linked. The cross-linked complex can then be directly detected by MS or separated from the reaction solution (electrophoretic gel, size exclusion chromatography) prior to proteolytic digestion and MS analysis, usually LC-MS (see Fig. I.12). Cross-linked sites can be determined by comparing the mass values of the peptide sets obtained after enzymatic digestion of the cross-linked complex. The interface sites of protein complex can be estimated, and thereby, the structure of the complex can be predicted [100] [101] [102]. However, identification of cross-linked proteolytic peptides can be hampered by the complexity of the protein mixture, as the cross-linking reaction yield is not very high.

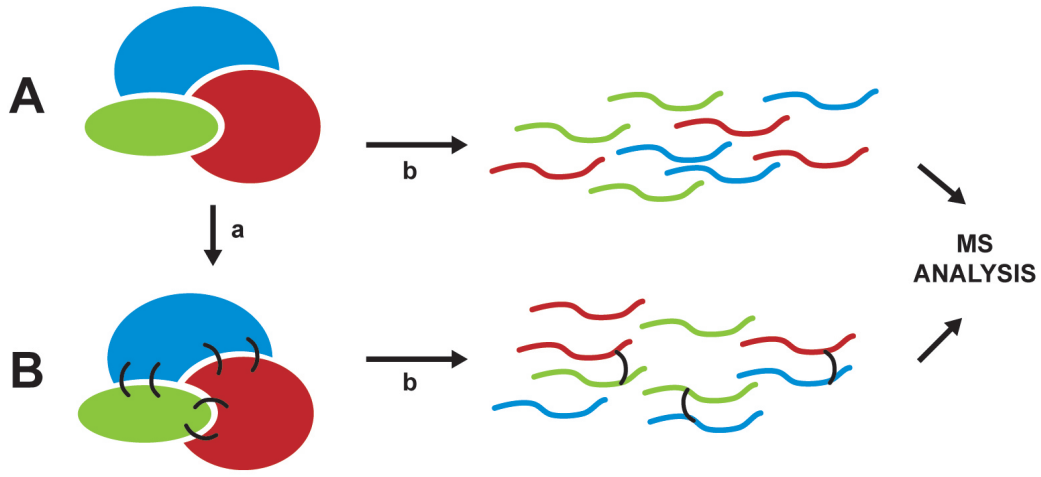


Figure I.12: Cross-linking based methods for the identification of contact residues in protein interaction studies. (A) Proteolytic digestion of the protein complex alone (b) and MS analysis of peptides; (B) after cross-linking experiments (a) the cross-linked protein complex digested (b) and peptides are analyzed by MS.

[97] T B Farmer and R M Caprioli. *Biol Mass Spectrom*, 20(12):796–800, 1991.

[98] K L Bennett, M Kussmann, P Bjork, M Godzwon, M Mikkelsen, P Sorensen, and P Roepstorff. *Protein Sci*, 9(8):1503–1518, 2000.

[99] A Sinz. *J Mass Spectrom*, 38(12):1225–1237, 2003.

[100] M Trester-Zedlitz, K Kamada, SK Burley, D Fenyo, BT Chait, and TW Muir. *J Am Chem Soc*, 125(9):2416–2425, 2003.

[101] JW Back, L de Jong, AO Muijsers, and CG de Koster. *J Mol Biol*, 331(2):303–313, 2003.

[102] S Kalkhof, C Ihling, K Mechtler, and A Sinz. *Anal Chem*, 77(2):495–503, 2005.

2.2.3 Limited proteolysis

Limited proteolysis coupled with MS is widely used for protein structure determination (See Results, Chapter I, page 47). It can be achieved by applying sequence specific protease, such as trypsin or chymotrypsin, and by performing MS analysis on the resulting proteolytic peptides. Indeed, if a protein is subjected to proteolysis under non-denaturing conditions, only surface-exposed and flexible sites can be cleaved by the protease, while cleavage sites of contact region of the protein subunits remain protected. By comparison of the proteolytics fragments obtained under denaturing (all the protein is digested) and non-denaturing conditions, structural domains of the protein can be found.

If a protein is associated with other molecules, limited proteolysis can be used for the determination of interface regions of protein complexes. Under non-denaturing conditions, the interface regions are protected from the access of the protease. Comparison of proteolytic fragments reveals a contrast between associated and unassociated state of the complex [103] (see Fig. I.13).

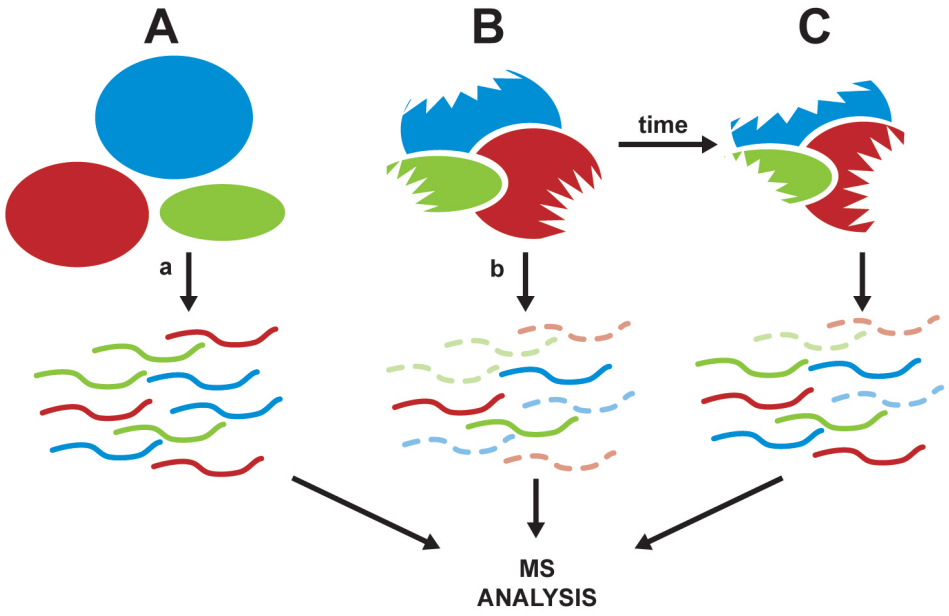


Figure I.13: Limited proteolysis based methods for the identification of contact residues in protein complex. (A) protein complex is digested under denaturing conditions giving a set of proteolytic peptides that map all protein sequences; (B) Protein complex is digested under non-denaturing conditions, the peptides identified correspond to the peptides on surface exposed regions while the peptides not found (dashed line) correspond to the peptides that are not accessible to the protease. (C) longer digestion times refine the determination of contact regions by giving larger set of proteolytic peptides.

Limited proteolysis experiments can be performed by using time-resolved proteolysis: a

[103] C Hager-Braun and K B Tomer. *Methods Mol Med*, 94:109–120, 2004.

certain quantity of protease is added to a protein/protein complex and MS analysis is done at several different times; or using protease-concentration-resolved proteolysis: the protein or protein complex are subjected to different quantities of protease for a certain time. The use of sequence specific proteases reduces the number of fragments that is produced and thus simplifies mass spectra analysis. Limited proteolysis can also be used to probe the conformational changes of protein induced by ligand binding [104]. Since proteolysis is performed in solution and can detect different conformers, this method can also contributes to the understanding of the dynamic domains within a protein.

[104] C Mc Donald and L Li. *Ananlytica Chimica Acta*, 534:3–10, 2005.

Chapter II

Atomic Force Microscopy

1 A brief history of AFM

When the optical microscope was developed in the 17th century, new insights into the biological world were made possible. Suddenly, individual cells and their sub-components, the very basic units of tissues, could be observed. However the resolution limit of the optical microscope is determined by the diffraction limit, which means that objects smaller than 1/3 of the working wavenlenght cannot be distinguished.

In the 1930 another jump in the world of microscopy was made, primarily by Ernst Ruska, with the development of the electron microscope. This new microscope has since been a key tool for investigation of sub-cellular components as well as proteins and virus structure.

In 1982, a new generation of microscopes called Scanning Probe Microscopes arised. Two physicists, Binn and Roh, developed the first of this family, Scanning Tunneling Microscope (STM)[105]. Its breathtaking capability was demonstrated by the direct imaging of single atoms[106] and the ability to move and manipulate single atoms on a surface[107]. Interestingly, the ability to do this was hypothesized two decades ago by the Nobel laureate Richard Feynman[108]. Binning and Rohrer later shared the Nobel Price in Physics with Ernst Ruska for their developments in microscopy.

The principle behind the STM is based on the quantum physical property whereby electrons are capable of tunnelling between two conductors provided that are sufficiently close, but not physically in contact. The original invention was received with much interest in the physics community, but found little applications in other fields such as biology. The requirement for a conducting surface severely limited its use for biological studies.

In 1986 Binnig, Quate and Gerber developed the second microscope of the SPM family, the

[105] G Binnig, H Rohrer, Ch Gerber, and E Weibel. *Appli Phys Lett*, 40(178-180), 1982.
[106] G Binnig, H Rohrer, C Gerber, and E Weibel. *Phys Rev Lett*, 50(120–123), 1983.
[107] DM Eigler and EK Schweitzer. *Nature*, 344:524–526, 1990.
[108] RP Feynman. *Meeting of the American Physical Society, Caltech (California)*, 1959, December 29th.

atomic force microscope (AFM)[109]. The AFM had no requirements for a conducting sample thus opening possibilities in new fields such as polymer and biological sciences. A very fine stylus mounted on a microcantilever is used following the same principle of a gramophone; the vertical movement of the microcantilever records then the topography of the surface. After some technical improvements concerning detecion of microcantilever movement, robustness, development of a huge probe market, etc, the technique became a desirable tool among biologists.

It is worth remembering that the AFM is is a mechanical nanoprofilometer. There are no optics focusing radiation on the sample, whether it be light or electrons. It is a mechanical microscope. The imaging is based purely on a physical rendering of the surface topography by raster scanning (zigzag movements) of a stylus across the sample. This gives certain limits to the technique, but also a range of advantages as described below. Whereas STM imaging was based on the quantum physical principle of electron tunnelling between tip and sample, the AFM images are a result of a multitude of forces interacting between the stylus tip and the sample, e.g., Van der Waals and electrostatic forces. See review[110].

2 Apparatus

AFM is not a conventional microscope designed to look at something, but it was rather designed to ”feel” something. Indeed the AFM gives a very detailed picture, not only of the topography at the nanoscale, but also about the mechanical properties of the sample surface, thanks to a very sharp probe that follows the surface of the sample. The vertical deflection of the microcantilever is measured by the reflection of a laser bean focused on the back face of the cantilever surface into a photodiode. The change in the vertical (z axis direction) position on the photodiode is directly related to the topography of the surface and the forces applied. A feedback mechanism maintains the cantilever at a constant distance through a piezoelectric (tube) scanner that displaces the tip in the Z direction and a real topographic profile is recorded.

2.1 Tips and cantilevers

The tip consists of a micro fabricated, extremely sharp spike mounted at the end of a microcantilever(see Fig.II.2). The tips are commonly made of one or two materials: silicon or silicon nitride. Silicon tips are usualy stiffer and more brittle, they can they can be coated by different materials with different purposes, i.e. measuring conductance, thermal gradients, mechanical properties, chemical interaction, magnetic domains, etc.. If optical sensing methods are to be

[109] G Binnig, CF Quate, and C Gerber. *Phys Rev Lett*, 56(9):930–933, 1986.

[110] Mark A Poggi, Elizabeth D Gadsby, Lawrence A Bottomley, William P King, Emin Oroudjev, and Helen Hansma. *Anal Chem*, 76(12):3429–3443, 2004.

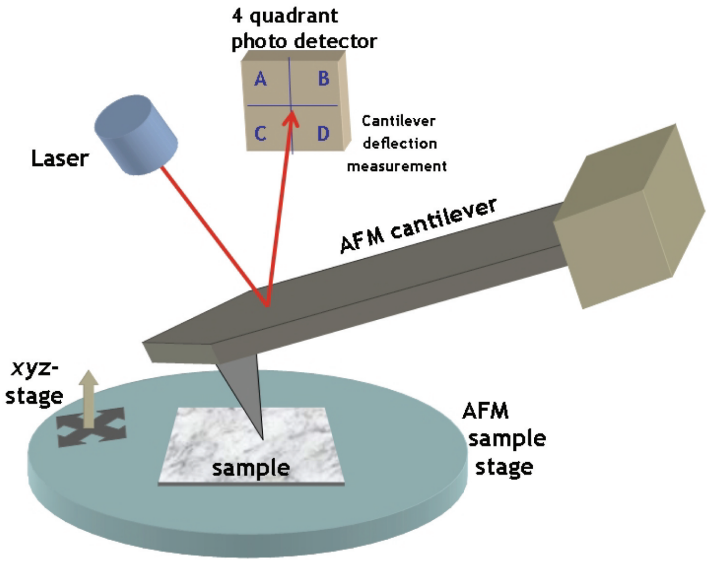


Figure II.1: Schematic representation of the atomic force microscope (Image from the Open source Handbook of Nanoscience and Nanotechnology).

used to monitor cantilever deflection then they are coated with a thin layer of gold to improve their reflectivity, or if magnetic sensitivity is required a ferromagnetic coating may be applied.

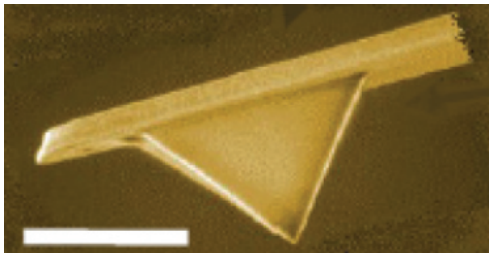


Figure II.2: AFM tip, bare scale at 3μm

The macroscopic shape of the tip is important. A tip with a relatively large cone angle will smooth the edges of a sample leading to an overestimation of the lateral size. However the height can be recorded very accurately (see Fig.II.3).

The geometry of the cantilever is also important depending on the applications. A triangular geometry (”V” shaped lever) is designed to minimize torsional motions, or twisting of the cantilever while scanning a sample, and is the cantilever of choice for purely topographical imaging. To measure the frictional properties of a sample, a rectangular geometry is the best, giving a greater degree rotation and making the cantilever sensitive to lateral forces. The working mode of the AFM (friction, force modulation, tapping, magnetic,...) will determine the cantilever choice.

Tips can also be functionalized to enable force measurements between the molecule coating the tip and the molecule onto the surface (see section imaging modes....)

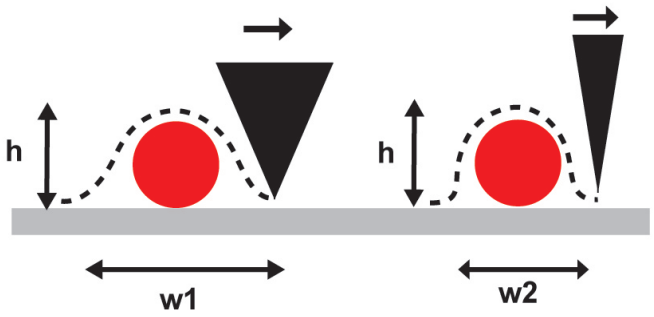


Figure II.3: Schematic representation of a sample imaging by two tips having different cone angle. A first image is obtained and the height (h) and the width (w1) of the sample is measured; the same sample is then image with sharper tip: resolution improved (w2) but height measurements stay uniform (h)

2.2 Piezoelectric scanners

There are two basic types of scanning mechanisms : some that scan the sample, and others that scan the tip. Both, however, rely upon piezoelectric transducers. The scanner tube consists of a thin-walled hard piezoelectric ceramic which is polarized. Electrode are stuck to the internal and external faces of the tube, and the external face of the tube is split into quarters parallel to the axis. By applying a bias voltage between electrodes, the tube will expand or contract (z direction). By applying a bias voltage in just one of the outer electrode the tube will bend (x and y directions).

2.3 Optical detection

Laser beam deflection is the most common detection method used in commercial AFMs. The principe is simply based on measurement of the deflection of the laser bean on the cantilever . This deflection is detected on photo-detector (usually a photodiode) which turns light into an electrical signal. The photo-diode is split into four sections enabling both vertical and lateral movements to be detected. motions of the tip to be differentiated. By comparing the relative intensity of the reflected laser light in each quadrant quantification of tip displacement can be achieved.

2.4 Control system - feedback system

Feedback control is used commonly for keeping the motion of an object in a fixed relationship to another object. In the AFM, feedback control is used to keep the probe in a ”fixed” relationship with the surface while a scan is measured.

3 Mode of operation

There are many imaging modes available when using AFM. These are often differentiated by the interaction forces involved in each case. Each mode has its own range of application.

3.1 AFM imaging modes

FigII.4 represents how force between tip and sample varies with distance together with an indication of the different regions of the force curve, which correspond to the three main imaging modes (contact, intermittent contact (known also as ”tapping mode”) and non-contact).

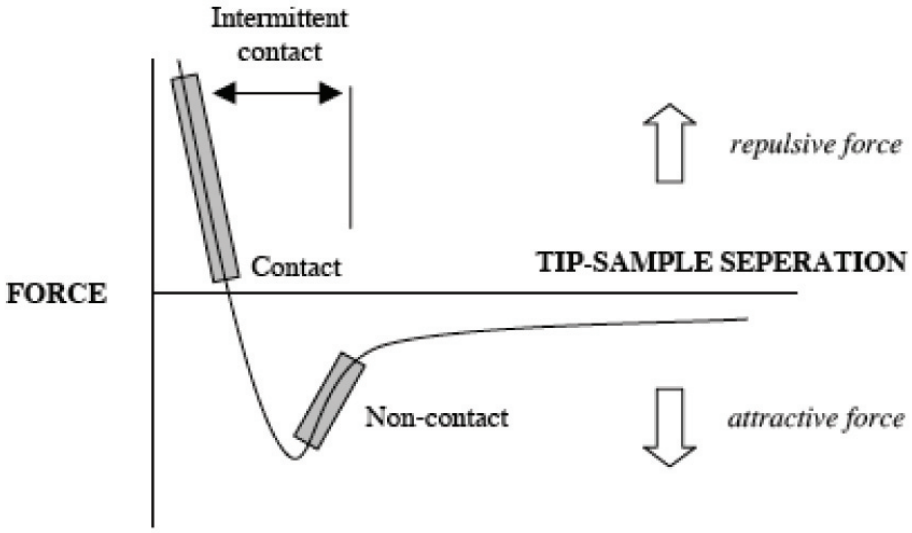


Figure II.4: Variation of the forces with tip-sample separation during the three main AFM imaging modes

3.1.1 Contact mode

Contact mode is regarded as the original imaging mode developed in AFM. In this mode, the microscope can be operated in either height or deflection mode.

The former utilizes an electronic feedback mechanism to continually adjust the height of the tip (or sample, depending on the AFM set-up) to maintain a constant force on the sample whilst the tip is raster scanned across the surface (see Fig II.5). When operated in this mode, the system monitors the changes in piezo height, subsequently using this information to form accurate three-dimensional images of the surface.

When operated in deflection mode, the z-piezo remains stationary whilst the deflection data from the movement of the laser in response to changes in topography are recorded. Images produced in deflection mode highlight rapid changes in topography in greater detail. This process can be performed either in air or under a liquid, and no special tips are required. Contact mode imaging is almost always preferred when imaging hard and stable samples. Additionally,

as contact mode imaging involves applying a relatively high force to the sample it generally offers the best option for achieving higher resolution images, as the probe is continually in contact with the sample surface (see Fig II.4). However, the inevitable offset to this is the higher applied force, the greater the likelihood of damage occurring to the surface via unwanted shear force between tip and sample effects such as frictional forces. While such forces may not degrade hardier samples, they are often of a magnitude sufficient to cause damage to softer, biological samples, and could also have a dramatical effect on the state of the tip.These limitations led to the development of intermittent and non-contact imaging modes.

If the scanner moves the sample perpendicular to the long axis of the cantilever, friction between the tip and sample causes the cantilever to twist. A photodetector position-sensitive in two dimensions can distinguish the resulting left-and-right motion of the reflected laser beam from the up-and-down motion caused by topographic variations. Therefore, AFMs can measure tip-sample friction while imaging sample topography. Besides serving as an indicator of sample properties, friction (or ”lateral force,” or ”lateral deflection”) measurements provide valuable information about the tip-sample interaction.

Figure II.5: Schematic representation of contact imaging mode.

3.1.2 Intermittent imaging mode

Intermittent mode was developed to help alleviate some of the limitations associated to contact mode imaging [111]. In this mode, the cantilever is allowed oscillating at its resonance frequency above the surface so only the lower part of the cantilever is in contact with the surface (see FigII.6). As in contact mode, the AFM feedback system constantly adjusts the z-piezo position to maintain the amplitude of the freely oscillating probe as a constant pre-set value [112], subsequently using this data to form accurate three dimensional images. Intermittent imaging mode greatly reduces lateral forces involved in imaging[113], thus minimizing sample damage. As such, it has been widely implemented in the imaging of easily deformable materials such as

[111] Q Zhong, K Innis, VB Kjoller, and V Elings. *Surf Sci Lett*, 290(L688–L692), 1993.

[112] DK Jandt. *Surf Sci*, 491(303–332), 2001.

[113] S H Leuba, G Yang, C Robert, B Samori, K van Holde, J Zlatanova, and C Bustamante. *Proc Natl Acad Sci U S A*, 91(24):11621–11625, 1994.

polymers, proteins and cells [114],[115].

As the tip oscillates up and down, extra information can be extracted[116]. By comparing the difference between the applied oscillation and the actual oscillation, a phase difference can be measured[117]. This signal is the result of the mechanical properties of the surface as well as the force interaction between the tip and the surface. The phase shift can be used to differentiate areas on a sample with such differing properties as friction, adhesion, and viscoelasticity. Nevertheless the actual meaning of the signal is complicated and its interpretation is still controversial.

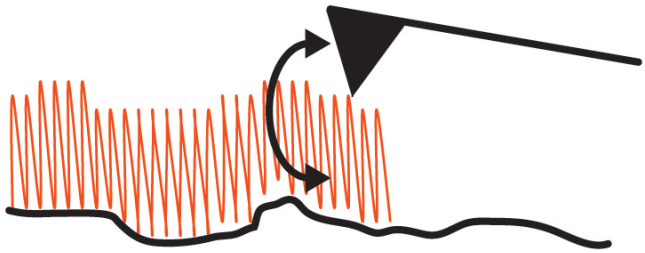


Figure II.6: Schematic representation of intermittent imaging mode.

3.1.3 Non-contact mode

The magnitude of the forces applied to the sample by the AFM tip can be further reduced by applying non-contact mode (NC-AFM)[118]. This mode operates by bringing a probe, which is vibrating at its resonant frequency, into the attractive force region (see FigII.4 and FigII.7). It does not come into physical contact with the surface, but is able to detect force gradients as it is raster scanned across the surface by either shifts in the resonant frequency or oscillation amplitude of the cantilever.

NC-AFM is desirable as it offers a means of investigating the sample topography with a complete lack of sample damage. However, the long range attractive forces that operate between the sample and the probe are relatively low and less sensitive to probe-sample separation. Such images obtained from NC-AFM inevitably display a lower spatial resolution.

---

[114] M Radmacher, M Fritz, and P K Hansma. *Biophys J*, 69(1):264–270, 1995.

[115] N H Thomson, M Fritz, M Radmacher, J P Cleveland, C F Schmidt, and P K Hansma. *Biophys J*, 70(5):2421–2431, 1996.

[116] O Sahin, S Magonov, S Chanmin, C F Quate, and O Solgaard. *Nat Nanotechnol*, 2(507–514), 2007.

[117] J Tamayo and R Gracia. *Appl Phys Lett*, 71:2394, 1997.

[118] R Luthi, E Meyer, H Haefke, L Howald, W Gutmannsbauer, and HJ Guntherodt. *Science*, 266(5193):1979–1981, 1994.

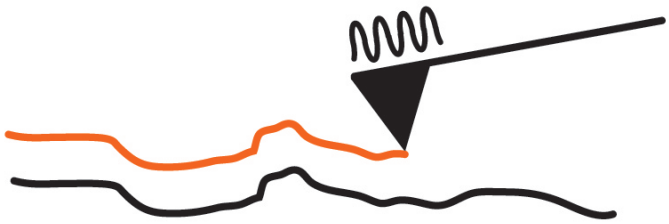


Figure II.7: Schematic representation intermittent imaging mode.

3.2 AFM force measurements

In addition to its imaging capabilities, AFM is also able to quantitatively measure the forces that exist between the probe and the sample. Experiments of this nature are commonly refereed to as force-distance measurements[119]. Force-distance measurements are recorded by monitoring the deflections of the cantilever while the probe is brought into contact with the sample at a constant z piezo velocity, and subsequently separated from the sample. Fig II.8 displays a scheme of a typical force-distance plot, which is constructed from the cantilever deflection force (y-axis) versus the distance travelled by the z-piezo measured in nm (x-axis).

At (A) the probe-sample distance is considerable and, as such, there is zero cantilever deflection. From this position, the probe approaches the sample at a constant velocity until the probe is close enough to the surface to begin experiencing weak long-range attractive forces. When the magnitude of these forces exceed the stiffness of the cantilever, the probe suddenly jumps into contact with the sample surface (B). Further movements of the tip towards the sample results in repulsive forces between the two, thus bending the cantilever in the opposite direction. This forward motion continues until (C), which represents the predetermined point of maximum load. The tip often remains engaged beyond the jump to contact distance and the force required for disengagement at (D), commonly referred to as the pull-off or adhesion force, exceeds that obtained at jump-to-contact. Eventually the tip disengages completely and returns to its original start position (E).

By using the AFM as a force-sensing instrument, considerable progress has been made in the understanding of the forces that govern the fundamental properties of many materials [120] [121][122].

[119] NA Burnham, X Chen, CS Hodges, GA Matei, EJ Thoreson, CJ Robert, CJ Davies, MC Davies, and SJB Tendler. *Nanotechnology*, 14:1–6, 2003.

[120] Rb Best, DJ Brockwell, JL Toca-Herrera, AW Blake, DA Smith, SE Radford, and J Clarke. *Ana Chim Acta*, 479(1):87–105, 2003.

[121] B Cappella and G Dietler. *Surf Sci Rep*, 34:1–104, 1999.

[122] S Allen, MC Davies, CJ Roberts, SJB Tendler, and PM Williams. *Tibtech*, 15:101–105, 1997.

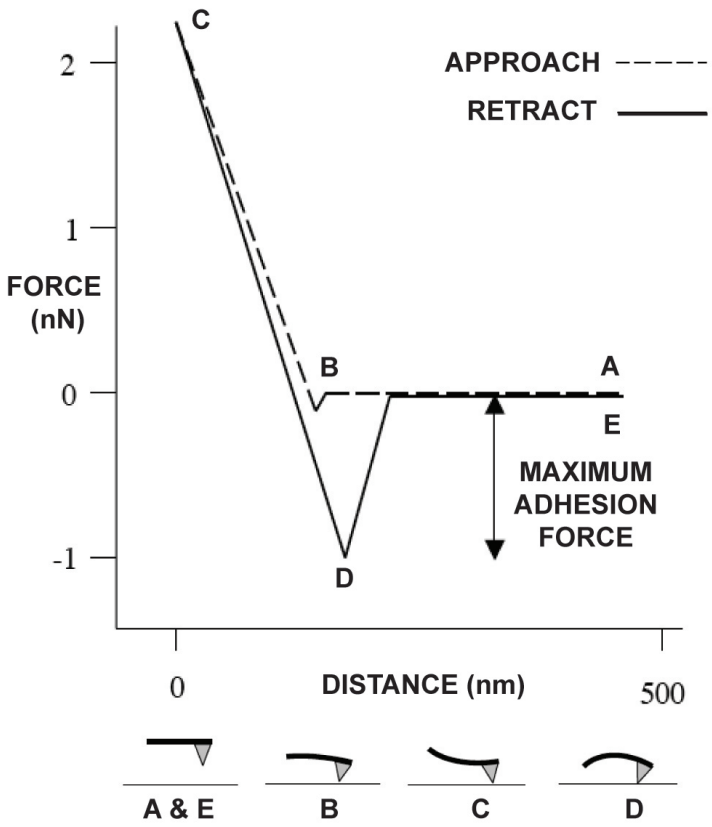


Figure II.8: Scheme of a typical force-distance curve observed with the AFM. (A) Tip approaches sample surface, (B) jump to contact due to long range attractive forces, (C) point of maximum load, which is set by the user, (D) disengagement (or pull-off) as tip is retracted from surface and (E) tip returns back to it s starting position out of the range of any forces.

4 Application of AFM to biology

Since its invention in the late 1980s, the AFM has increasingly been used for the visualization of biomolecules and complex biological structures [123][124][125][126]. In addition to high-resolution proteins imaging, the tip of the microscope can be used as a tool the inter and intramolecular forces[127][128][129][130].

4.1 Working with biological samples

4.1.1 Intermittent mode under liquid condition

As seen previously, intermittent-mode is the method of choice to image biological samples. However, in ”air” intermittent-mode imaging, biological samples are dried and fixed onto the surface. This situation is far to be representative of the biological environment. The application of intermittent-contact-mode-AFM under liquid offers a unique possibility to image biological samples in near native conditions, preserving the biological structure, and has opened up a new door for imaging biomolecular structures [131][132][133].

Working in liquids requires the use of a liquid cell. Basically, liquid cell performs different functions: it contains the sample, it contains the liquid, it provides a stable optical path for the laser beam which is reflected off the cantilever and it keeps the sample surface away from environmental contaminants.To avoid any interaction with the sample and contamination, liquid cells must be made of an inert material.

If the substrate is not readily used after its preparation, when exposed to air it will be quickly covered with contaminants, which will modify the sample adsorption and the AFM measurement. All the aqueous solutions used for the sample and substrate preparation for the AFM analysis must be prepared with ultra-pure water in order to minimize levels of hydrocarbons or other contaminants. As a general rule forAFM biological studies, it is recommended to keep the samples hydrated and in conditions where their functionality remains intact.

[123] J K H Horber and M J Miles. *Science*, 302(5647):1002–1005, 2003.

[124] F Kienberger, H Mueller, V Pastushenko, and P Hinterdorfer. *EMBO Rep*, 5(6):579–583, 2004.

[125] A Engel and D J Muller. *Nat Struct Biol*, 7(9):715–718, 2000.

[126] T Ando, N Kodera, Y Naito, T Kinoshita, K Furuta, and YY Toyoshima. *Chemphyschem*, 4(11):1196–1202, 2003.

[127] J Zlatanova, S M Lindsay, and S H Leuba. *Prog Biophys Mol Biol*, 74(1-2):37–61, 2000.

[128] B Isralewitz, M Gao, and K Schulten. *Curr Opin Struct Biol*, 11(2):224–230, 2001.

[129] T E Fisher, P E Marszalek, and J M Fernandez. *Nat Struct Biol*, 7(9):719–724, 2000.

[130] M Carrion-Vazquez, A F Oberhauser, T E Fisher, P E Marszalek, H Li, and J M Fernandez. *Prog Biophys Mol Biol*, 74(1-2):63–91, 2000.

[131] P K Hansma, JP Cleveland, M Radmacher, DA Walters, PE Hillner, M Bezanilla, M Fritz, D Vie, HG Hansma, CB Prater, J Massie, L Fukunuga, J Gurley, and Elings V. *Appl Phys Lett*, 64:1738–40, 1994.

[132] CAJ Putman, KO Vanderwerf, BG Degrooth, NF Vanhulst, and J Greve. *Appl Phys Lett*, 64:2454–2456, 1994.

[133] J Yang, L K Tamm, A P Somlyo, and Z Shao. *J Microsc*, 171(Pt 3):183–198, 1993.

4.1.2 Choice of the substrate

As in any other types of microscopy, the biological sample has to be deposited on a solid substrate. The ideal substrate has to be smooth at the atomic level and should have affinity for the studied molecules.

Hydrophilic substrates like mica, glass and silcon oxyde, are the most common used substrates for AFM studies. Muscovite mica is a nonconducting layered mineral composed of multiple thick silicate layers[134]. It can be cleaved by the help of adhesive tape to produce clean, hydrophilic, atomically flat surfaces that are negatively charged. It can be modified with silanes either to promote adsorption or to allow covalent binding of the target biomolecules[135].

Glass substrates are usually flat enough for imaging cells and other large structures, but are usually too rough to image small molecules. The glass surface is easily contaminated and must be washed carefully before use (in acidic solution followed by ultrasonication in water solution). Glass can also be modified with silanes[136]. For a smoother surface, silicon oxyde wafers can be used instead of glass.

Hydrophobic substrates may sometimes be preferred. For instance, highly oriented pyrolic graphite (HOPG) which offers atomically flat large areas or mica coated with carbon [137].

For some specific surface chemistries, gold surfaces are the surfaces of choice. Gold is chemically inert against oxygen and stable against radicals. It can be easily modified with self-assembled monolayers of organic alkanethiols that can be further used to adsorp or to attach biomolecules[138].

4.2 Immobilization of proteins: the role of SAMs

Immobilization of proteins to solid surfaces is crucial in the development of solid-phase based bioanalytical techniques, biosensors, and biocompatible materials. A key requirement is to attach protein readily onto surface without sacrificing their bioactivities.

SAMs exhibit a great potential for use in a wide range of areas from biotechnology to molecular electronics[139]. Its large variety of terminal groups makes SAMs a unique procedure to immobilize biomolecules. For example, alkanethiol SAMs on gold functionalized with hydroxyl (OH) termini provide a hydrophilic surface environment for bio-application, while carboxyl (COOH) and aldehyde (CHO) functionalities have been utilized as support for protein

[134] SW Bailey. *Mineralogical Society of America, Washington DC*, 1984.

[135] Y L Lyubchenko, A A Gall, L S Shlyakhtenko, R E Harrington, B L Jacobs, P I Oden, and S M Lindsay. *J Biomol Struct Dyn*, 10(3):589–606, 1992.

[136] S Karrasch and A Engel. *Procedures in scanning probe microscopies*, pages 433–439, 1998.

[137] J Yang, K Takeyasu, and Z Shao. *FEBS Lett*, 301:173–176, 1992.

[138] A Ulman. *Chem Rev*, 96(4):1533–1554, 1996.

[139] NJ Tao. *Nature*, 1:173–181, 2006.

immobilization via electrostatic and covalent interactions, respectively. The use of aldehyde termini has the advantage of forming strong covalent bonds with primary amines of the side chains of the amino acids in the proteins. SAMs of alkanethiols on gold has known to have a dense and well-ordered structure[140].

Small terminal groups such as -CH3, -CN, do not disturb the organization but larger groups can modify slightly the organization of the organic layer (ref). In a general rule, large terminal groups (peptides, proteins, antibodies) are sterically unable to adopt a well-ordered conformation and the resulting structures are more disordered and less dense than those formed by alkanethiols terminated with small groups. AFM is able to characterize SAMs by topography imaging which can clearly reveal weaky-packed areas (disordered domains), steps on the substrates or tighty-packed areas (ordered domains) (see FigII.9). AFM can thus be used to estimate the degree of packaging and organization of a mixed SAM.

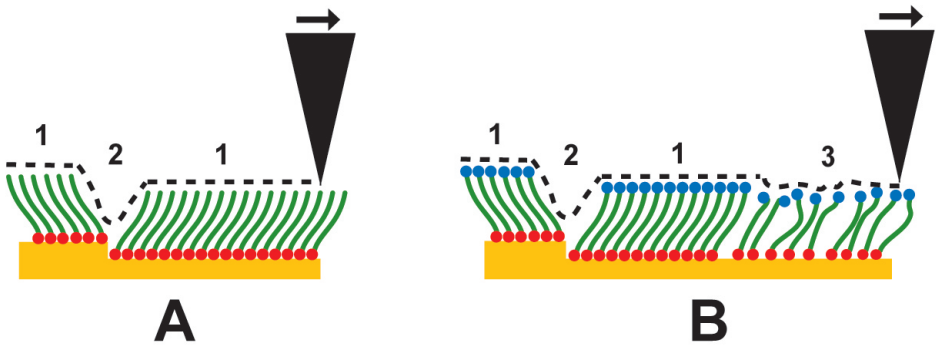


Figure II.9: Schematic representation of SAMs structures; (A) typical morphology of an alkanethiols SAM showing ordered domains (1) and defects on the surface substrate (2); (B) morphology of a terminal-modified alkanethiols SAM showing ordered domains (1), defects on the surface substrate (2) and disordered domains (3)

4.2.1 Adsorption

In the majority of the situations, the adsorption is obtained through physical interactions (hydrogen bond, electrostatic, polar or van der Waals forces). However, this technique is dependent on the properties of the surface (hydrophilic or hydrophobic), on the biochemical features of the protein itself and on the experimental conditions (pH, buffer) [141]. Moreover, physical adsorption involves no specificity in the immobilization of the protein, a random distribution throughout the surface, as well as random orientation of the immobilized proteins. Finally, weakly attached proteins can be easily removed by either washing step or by lateral forces applied with the tip (see FigII.10).

[140] C Vericat, M E Vela, and R C Salvarezza. *Phys Chem Chem Phys*, 7(18):3258–3268, 2005.
[141] K Wadu-mesthridge, N A Amro, and Lius G Y. *Scanning*, 22:380–388, 2000.

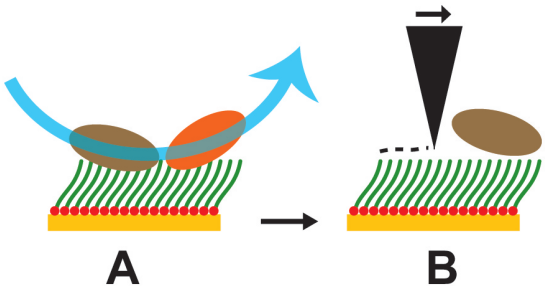


Figure II.10: Schematic representation of physical adsorption of proteins onto SAMs: some protein can be removed with washing steps, while some other proteins can be displaced or removed by the scanning tip.

4.2.2 Covalent binding

Thus, to observe biological structures in their native states by AFM, they must be well attached to the substrate. A covalent binding can be achieved for instance via the primary amines of the protein and an aldehyde-terminated SAM, forming aldimine[142]. This method present the advantage of overcoming the washing steps and displacement of the sample by lateral forces problems (see Fig II.11. Nevertheless, the specificity of the binding and the oritentation of the molecule immobilized still remains hazardous.

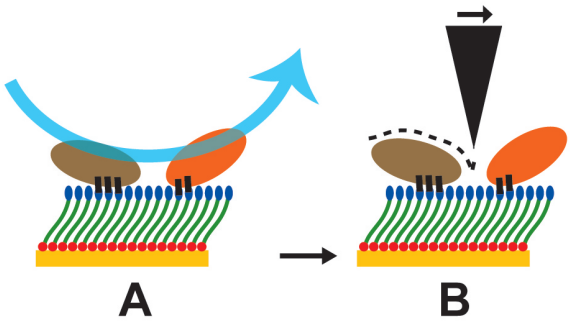


Figure II.11: Schematic representation of covalent binding of proteins onto SAMs

4.2.3 Capture ligand strategy

A capture ligand strategy was also developed in order to selectively attach desired protein on the substrate[143]. This capture ligand strategy allows a selective covalent binding between a ligand exposed on the SAM and a capture -protein binding to the protein of interest (see FigII.12). The use of mixed SAMs on the surface is the key point of the methodology. AFM topography measurements verify the uniform orientation of the immobilized protein.

[142] A Baker, L Zidek, D Wiesler, J Chmelik, M Pagel, and M V Novotny. *Chem Res Toxicol*, 11(7):730–740, 1998.
[143] C D Hodneland, Y S Lee, D H Min, and M Mrksich. *Proc Natl Acad Sci U S A*, 99(8):5048–5052, 2002.

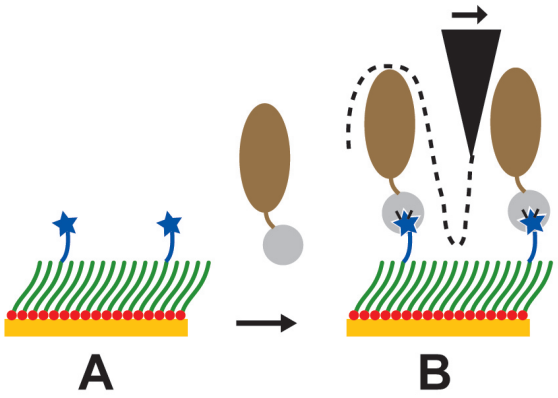


Figure II.12: Schematic representation of capture ligand strategy onto SAMs

4.2.4 Streptavidin-biotin system

An alternative to the capture ligand startegy is the streptavidin-biotin system which is one of the most widely used in bioconjugation chemistry [144]. The strong affinity and high specificity of the interaction is the strongest among all noncovalent interactions. The presence of four binding sites on each streptavidin molecule makes it possible to link a biotin-tagged molecule to a biotinylated surface (See Fig II.13). The exceptional strong nature of this interaction ensures the integrity of the system under a wide range of experimental conditions. When this interaction is used for the immobilization of a biotin-tagged protein/ molecule to a solid surface, the binding occurs trough the tag, thus minimizing disturbance to protein structure and ensuring its activity.

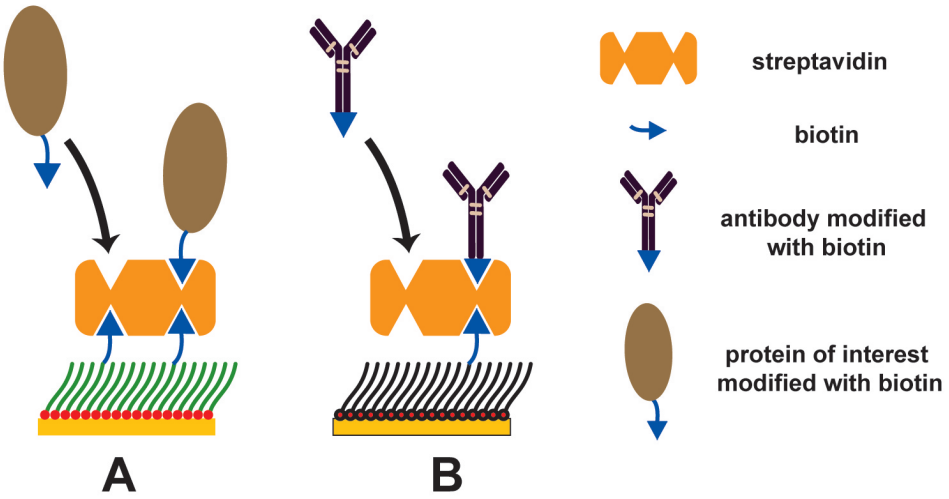


Figure II.13: Schematic representation of the strepatvidin-biotin system onto SAMs.

[144] J Turkova. *J Chromatogr B Biomed Sci Appl*, 722(1-2):11–31, 1999.

4.2.5 Microcontact printing

Micro-contact printing (μ CP) is a method of patterning SAMs on surfaces and specially SAMs of alkanethiol[145]. μ CP uses elastomeric stamps made usually of polydimethylsiloxane (PDMS) which exhibits relief patterns at the micron and nanoscale. These stamps let to parallel deposition of molecules on a target surface.

The fabrication of a PDMS-stamp consists in generating a silicon master. This master is used as a mold to form the PDMS-stamp. A silicon master can be reused more than 50 times and each stamp can be used for a large number of prints (around 100 times).

The PDMS-stamp is then usually incubated with an ethanolic solution containing the molecules to print for a short time (typically 10-15 minutes). At this stage, alkanethiols, especially non polar alkanethiols such as n-alkanethiols, diffuse into the bulk of the hydrophobic stamp. Polar molecules, however, are known to remain entirely at the surface of the stamp[146]. The stamp is then blow dried under nitrogen beam and immediately placed in contact with the substrate surface. Alkanethiols diffuse from the stamp to the surface where they assemble into an ordered structure. A common strategy in biological studies using μCP, is to fill the bare surface with another alkanethiol solution. This will create a new SAM,which can be for example modified with ethylene-glycol group in order to avoid unspecific interaction on this area.The surface is then ready to be used and protein can be immobilized on the terminated modified SAM area (see Fig II.14).

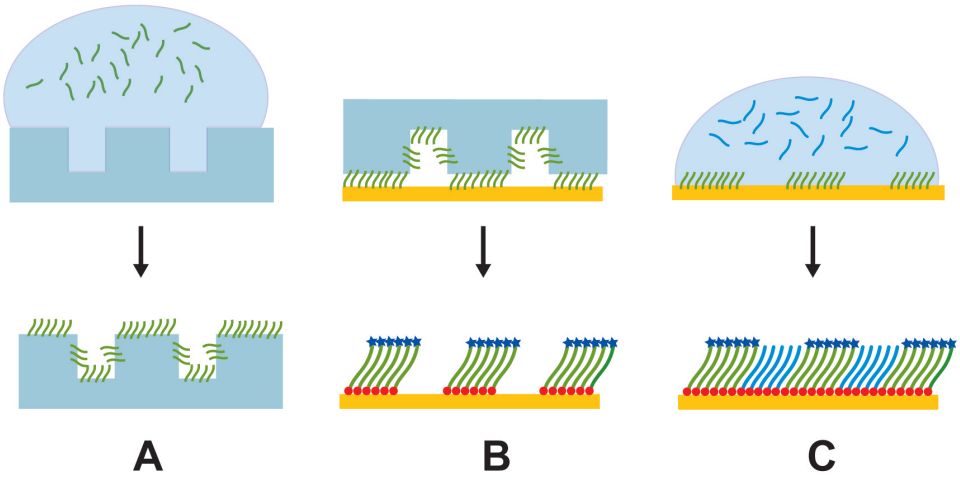


Figure II.14: Schematic representation of patterning SAM by microcontact printing. (A) the stamp is incubated in a solution containing the alkanethiol and then dried; (B) the stamp is placed in contact with the surface and then removed, leading a SAM at the contact areas; (C) the surface can be incubated with another alkanethiol solution that will fill the bare surface with a new inert SAM.

[145] JC Love, DB Wolfe, ML Chabinyc, KE Paul, and GM Whitesides. *J Am Chem Soc*, 124(8):1576–1577, 2002.
[146] JN Lee, C Park, and GM Whitesides. *Anal Chem*, 75(23):6544–6554, 2003.

The composition, mass coverage, and organization of SAMs formed by μCP were studied by AFM [147]. Direct data from these studies indicate that the SAMs formed by μCP are usually a complex mixture of phases but can reach a state of organization that is spectroscopically indistinguishable from SAMs formed by adsorption from solution. Nevertheless, the quality of the μCP is greatly depending on the concentration of the alkanethiols solution, the pressure applied to the stamp and duration of the contact time.

Not only small molecules, but also proteins and peptides can be deposited on a substrate surface by microcontact printing (μ CP)[148] [149].

4.3 Imaging protein and protein complexes by AFM

Basically the strategy remains the same that when imaging one protein. A protein is immobilized on a surface and first imaged by AFM. The height of the immobilized protein is accurately measured. The same surface is incubated with a solution containing a protein partner. If the surface is designed with a background to avoid unspecific interaction (i.e. ethylene glycol), a mixture of protein can be incubated. The protein partner interact with the protein immobilized and the surface is washed carefully to remove any contaminants (or unbound proteins) and immediately analyzed by AFM. As a result, the binary and tertiary complexes of partner proteins are revealed based on the increase object's height upon complex formation (see Fig.II.15). The use of an antibody-antigen system in the characterization of these protein interactions brings additional information regarding the specificity of the interaction.

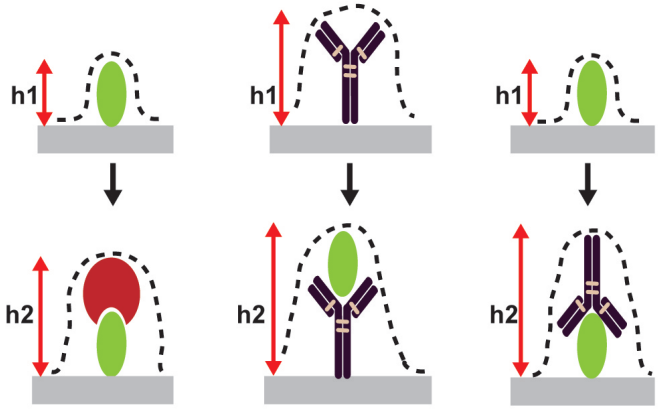


Figure II.15: Schematic representation of AFM measurements to visualize protein-protein interactions; the system can be formed by either protein-protein or antibody-antigen interactions.

An interesting application is to perform in-situ binding studies. Indeed, under liquid con-

[147] M Fujihira, M Furugori, U Akiba, and Y Tani. *Ultramicroscopy*, 86(1-2):75–83, 2001.

[148] JP Renault, A Bernard, D Juncker, B Michel, HR Bosshard, and E Delamarche. *Angew Chem Int Ed Engl*, 41(13):2320–2323, 2002.

[149] J Foley, H Schmid, R Stutz, and E Delamarche. *Langmuir*, 21(24):11296–11303, 2005.

ditions, AFM allows to monitor protein-protein interaction studies in-situ and in real time [150]. The principle is based on using the liquid cell as an incubator where the liquid can be injected into or removed from the liquid cell without perturbation of the system, which means without removing the AFM tip or moving the surface (see Fig.II.16). In situ studies give informations about protein-protein reaction kinetics, as well as information regarding the stability in time of the complex formed.

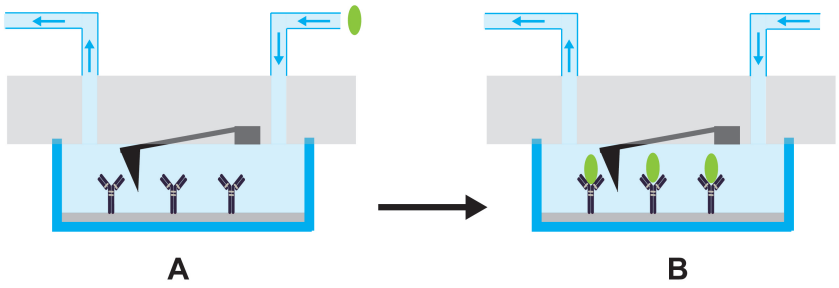


Figure II.16: Schematic representation of an experimental set-up to perform in-situ AFM studies under liquid conditions; liquid can be injected into or removed from the liquid cell without perturbation to the system.

4.4 Force measurements

In addition to high-resolution imaging of proteins, nucleotides, membranes, and living cells, the force measurements at the molecular level provides details insights into the function and structure of biomolecular system.

A typical application, is the study for instance of protein membranes and lipid bilayers. A jump in the approaching force curve can be detected when the tip penetrates the membrane. The force measured is the maximum force that can resist the membrane without breaking [151][152].

Intermolecular forces, between tip-bound ligands and surface-immobilized receptors, can be study by AFM. The tip approaches to the surface and is subsequently withdrawn, while the bending (i.e. deflection) of the cantilever is recorded. The bending of the lever, which is proportional to the force, is plotted versus tip-surface separation (i.e. distance). Upon retraction of the cantilever, and in the case there is a binding between the ligand and the receptor, the physical connection between tip and surface exerts an increasing force until the ligand-receptor complex abruptly dissociates at a certain critical force, termed the unbinding force. Specific

[150] ME Browning-Kelley, K Wadu-Mesthridge, V Hari, and Liu G Y. Atomic force microscopic study of specific antigen/antibody binding. *Langmuir*, 13:343–350, 1997.

[151] V Franz, S Loi, H Muller, E Bamberg, and H H Butt. *Colloids Surf. B: Biointerfaces*, 23:191–200, 2002.

[152] S Garcia-Manyes, O Domenech, F Sanz, M T Montero, and J Hernandez-Borrell. *Biochim Biophys Acta*, 1768(5):1190–1198, 2007.

recognition signal in the retraction curve can be recorded (see Fig.II.17B).

For studying intramolecular forces, a common method of mechanical manipulation by AFM is to pull a single molecule by a tip attached to a cantilever and probe the effect of stretch forces on protein stabiility. The retraction of the tip induces an unfolding of the suspended molecule [153] [154]. Specific signals can be recorded on the force distance curve (see Fig.II.17C).

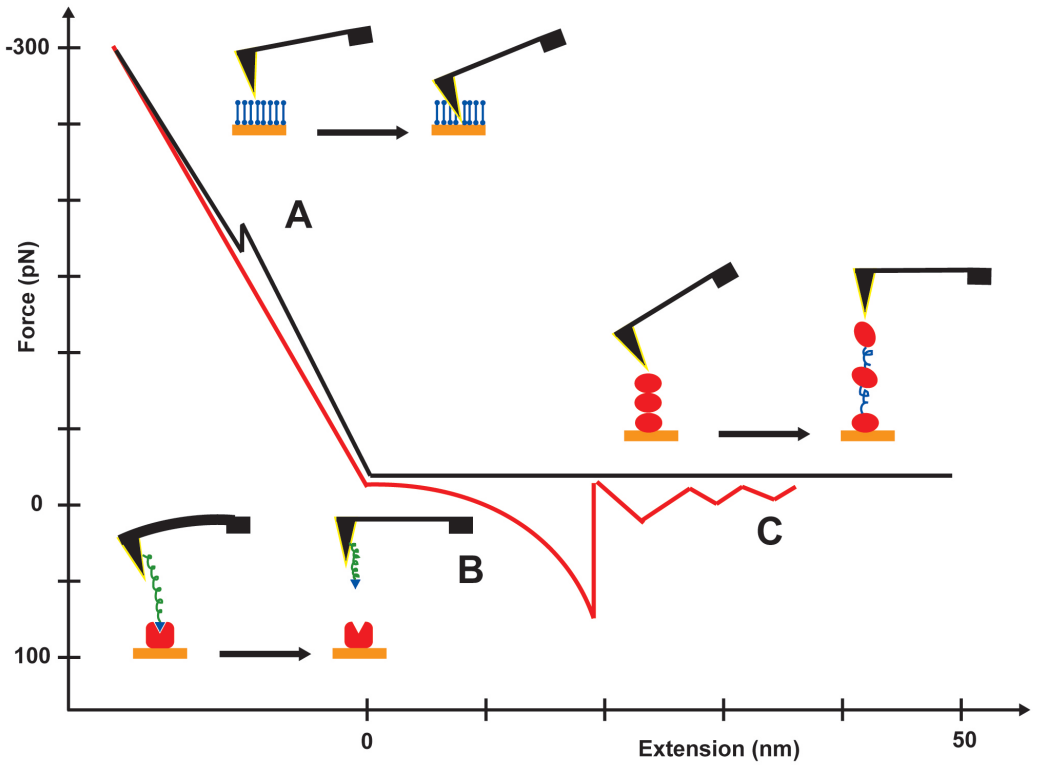


Figure II.17: Schematic representation of force curve with 3 areas of force measurement study; (A); (B)intermolecular forces (ligand-protein); (C) intramolecular forces (unfolding).

By combination of imaging with force measurements, receptor sites on a surface are localized with nanometer positional accuracy [155] [156] [157]. This technique can map composition on top of topographical image and can detect compositional changes occurring during biological process. This simultaneous investigation of both topography and recognition will probably open a wide field of applications for investigating biological structure-function relationships in native environments on the nanometer scale.

[153] B Samori, G Zuccheri, and R Baschieri. *Chemphyschem*, 6(1):29–34, 2005.

[154] S Furuike, T Ito, and M Yamazaki. *FEBS Lett*, 498(1):72–75, 2001.

[155] CM Stroh, A Ebner, M Geretschlager, G Freudenthaler, F Kienberger, AS Kamruzzahan, SJ Smith-Gill, HJ Gruber, and P Hinterdorfer. *Biophys J*, 87(3):1981–1990, 2004.

[156] A Ebner, F Kienberger, G Kada, CM Stroh, M Geretschlager, AS Kamruzzahan, L Wildling, WT Johnson, B Ashcroft, J Nelson, SM Lindsay, HJ Gruber, and P Hinterdorfer. *Chemphyschem*, 6(5):897–900, 2005.

[157] CM Stroh, A Ebner, M Geretschlager, G Freudenthaler, F Kienberger, AS Kamruzzahan, SJ Smith-Gill, HJ Gruber, and P Hinterdorfer. *Biophys J*, 87(3):1981–1990, 2004.

Objectives

This thesis has as a general objective the structural study of proteins and protein interactions by mass spectrometry and atomic force microscopy.

In order to accomplish this general goal, the work has been divided in five more focused objectives:

1. To develop a strategy to study and identify the folded domain of a protein by coupling limited proteolysis experiments with mass spectrometry analysis (see Chapter I, page 47).

2. To identify a mutant of the hemoglobin beta chain by the combination of liquid chromatography, mass spectrometry and mass spectrometry in tandem (see Chapter II, page 61).

3. To evaluate the ability of a synthetic beta-tetrapeptide to self-assemble in solution by atomic force microscopy (see Chapter III, page 71).

4. To develop a beta-amyloid functionalized gold surface that is bioactive and compatible with atomic force microscopy and mass spectrometry for the study of protein-protein interactions (see Chapter IV, page 77).

5. To study a specific protein-protein interaction on a functionalized gold surface by proteomics (see Chapter V, page 97).

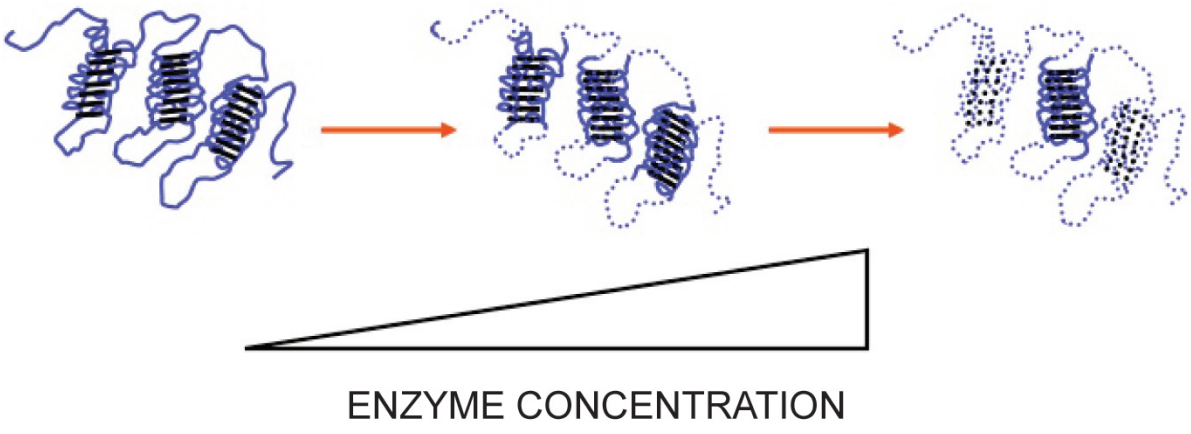
Results

Chapter I

Limited Proteolysis and Mass Spectrometry

Domain Architecture of the p62 Subunit from the Human Transcription/Repair Factor TFIIH Deduced by Limited Proteolysis and Mass Spectrometry Analysis

Biochemistry 2004, 43, 14420-14430



Domain architecture of the p62 subunit from the human transcription/repair factor TFIIH deduced by limited proteolysis and mass spectrometry analysis

Anass Jawhari, Stéphanie Boussert, Valérie Lamour, R. Andrew Atkinson, Bruno Kieffer, Olivier Poch, Noelle Potier, Alain van Dorsselaer, Dino Moras, and Arnaud Poterszman

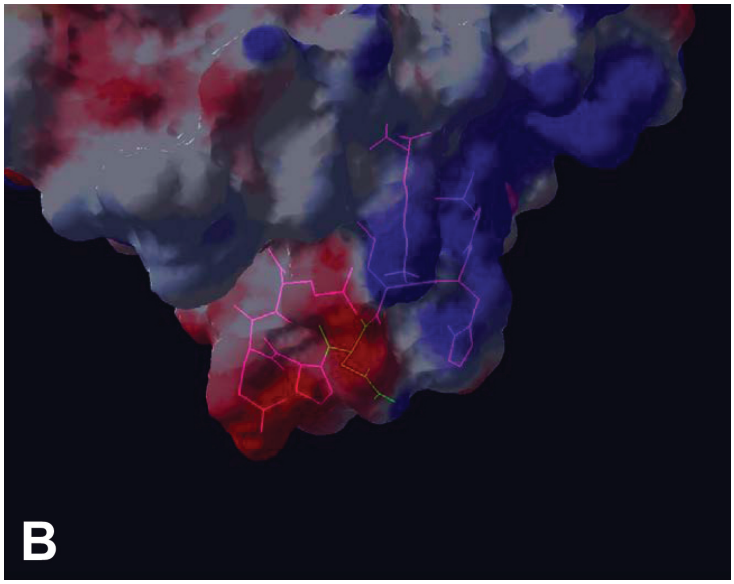
Biochemistry, 2004, Vol. 43, No. 45, Pages 14420 -14430

Chapter II

Human Hemoglobin and Mass Spectrometry

Characterization of hemoglobin Wurzburg α_2 β_2 4(A1)Thr - Asn), a new electrophoretically silent variant, by mass spectrometry and molecular modeling studies

Journal of Chromatography A, 1115 (2006) 118124



Characterization of hemoglobin Würzburg (α2ß2 4(A1)Thr →Asn), a new electrophoretically silent variant, by mass spectrometry and molecular modeling studies

Emmanuel Bissé, Nathalie Zorn, Stéphanie Boussert, Thomas Epting, Alain Van Dorsselaer, Jürgen Horst, Manfred Baumstark, Heinrich Wieland

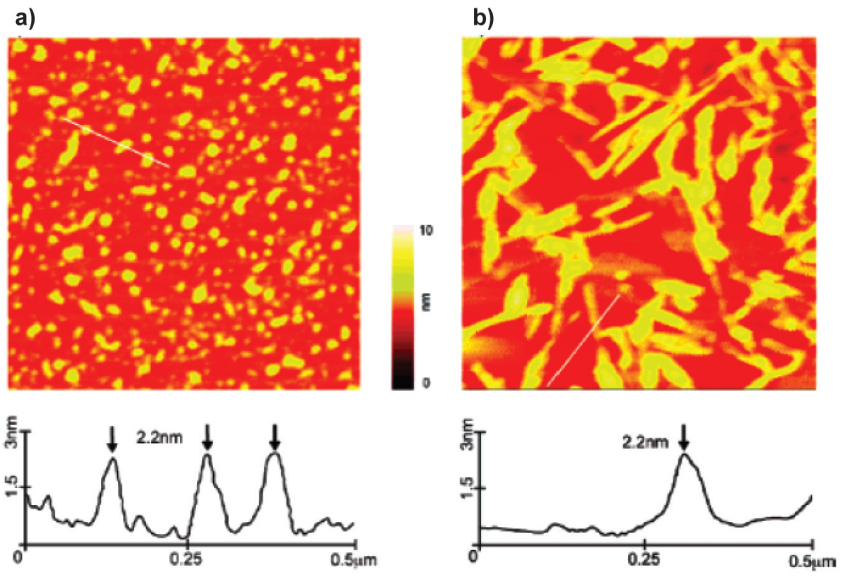
Journal of Chromatography A, 2006, Vol. 1115, Numéro 1-2, Pages 118-124

Chapter III

β-peptide and Atomic Force Microscopy

Self-Assembly of a Cyclobutane β-Tetrapeptide To Form Nanosized Structures

Organic Letters 2007 Vol. 9, No. 18 3643-3645



Self-assembly of a cyclobutane β-tetrapeptide to form nanosized structures

Federico Rùa, Stéphanie Boussert, Teodor Parella, Ismael Dìez-Pérez, Vicenç Branchadell, Ernest Giralt, and Rosa M. Ortuño

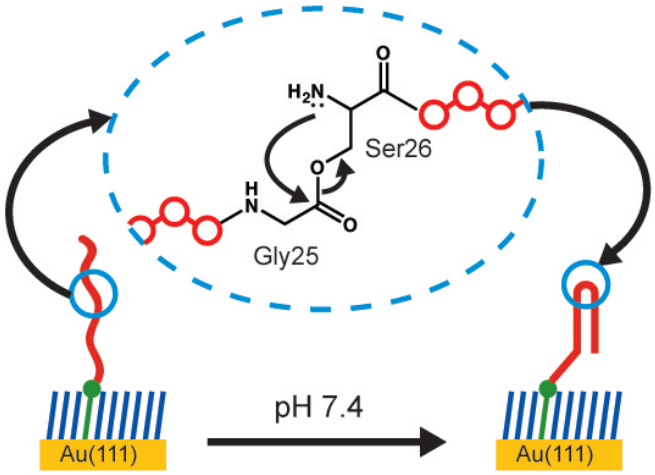
Organic Letters, 2007, Vol. 9, Numéro 18, Pages 3643 -3645

Chapter IV

β-Amyloid Peptide and Atomic Force Microscopy

An intramolecular O-N migration reaction on gold surfaces: toward the preparation of well-defined amyloid surfaces

in preparation



An intramolecular O-N migration reaction on gold surfaces: toward the preparation of well-defined amyloid surfaces

Stephanie Boussert, Ismael Diez-Perez, Marcelo Kogan,
Eliandre de Oliveira, Ernest Giralt.

in preparation

Amyloid is a family of self-aggregating proteins that are related to a variety of central nervous system disorders, including Alzheimers disease (AD). Neuritic plaques, which mainly contain insoluble aggregates of the beta amyloid peptide (Aβ), and neurofibrilary tangles of abnormally phosphorylated Tau proteins are the two most distinctive brain pathologies of AD [1].Cleavage of the beta amyloid precursor protein (APP) by proteases such as beta-secretase and gamma-secretase produces a predominant product of 40 amino acids residues: beta amyloid peptide (Aβ40) [2]. Other shorter or longer Aβ species are also produced, including a fibrilogenic peptide of 42 amino acids (Aβ42). The deposition of Aβ40 and Aβ42 into cerebral plaques begins with nucleation of soluble Aβ42 into fibrils, followed by accumulation of soluble Aβ40 [3]. It is thought that pre-fibrilar soluble forms of amyloid peptides, including oligomers, may be the main pathogenic factor in AD [4], [5], [6], [7].

At certain crucial concentrations, Aβ peptides strongly tend to aggregate and form fibrils. These concentrations vary with peptide structure and medium conditions. Moreover, an ensemble of different aggregate states that fall between nucleates and fibrils, called *protofibrils*, are present in solution. This heterogeneity in the assembly states of Aβ peptides and their aggregating behavior complicate in-solution studies such as structure determination and characterization of protein-protein interactions involving Aβ . Therefore, the development of a technique to obtain intact and soluble Aβ monomers under physiological conditions is crucial for being able to study their role in AD pathogenesis.

Solid phase peptide synthesis (SPPS) is a known technique pioneered by Bruce Merrifield [8] to obtain peptide in small-medium amounts. Nevertheless, peptides which are strongly hydrophobic or that tend to aggregate can be difficult to synthesize on solid-phase. One of the methods proposed to solve this problem included the use of special building blocks during the synthesis [9]. More recently, Sohma, *et al.* showed that using an O-acyl residue instead of an N-acyl residue in the peptide sequence could overcome the solubility problems of large peptides with difficult sequences [10]. The resulting O-acyl isopeptide can then be easily converted,

under physiological conditions (pH 7.4), to the native peptide via an intramolecular O-N acyl migration reaction. Because of the convenient SPPS reaction conditions, this last method was chosen to synthesize the soluble Aβ40 we used in the present work.

Typically, studies involving protein-protein interactions are done in-solution. Nevertheless, the employment of functionalized surfaces such microarrays and microchips have been a surge for the analysis of the activities and interactions of proteins and their complexes [11], [12], [13], [14]. Self-assembled monolayers (SAMs) are highly effective for modifying surfaces for these applications [15] [16]. They enable the immobilization of macromolecules, including DNA [17], proteins [18],[19] and cells [20], [21]. The SAMs of alkanethiols on gold (111) are probably the most studied class and provide a dense, well-defined and readily functionalizable monolayer [22]. For instance, alkanethiols can be modified with oligoethylene or polyethylene glycol (PEG) groups, which are widely used to generate inert surfaces, since they confer high resistance to nonspecific adsorption of biomolecules or cells to the surfaces [23],[24]. However, the synthesis of PEGylated alkanethiols is challenging by conventional organic chemistry [16].

The combination of SAM and matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS), named SAMDI-TOF MS, is a recent and a powerful analytical tool for characterizing surfaces. It has been employed for enzymatic activity assays [25], screening of peptides librairies [26] or characterization of products generated by electrochemical reactions [27]. This technique is also very useful for verifying immobilization of a ligand on a surface [28]. Individual proteins and their complexes can also be detected and characterized on surface and under physiological conditions by other biophysical approaches such as atomic force microscopy (AFM) [29], [30], [31].

In a first step for characterizing Aβ surfaces and directly observing the Aβ peptide-antibody complex, we report here the solid phase synthesis of Aβ40 isopeptide, a non-aggregating precursor form of the amyloid peptide, its immobilization onto a gold surface using a mixed SAM of penta-PEGylated alkanethiols, and subsequent on-surface intramolecular O-N acyl migration to convert the Aβ40 isopeptide. Two complementary techniques, AFM and MS, were used to characterize the functionalized gold surface and to follow the interaction between the Aβ and a model protein.

The Aβ40 was synthesized via Fmoc strategy of SPPS using the O-Acyl isopeptide method [32]. The main advantage when using this method is the improvement of the Aβ40 isopeptide solubility in comparison with native Aβ40. The obtention of a soluble product allowed chromatographic purification and further in-solution studies. For specific attachment of Aβ40 to our SAM we added an N-terminal cysteine to the peptide sequence (Fig. 1). We reasoned that, since the N-terminus of the amyloid peptide sequence is structurally disordered, then surface attachment via this terminus would not disrupt peptide folding.

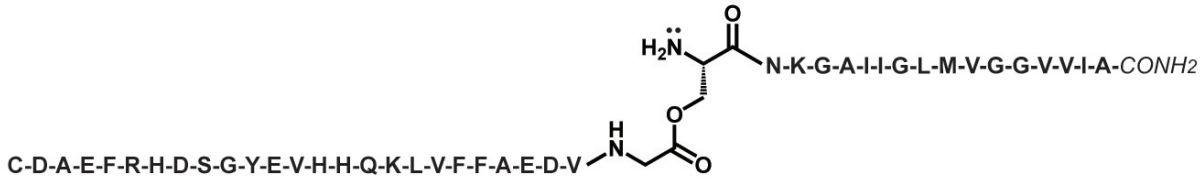


Figure 1: βamyloid 40 isopeptide

The construction of the functionalyzed surface was done in three steps. In the first one we covered a gold surface with SAMs of two penta-PEGylated alkanethiols, one of which contained a binding site for the cystein-containing Aβ40 isopeptide (Cys-Aβ40 isopeptide) (see Fig 2). Synthesis of these alkanethiols was greatly simplified on solid phase and only two or three synthesic steps were required (depending on the incorporation of the peptide binding site). Formation of the desired SAM was done by the incubation of a mixed solution of these two modified alkanethiols on the gold surface. In the second step, the free amino group of the lysine side chain was reacted with a bi-functional 3-(maleimido)propionic acid N-hydrosuccinimide ester linker [28].

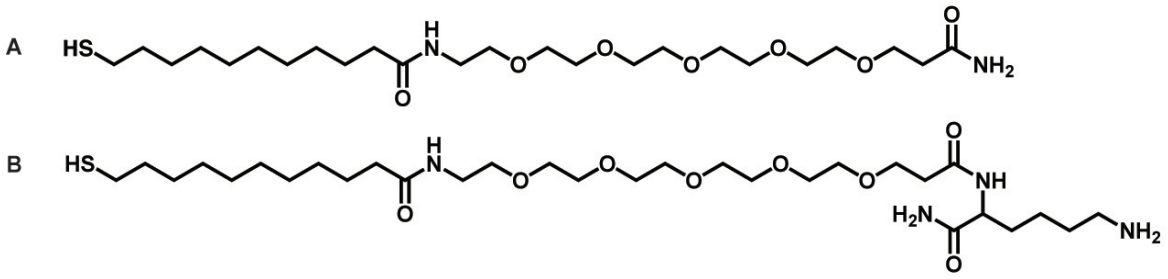


Figure 2: Alkanethiols

Finally, the Cys-Aβ40 isopeptide was immobilized to the SAM in acid solution to prevent the intramolecular O-N acyl migration reaction and then enable surface binding of the Aβ40 isopeptide, primarily in monomeric form. Following immobilization of the Aβ40 isopeptide, the surface was washed and incubated with phosphate buffer saline (PBS, pH 7.4) in order to remove traces of acid solution on the chip and leave the Aβ40 in physiological conditions. Moreover, the incubation with PBS allowed the ON-N acyl migration reaction (see Fig. 3). The surface was then immediately analyzed by AFM.

AFM analysis showed a background of a compact and dense monolayer corresponding to the adsorption of the mixed SAM. We also observed the presence of many bright spots on the surface corresponding to the attached Aβ40 (see Fig.5B2). The measured height of each bright spots was ca. 6 nm (see Fig.5A3). As the theoretical length of the Cys-Aβ40 in a single β-strand would be 13.4 nm, this result suggests that our Cys-Aβ40 adopts a β-hairpin conformation and

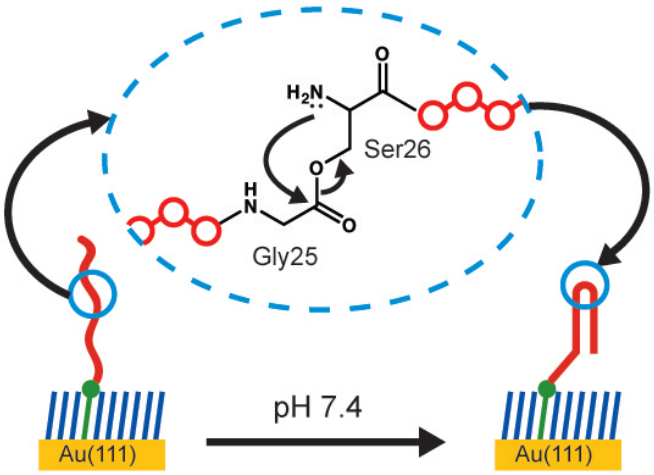


Figure 3: O-N intramolecular migration reaction on the functionalyzed gold surface.

that this on-surface version of the O-N intramolecular acyl migration reaction was successful. The experimental height is in good accordance with the dimensions given in the structural model for Aβ40 molecules (6 nm) [33]. The homogeneity in the measured heights of the bright spots confirm our hypothesis that the Aβ40 is mainly attached to the surface in monomeric form (see Fig.5A1). Moreover, the homogenous distribution of the spots on the surface without segregation into islands indicates that the lysine amino group side chain, present in one of the modified alkanethiols, does not interfere with the previous organization of the SAM. The same functionalized gold surface was also characterized by MS. We were able to directly confirm the presence of the Aβ40 on the surface using SAMDI-TOF-MS (see Fig.4).

We then performed a protein-protein interaction assay directly on the surface, which was tracked by AFM and chemiluminescence. AFM is advantageous in its ability to capture images of biological samples in solution and thus preserve the native structure of protein complexes.

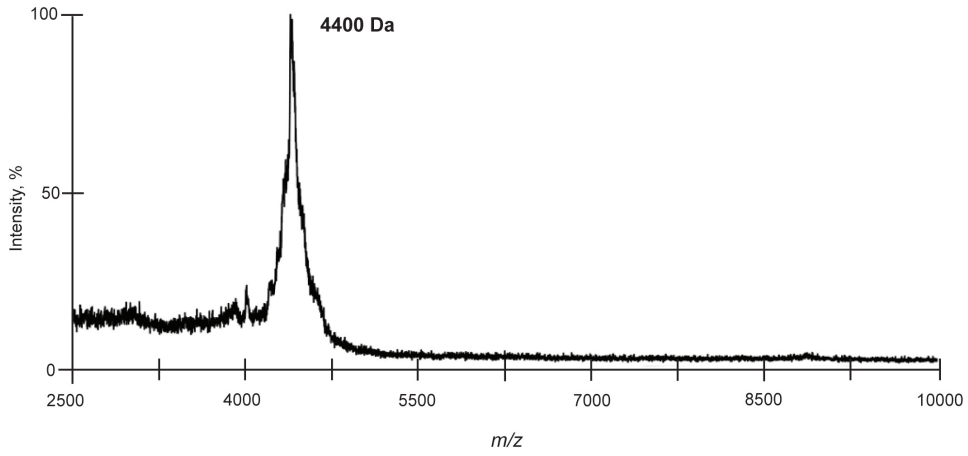


Figure 4: SAMDI-TOF-MS spectra of the βA functionalyzed gold surface.

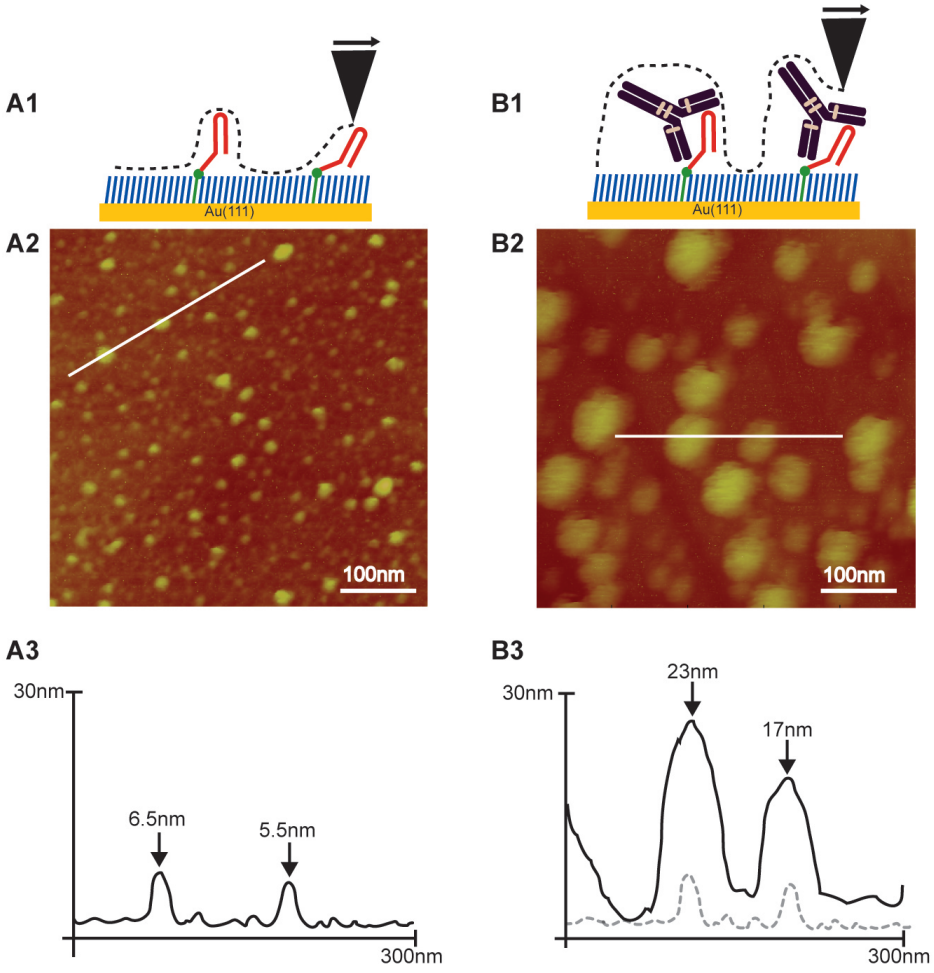


Figure 5: (A) AFM of the Aβ functionalyzed gold surface before incubation with an anti βA antibody, (B) AFM of the Aβ functionalyzed gold surface after incubation with an anti Aβ antibody.

Our Aβ40 functionalized gold surface was incubated with an anti-Aβ monoclonal antibody (anti-Aβ) described to react with amino acid residues 1-17 of human Aβ. AFM measurements showed a clear change in the surface morphology after antibody incubation (see Fig.5B2).

Topographic profiles revealed heights from 17 nm to 23 nm (see Fig.5B3), which indicate the formation of an Aβ40/anti-Aβ complex. The measurements are in close agreement with dimensions of IgG antibodies [34], [35]. All the AFM measurements were done in liquid conditions, which allow free rotation of the Aβ on the surface. Therefore, the range of heights observed suggests that the highest and lowest spots on the AFM images correspond to antigen/antibody complexes in standing pose and in lying pose, respectively (see Fig.5B1). This Aβ40-anti-Aβ interaction was also directly observed on the surface by chemiluminescence. After incubation with a secondary antibody labeled with peroxidase, chemiluminescence was detected on autoradiography film, indicating the presence of anti-Aβ (see Fig.6A)

To confirm the specificity of the antigen/antibody interaction on surface, an identical Aβ40 surface was incubated with a secondary antibody labeled with peroxidase enzyme. The AFM

images resulting from the analysis of the surface did not show any evidence of the formation of non-specific complex Aβ/secondary antibody (see Fig.6B). Moreover, no resulting light was detected by chemiluminescence.

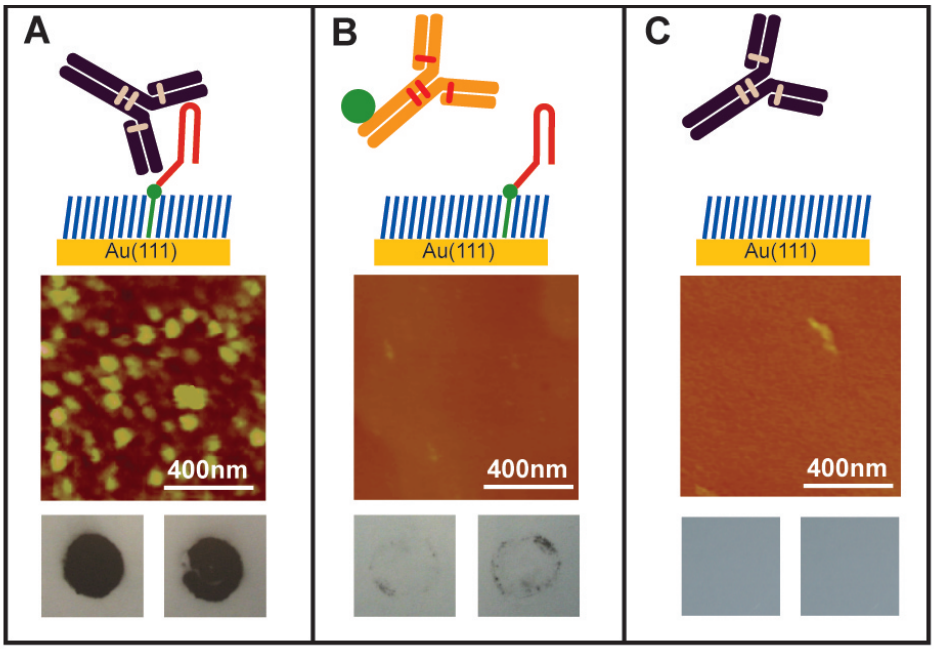


Figure 6: AFM and chemiluminescence reactions; (A) Aβ functionalized gold surface after incubation with an anti Aβ antibody was imaged by AFM, resulting light was detected after incubation with a peroxydase labeled antibody and chemiluminescence reaction indicating the presence of the anti Aβ antibody; (B) Aβ gold surface after incubation with a peroxydase labeled antibody was imaged by AFM, no resulting light was detected by chemiluminescence reaction; (C) penta-PEGylated alkanethiol gold surface after incubation with aAβ antibody was imaged by AFM, no resulting light was detected by chemiluminescence reaction.

One of the crucial points in our study was to avoid non-specific interactions with the modified surface and be sure that the interactions we observed were specific. A monolayer surface containing only the penta-EGylated alkanethiol (1-[11-mercaptoundecanamido]-3,6,9,12,15-pentaoxaoctadecan-18-amide) was incubated with anti-Aβ, and as expected, no non-specific interactions between antibody and the monolayer were detected by either AFM or chemiluminescence (see Fig.6C). These observations validated the ability of our penta-EGylated alkanethiols to prevent non-specific adsorption of proteins onto the surface.

In summary, the methodology reported here represents an important step towards the preparation of well-defined Aβ peptide-functionalized surfaces and opens the way to general preparation of surfaces modified with other proteins that have a strong tendency to aggregate. We have shown that our Aβ surface is functionally active and is compatible with AFM and MS techniques for tracking protein-protein interactions. Having the support of both AFM and MS data adds to the robustness of the methodology. We believe that the amenability of the sur-

faces prepared here will be invaluable to the future study of specific protein-protein interactions by proteomics MS studies.

References

[1] D J Selkoe. Alzheimer's disease: genes, proteins, and therapy. *Physiol Rev*, 81(2):741–766, 2001.

[2] Michael S Wolfe. Therapeutic strategies for alzheimer's disease. *Nat Rev Drug Discov*, 1(11):859–866, 2002.

[3] Catherine A Rottkamp, Craig S Atwood, James A Joseph, Akihiko Nunomura, George Perry, and Mark A Smith. The state versus amyloid-beta: the trial of the most wanted criminal in alzheimer disease. *Peptides*, 23(7):1333–1341, 2002.

[4] Sergio T Ferreira, Marcelo N N Vieira, and Fernanda G De Felice. Soluble protein oligomers as emerging toxins in alzheimer's and other amyloid diseases. *IUBMB Life*, 59(4-5):332–345, 2007.

[5] Charles C Glabe. Amyloid accumulation and pathogensis of alzheimer's disease: significance of monomeric, oligomeric and fibrillar abeta. *Subcell Biochem*, 38:167–177, 2005.

[6] Christian Haass and Dennis J Selkoe. Soluble protein oligomers in neurodegeneration: lessons from the alzheimer's amyloid beta-peptide. *Nat Rev Mol Cell Biol*, 8(2):101–112, 2007.

[7] Dominic M Walsh and Dennis J Selkoe. A beta oligomers - a decade of discovery. *J Neurochem*, 101(5):1172–1184, 2007.

[8] R B Merrifield. Solid phase peptide synthesis. i. the synthesis of a tetrapeptide. *J Am Chem Soc*, 85(11):2149–2154, 1963.

[9] M Mutter, A Nefzi, T Sato, X Sun, F Wahl, and T Wohr. Pseudo-prolines (psi pro) for accessing "inaccessible" peptides. *Pept Res*, 8(3):145–153, 1995.

[10] Youhei Sohma, Yoshio Hayashi, Mariusz Skwarczynski, Yoshio Hamada, Masato Sasaki, Tooru Kimura, and Yoshiaki Kiso. O-n intramolecular acyl migration reaction in the development of prodrugs and the synthesis of difficult sequence-containing bioactive peptides. *Biopolymers*, 76(4):344–356, 2004.

[11] T Kodadek. Protein microarrays: prospects and problems. *Chem Biol*, 8(2):105–115, 2001.

[12] D J Cahill. Protein and antibody arrays and their medical applications. *J Immunol Methods*, 250(1-2):81–91, 2001.

[13] David S Wilson and Steffen Nock. Functional protein microarrays. *Curr Opin Chem Biol*, 6(1):81–85, 2002.

[14] Heng Zhu and Michael Snyder. Protein chip technology. *Curr Opin Chem Biol*, 7(1):55–63, 2003.

[15] M Mrksich and G M Whitesides. Using self-assembled monolayers to understand the interactions of man-made surfaces with proteins and cells. *Annu Rev Biophys Biomol Struct*, 25:55–78, 1996.

[16] J Christopher Love, Lara A Estroff, Jennah K Kriebel, Ralph G Nuzzo, and George M Whitesides. Self-assembled monolayers of thiolates on metals as a form of nanotechnology. *Chem Rev*, 105(4):1103–1169, 2005.

[17] A G Frutos, Q Liu, A J Thiel, A M Sanner, A E Condon, L M Smith, and R M Corn. Demonstration of a word design strategy for dna computing on surfaces. *Nucleic Acids Res*, 25(23):4748–4757, 1997.

[18] G B Sigal, C Bamdad, A Barberis, J Strominger, and G M Whitesides. A self-assembled monolayer for the binding and study of histidine-tagged proteins by surface plasmon resonance. *Anal Chem*, 68(3):490–497, 1996.

[19] W Muller, H Ringsdorf, E Rump, G Wildburg, X Zhang, L Angermaier, W Knoll, M Liley, and J Spinke. Attempts to mimic docking processes of the immune system: recognition-induced formation of protein multilayers. *Science*, 262(5140):1706–1708, 1993.

[20] M Mrksich, C S Chen, Y Xia, L E Dike, D E Ingber, and G M Whitesides. Controlling cell attachment on contoured surfaces with self-assembled monolayers of alkanethiolates on gold. *Proc Natl Acad Sci U S A*, 93(20):10775–10778, 1996.

[21] B T Houseman and M Mrksich. The microenvironment of immobilized arg-gly-asp peptides is an important determinant of cell adhesion. *Biomaterials*, 22(9):943–955, 2001.

[22] C Vericat, M E Vela, and R C Salvarezza. Self-assembled monolayers of alkanethiols on au(111): surface structures, defects and dynamics. *Phys Chem Chem Phys*, 7(18):3258–3268, 2005.

[23] K L Prime and G M Whitesides. Self-assembled organic monolayers: model systems for studying adsorption of proteins at surfaces. *Science*, 252(5010):1164–1167, 1991.

[24] R R Siegel, P Harder, R Dahint, M Grunze, F Josse, M Mrksich, and G M Whitesides. On-line detection of nonspecific protein adsorption at artificial surfaces. *Anal Chem*, 69(16):3321–3328, 1997.

[25] Dal-Hee Min, Jing Su, and Milan Mrksich. Profiling kinase activities by using a peptide chip and mass spectrometry. *Angew Chem Int Ed Engl*, 43(44):5973–5977, 2004.

[26] Dal-Hee Min, Wei-Jen Tang, and Milan Mrksich. Chemical screening by mass spectrometry to identify inhibitors of anthrax lethal factor. *Nat Biotechnol*, 22(6):717–723, 2004.

[27] Woon-Seok Yeo and Milan Mrksich. Electroactive substrates that reveal aldehyde groups for bio-immobilization. *Advanced Materials*, 16:1352–1356, 2004.

[28] B T Houseman, Gawalt E S, and M Mrksich. Maleimide-functionalyzed self-assembled monolayers for the preparation of peptide and carbohydrate biochips. *Langmuir*, 19:1522–1531, 2003.

[29] J Yang, J Mou, and Z Shao. Molecular resolution atomic force microscopy of soluble proteins in solution. *Biochim Biophys Acta*, 1199(2):105–114, 1994.

[30] Simon Scheuring. Afm studies of the supramolecular assembly of bacterial photosynthetic core-complexes. *Curr Opin Chem Biol*, 10(5):387–393, 2006.

[31] Simon Scheuring, Thomas Boudier, and James N Sturgis. From high-resolution afm topographs to atomic models of supramolecular assemblies. *J Struct Biol*, 159(2):268–276, 2007.

[32] Youhei Sohma, Yoshio Hayashi, Maiko Kimura, Yousuke Chiyomori, Atsuhiko Taniguchi, Masato Sasaki, Tooru Kimura, and Yoshiaki Kiso. The 'o-acyl isopeptide method' for the synthesis of difficult sequence-containing peptides: application to the synthesis of alzheimer's disease-related amyloid beta peptide (abeta) 1-42. *J Pept Sci*, 11(8):441–451, 2005.

[33] Aneta T Petkova, Yoshitaka Ishii, John J Balbach, Oleg N Antzutkin, Richard D Leapman, Frank Delaglio, and Robert Tycko. A structural model for alzheimer's beta -amyloid fibrils based on experimental constraints from solid state nmr. *Proc Natl Acad Sci U S A*, 99(26):16742–16747, 2002.

[34] Jerry Yang, Michael Mayer, Jennah K Kriebel, Piotr Garstecki, and George M Whitesides. Self-assembled aggregates of iggs as templates for the growth of clusters of gold nanoparticles. *Angew Chem Int Ed Engl*, 43(12):1555–1558, 2004.

Material and Method, Supporting Informations

1 Abbreviations used:

AA, amino-acid;

AAA, amino-acid analysis;

Boc-AA, N-alpha-tert-butyloxycarbonylamino acid;

DCM, dichloromethane;

DIEA, diisopropylethyl amine

DMAP, 4-(N,N-dimethylamino) pyridine;

DMF, dimethylformamide;

DIPCDI, 1,3-diisopropylcarbodiimide;

EDT, ethane-1,2-dithiol;

EtOAc, ethyl acetate;

EtOH, ehtanol;

Fmoc-AA, N-fluorenylmethyloxycarbonylamino acid;

HCl, hydrogem chloride;

Hex, hexane;

HOBT, N-hydroxybenzotriazole;

HPLC, high-pressure liquid chromatography;

IgG, immunoglobulin G;

Lys, lysine-amino-acid;

MeCN, acetonitrile;

MeOH, methanol;

MALDI-MS, matrix-assisted laser desorption ionization mass spectrometry;

$MgSO_4$, magnesium sulfate;

MS, mass spectrometry;

PBS, phosphate buffered saline;

TBTU, 1-[bis(dimethylamino)methylene]-1H-benzotriazolium hexafluorophosphate 3 oxide;

RP-HPLC, reverse phase high-pressure liquid chromatography;

SAM, self-assembled monolayer;

SAMDI, self-assembled for matrix assisted laser desorption/ionization;

TFA, triflouroacetic acid;

TIS, triisopropylsilane;

2 Material:

PBS; 11-bromoundecanoic acid; sodium ethanolate; triphenylmethanethiol; alpha-cyano-4-hydroxycinnamic acid; and monoclonal anti human β-amyloid antibody 6E10, purified from mouse Igg1, were purchased from Sigma-Aldrich (Steinheim, Germany).

Protected amino acids, resins and HOBT were supplied by Neosystem (Strasbourg, France), Calbiochem-Novabiochem AG (Lafelfingen, Switzerland), Bachem AG (Budendorf, Switzerland) or Iris Biotech (Marktredwitz, Germany).

DIEA, DIPCDI, DMAP, TBTU, EDT, and 3-maleimidopropionic acid N-hydrosuccinimide ester were obtained from FlukaChemika (Buchs, Switzerland). TIS was obtained from AkrosPharma (Princeton, NJ, USA).

Solvents for peptide synthesis, RP-HPLC and analytical-HPLC were obtained from Scharlau or SDS (Barcelona, Spain). TFA was supplied by KaliChemie (Bad Wimpfen, Germany).

The gold (Au[111)]) crystal surface was purchased from Mateck (Juelich Germany). Tween20, anti-mouse IgG1 secondary antibody horseradish peroxidase, detection reagents, and ECL western blot reagents were obtained from Amersham Biosciences (UK). Autoradiography films were purchased from Fuji Medical X-Ray Film (Dusseldorf, Germany).

Manual syntheses were performed in polypropylene syringes, each fitted with a porous polyethylene disk. Solvent and soluble reagents were removed by suction. Between synthetic steps, the resins were washed with DMF (3 x 1 min) and DCM (3 x 1 min) using 5 mL/g of resin per wash. During coupling, the mixture was allowed to react with intermittent manual stirring. The Kaiser colorimetric assay was used for the detection of solid phase-bound primary amines (ref: Kaiser, E.: Colescott, R. L. and Bossiger, C. I.; Anal. Biochem; 1970; 34; 595-598).

Analytical HPLC was run on a Waters Alliance 2695 equipped with a Waters 996 photodiode array detector (Waters, Milford, MA, USA) using a Symmetry C18 column (4.6 mm x 150 mm x 5 μm), H_2O (0.045% TFA) and MeCN (0.036% TFA) as eluents (flow rate: 1 mL/min), and Millenium version 4.0 software.

Preparatice RP-HPLC was run on a Waters 600 with a double-wavelength UV detector using a Symmetry C18 column (100 x 30 mm x 5 μm, 300 Å), H_2O (0.1% TFA) and MeCN (0.05% TFA) as eluents (gradient: 15-60% MeCN in 30 min; flow rate: 20 mL/min).

MALDI-TOF mass spectra of all synthesized products were obtained on an Applied Biosystems 4700 Proteomics Analyzer (Applied Biosystems, Foster City, CA, USA). Mass spectra were acquired in the MS reflector positive-ion mode. Typical parameters were set to source and grid voltages of 20 kV and 14 kV, respectively; power laser from 5500 to 6000; signal to noise threshold of 5; and noise window width of 50.

SAMDI-MS of gold surfaces were performed on a Voyager DE-RP (Applied Biosystem) in positive linear mode with accelerating time of 25,000V; grid voltage of 92%; and extraction delay time of 300 nsec.

3 Synthesis of 11-(tritylthio)undecanoic acid:

The synthesis was based on synthesis J. Med. Chem. 2003, 46, 1989-1996; and Langmuir, Vol.20, No 21, 2004. To a solution of sodium ethanolate (21% in EtOH; 2 eq.) and triphenylmethanethiol (10 g in 50 mL of toluene; 1 eq.) was added 11-bromoundecanoic acid (9.5g dissolved in MeOH at 5-10C; 1 eq.). The solution was stirred at 50 ℃for 2 h (reflux), and then cooled to room temperature. It was acidified to pH 2 using 6M HCl, and then extracted with ethyl acetate (3 x 200 mL). The organic phase was washed with water (2 x 400mL), dried ($MgSO_4$), and filtered. The crude material was recrystallized, and then dissolved in EtOAc-Hex (1:4, v/v). This solution was frozen overnight at -20C, and then filtered to obtain the final product as a white solid. m/z 460 $[M + H]^+$. see Fig.1.

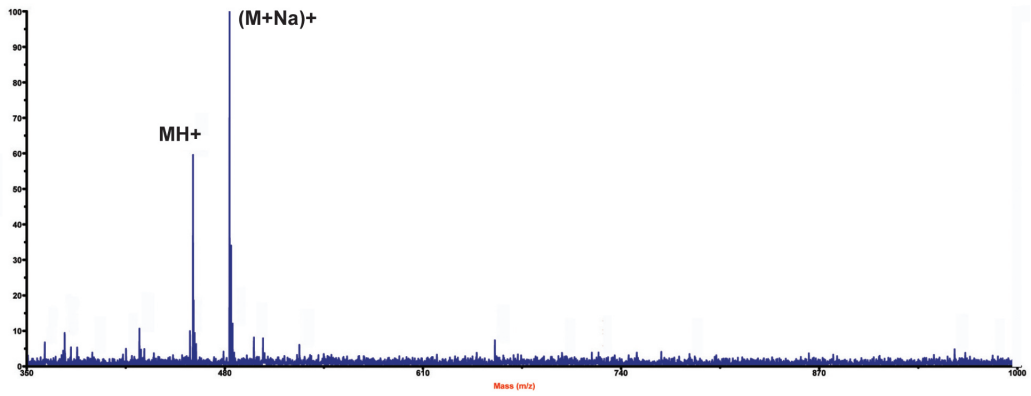


Figure 1: MS spectrum of 11-(tritylthio) undecanoic acid; Theoretical mass = MH+ 460 Da.

4 Synthesis of modified alkanthiols A and B:

(A) penta-PEGylated alkanethiol (1-(11-mercaptoundecanamido)-3,6,9,12,15-pentaoxaoctadecan-18-amide;

(B) the N-Lys-(penta-PEGylated) alkanethiol (N-(1,6-diamino-1-oxohexan-2-yl)-1-(11-mercaptoundecanamido)-3,6,9,12,15-pentaoxaoctadecan-18-amide) Resin Rink Amide-MBHA (o.6mmol/g).

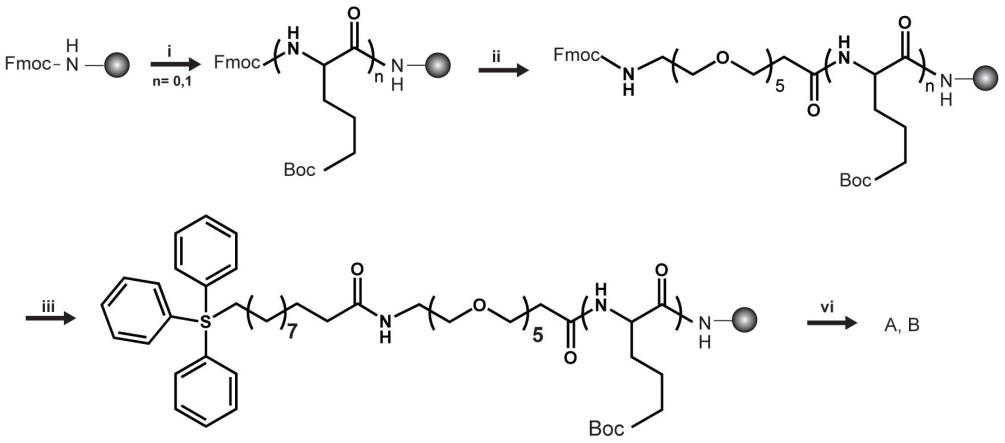


Figure 2: Scheme of the synthesis of the the penta-PEGylated alkanethiol (A, n=0) and the N-Lys-(penta-PEGylated) alkanethiol (B, n=1); (i) deprotection of the resin: 20% piperidine/DMF for 20min; coupling: Fmoc-Lys-(Boc)-OH (3 eq.), DIPCDI (3 eq.) and HOBT (3 eq.) in DMF for 1h 30 min; (ii) 20% piperidine/DMF for 20min; coupling: Fmoc-EG5-OH (2.5 eq.) (Neosystem, Strasbourg, France), TBTU (2.5 eq.), HOBT (2.5 eq.) and DIEA (5 eq.) in DMF for 2 h; deprotection: 20% piperidine/DMF, 20min; (iii) coupling: 11-(tritylthio)undecanoic acid (3 eq.), DIPCDI (3 eq.) and HOBT (3 eq.) in DMF for 10 to12 h; (iv) cleavage: mixture of TFA, EDT, H_2O and TIS (95:2:2:1) for 2 h.

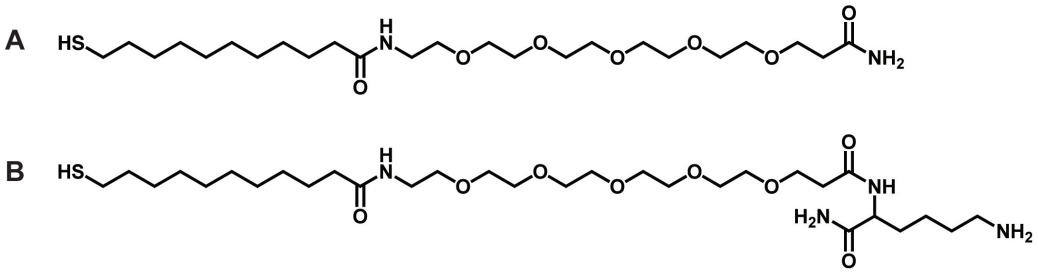


Figure 3: (A) penta-PEGylated alkanethiol; (B) N-Lys-(penta-PEGylated) alkanethiol

5 Synthesis of Cys-Aβ40 isopeptide:

AA40 was coupled manually to Rink-amide-MBHA (5 eq. Fmoc-AA, 1 eq. DMAP, and 5 eq. DIPCDI, 2 x 1 h). The remainder of the synthesis was then performed with an Applied Biosystems 433A automated synthesizer using FastMoc chemistry (10 eq. Fmoc-AA, 10 eq. TBTU, and 10 eq. HOBT),

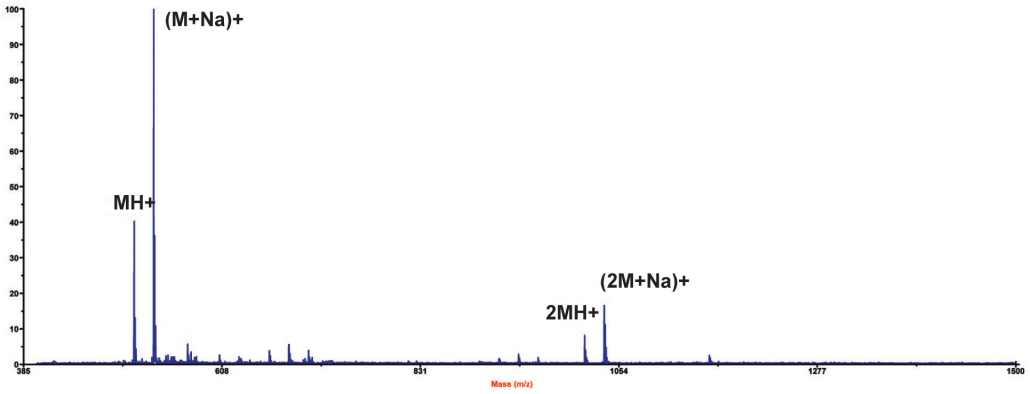


Figure 4: MALDI-MS spectrum of the penta-PEGylated alkanethiol; Theoretical mass = MH+ 509 Da

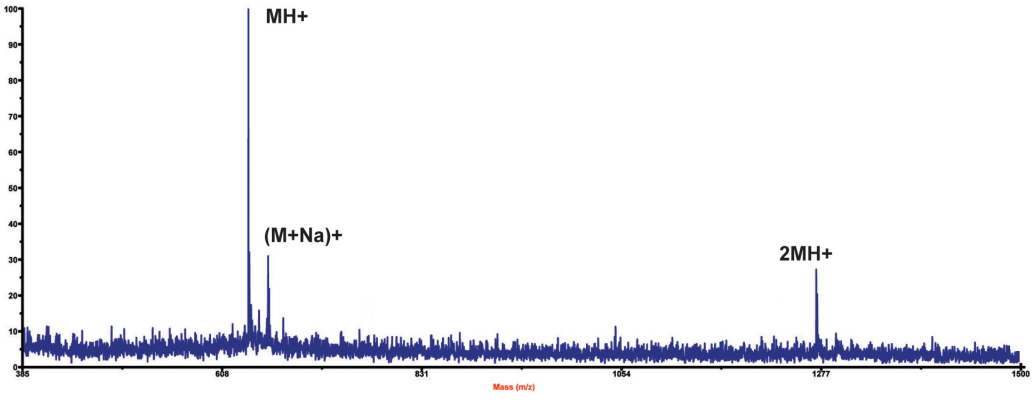


Figure 5: MALDI-MS spectrum of the N-Lys-(penta-PEGylated) alkanethiol; Theoretical mass = MH+ 637 Da.

except for AA26(3eq. Boc-Ser-OH, 4eq. HOBT, 4eq. DIPCDI, 1 h) and AA25(5eq. Fmoc-Gly, 1 eq. DMAP and 5 eq. DIPCDI for 1 x 10-12h, and then 1 x 1 h), which were coupled manually. The peptide was cleaved from resin with a mixture of TFA, H_2O, EDT and TIS (94.5:2.5:2.5:1) for 2 h, and then purified by RP-HPLC. The purified product was analyzed by analytical HPLC and MALDI-TOF MS.

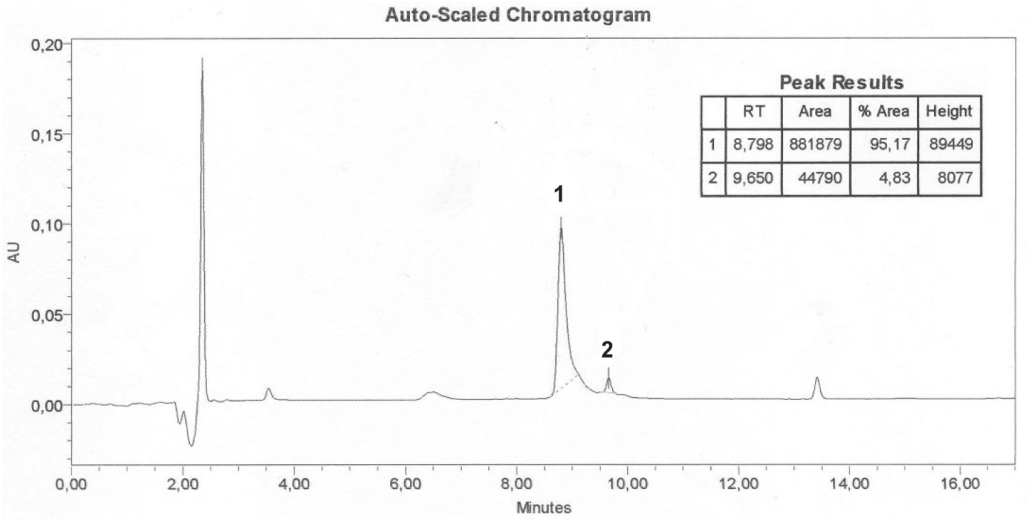


Figure 6: Analytical-HPLC chromatogram of the purified Cys-Aβ40 isopeptide

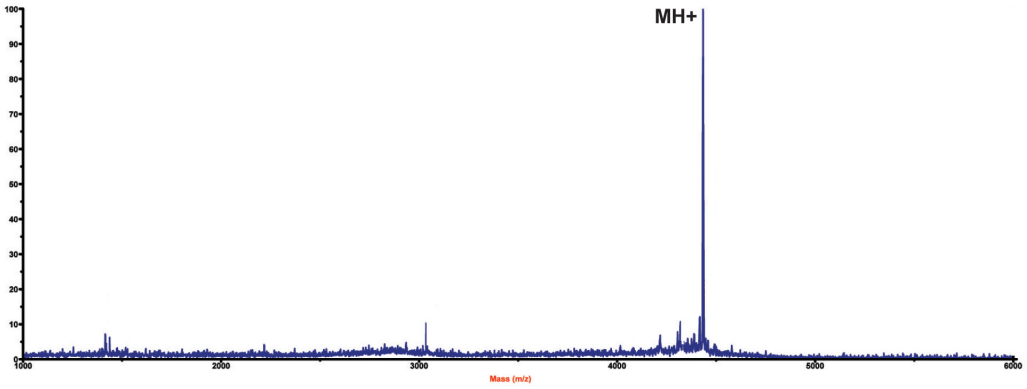


Figure 7: MS spectrum of the purified Cys-Aβ40 isopeptide; Theoretical mass = MH+ 4437 Da.

6 Preparation of the gold surface:

The crystalline gold (111) surface was cleaned and flattened using electrochemistry (45 seconds in 0.1 M H_2SO_4, voltage fixed at 10V, using an Au cathode and a Pt anode) whereby thick, hydrous gold (III) oxide was formed. The oxide was washed away using MQ H_2O followed by immersion in 1M HCl solution (1 min), to afford a fresh metallic gold surface. The surface as then washed again with MQ water before being annealed in a butane-propane flame (3 min) to increase the terrace size and remove any volatile contaminants. The surface was cooled under Ar for at least 15 minutes, and

then immediately used. After annealing, the surface exhibited atomically smooth gold (111) terraces separated by mono-atomic steps in height.

7 Functionalization of the gold:

A freshly prepared diluted solution of a mixture of the penta-PEGylated alkanethiol and the N-Lys-(penta-PEGylated) alkanethiol (1 μM in DMF, 65:35 [v/v]) was incubated on the freshly prepared gold surface for 14 h. The surface was then thoroughly rinsed with DMF to remove any unbound modified alkanethiols, dried under a strong stream of N_2, and then immediately incubated with a freshly prepared dilute solution of maleimide bi-functional group: 3-(maleimido)propionic acid N-hydrosuccinimide ester (10 μM in DMF, room temperature, 3 h 30 m, with agitation [200 rpm]). The surface was then rinsed thoroughly with DMF and dried under a strong stream of N_2. An acidic aqueous solution of Cys-Aβ40 (0.1 μM, 0.1% TFA) was added to the surface and incubated for 2 h at 4°C. The surface was then washed with an acid aqueous solution (0.1% TFA). Prior to AFM analysis, it was incubated for at least 30 min with PBS (pH 7.4). After AFM imaging, the Aβ functionalized surface was rinsed with PBS and incubated with a monoclonal anti human β-amyloid antibody (1 μL/60 μL PBS-[0.1%] Tween20, overnight, 4°C). After incubation, the gold surface was thoroughly rinsed with PBS-0.1% Tween (ca. 20 mL) and immediately analyzed by AFM.

8 AFM experiments:

Imaging was performed with a MultiMode instrument controlled by Nanoscope IV electronics (Digital Instruments, Santa Barbara, CA, USA) equipped with either a 12-m scanner (E-scanner) or a 120 m scanner (J-scanner). All images were recorded in solution (PBS) in tapping mode using a liquid cell without the O-ring seal.

9 MS experiments on gold surface:

Gold surface experiments: a saturated solution of alpha-cyano-4-hydroxycinnamic acid (5 mg/mL in 0.1% TFA/MeCN [1:1, v/v]) was deposited directly onto the βA functionalized gold surface, which was then analyzed by mass spectrometry. MS analysis was performed on a Voyager DE-RP (Applied Biosystem) in positive linear mode with accelerating time of 25,000V; grid voltage of 92%; and extraction delay time of 300 nsec.

10 Chemiluminescence experiments:

After AFM analysis, the functionalized gold surface was rinsed with PBS and incubated with anti-mouse IgG1 secondary antibody horseradish peroxidase (1 μL in a 5 mL mixture of PBS [98.9%], Tween20 [0.1%] and non-fat-milk [1%] at RT for 1 h). The gold surface was washed with PBS-Tween20 and incubated for 1 min with detection reagents following manufactured instructions. This elicits peroxidase-catalyzed oxidation of luminol and subsequently, enhanced chemiluminescence, whereby the peroxidase-labeled antibody is bound to the antigen on the primary antibody. The resulting light was detected on autoradiography films at 1 min and 3 min.

Chapter V

Interactions on Surface and Mass Spectrometry

Identification of specific protein-protein interactions on surface by proteomics approaches

in preparation

Identification of specific protein-protein interactions on surface by proteomics approaches.

Stephanie Boussert, Ernest Giralt, Eliandre de Oliveira.

in preparation

In this study, we demonstrate the ability of a gold surface functionalized with the beta-amyloid peptide (Aβ)(see Chapter IV, page 67) to identify protein-protein interactions using proteomics approaches. In a previous work, we described the preparation of the Aβ surface and we confirmed its correct assembly by atomic force microscopy (AFM) and self-assembled for matrix assisted laser desorption/ionization (SAMDI-MS). Serum amyloid P component (SAP) is a 23 kDa protein known to interact with Aβ[1][2]. SAP has been localized in neurofibrillar tangles, senile plaques and amyloid angiopathy of the Alzheimer's disease. SAP aggregates have also been observed in other neurodegenerative diseases including Creutzfeldt-Jakob disease, Pick's disease, Parkinson's disease and Lewy body disease [3][4][5]. SAP is also known to promote plaque formation by co-aggregating with Aβ peptide in presence of Ca^{2+}[6].

In addition to its affinity to Aβ, SAP is commercially available, which makes this protein a convenient model to evaluate the ability of our Aβ functionalized gold surface to fish Aβ partners and allow on-chip enzymatic digestion for MS analysis.

In a first part of the work, we performed a MS study of protein-protein interaction where only one target protein was incubated on the surface (see Fig.1A). In a second part, the MS study of protein-protein interaction was performed by incubating two proteins on the surface. The first protein is the target protein (SAP) and the second one is bovin serum albumin protein (BSA) that is not supposed to interact with our system (see Fig.1B).

The βA funcionalized gold surface was prepared as described in Chapter IV. Briefly, a self-assembled monolayer of two modified-alkanethiols (Lys-penta-EG-alkanethiols and penta-EG-alkanethiols) was formed on a gold surface. The Aβ40 was immobilized covalently to the surface via ligation to a bi-functional maleimide group incorporated into the SAM.

[1] I Liko, M Mak, E Klement, E Hunyadi-Gulyas, T Pazmany, KF Medzihradszky, and Z Urbanyi. *Neurosci Lett*, 412(1):51–55, 2007.

[2] Z Urbanyi, L Laszlo, TB Tomasi, E Toth, E Mekes, M Sass, and T Pazmany. *Brain Res*, 988(1-2):69–77, 2003.

[3] R N Kalaria and I Grahovac. *Brain Res*, 516(2):349–353, 1990.

[4] R N Kalaria, P G Galloway, and G Perry. *Neuropathol Appl Neurobiol*, 17(3):189–201, 1991.

[5] LS Perlmutter, E Barron, M Myers, D Saperia, and HC Chui. *J Comp Neurol*, 352(1):92–105, 1995.

[6] H Hamazaki. *J Biol Chem*, 270(18):10392–10394, 1995.

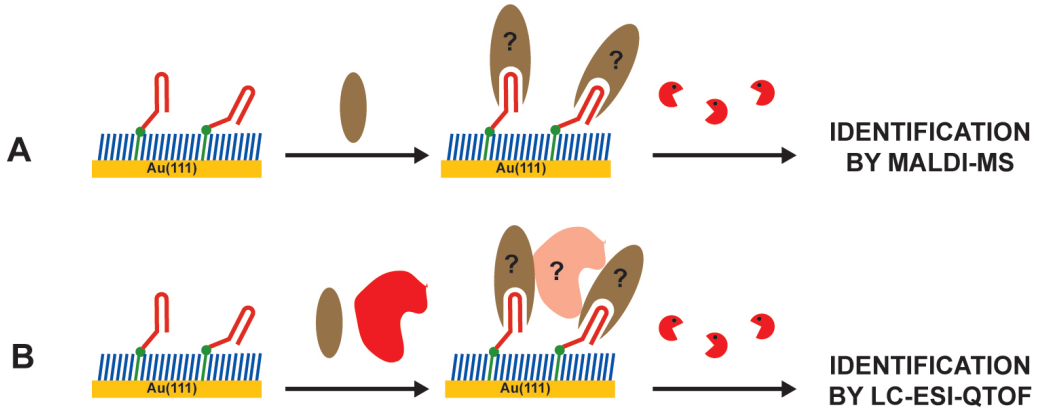


Figure 1: Schematic representation of the study performed on the Aβ40 functionalized gold surface; (A) SAP protein was incubated on the surface; (B) both SAP and BSA were incubated on the surface.

This surface was incubated 2 hours at 4℃ with a solution of SAP protein (0.5μg/μL, in buffer 140mM NaCl, 10mM TrisHCl, 0.1% NaN_3, 2mM $CaCl_2$ buffer). After the washing step (three times 5mL of binding buffer and water each), the surface was subjected to a tryptic digestion during 5 hours at 37℃ with 50 to 100 ng of trypsin in 50mM ammonium bicarbonate. The released peptides were extracted in 0.1%TFA- 50% acetonitrile, dried, resolubilized in 0.1%TFA, passed trough a RP-C18 micro-column to remove salts and eluted with matrix solution (3μL of a solution of 5mg alpha-cyano-4-hydroxy-cinnamic acid in 1mL 50% acetonitril - 0.1%TFA) onto a MALDI-plate.

The signals oberved in the MALDI-MS spectra, correspond to the peptide mass fingerprint (PMF) of the SAP protein (see Fig.2). These data indicate the presence of the SAP on the surface and thus confirm its interaction with Aβ40.

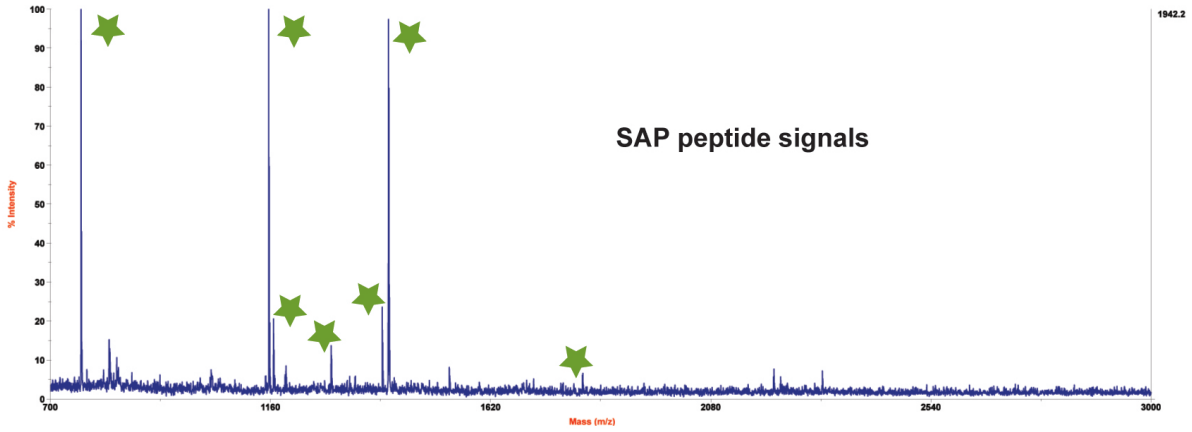
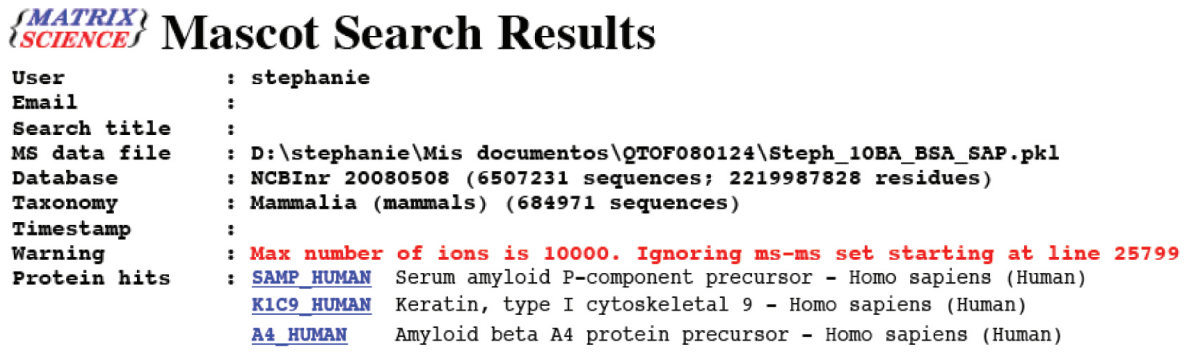


Figure 2: MALDI-MS spectrum of tryptic digested peptides after SAP incubation onto the surface. The stars indicate signals of SAP tryptic peptides.

In order to check the specificity of the interaction, we performed a SAP-Aβ interaction study

on the surface in the presence of BSA. BSA is a 66 kDA protein widely used as a control in proteomics experiments. Indeed, BSA is easily identified by MS as it generates a large amount of peptides when submitted to proteolytic digestion.

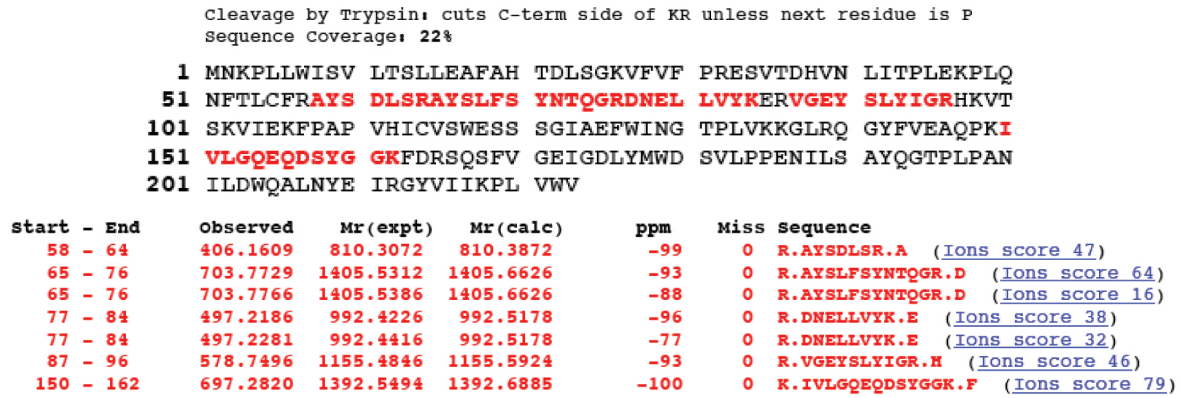
Next, both SAP (10μg) and BSA (10μg) were incubated on the Aβ40 functionalized gold surface. After incubation, washing steps and tryptic digestion, peptides were extracted and directly analyzed by LC-MS (on-line liquid chromatography tandem mass spectrometry, Cap-LC-nano-ESI-Q-TOF, Micromass-Waters). MS analysis confirmed the presence of SAP, while BSA was not detected (see Fig.3). Indeed, we observed peptides matching with up to 22% of the theoretical sequence of SAP (see Fig.4).



MATRIX SCIENCE **Mascot Search Results**

User	:	stephanie
Email	:	
Search title	:	
MS data file	:	D:\stephanie\Mis documentos\QTOF080124\Steph_10BA_BSA_SAP.pkl
Database	:	NCBInr 20080508 (6507231 sequences; 2219987828 residues)
Taxonomy	:	Mammalia (mammals) (684971 sequences)
Timestamp	:	
Warning	:	Max number of ions is 10000. Ignoring ms-ms set starting at line 25799
Protein hits	:	SAMP_HUMAN Serum amyloid P-component precursor - Homo sapiens (Human)
		K1C9_HUMAN Keratin, type I cytoskeletal 9 - Homo sapiens (Human)
		A4_HUMAN Amyloid beta A4 protein precursor - Homo sapiens (Human)

Figure 3: Mascot Search Results obtained from MS data of BSA and SAP proteins tryptic digestion on the Aβ functionalyzed gold surface.



Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: 22%

```
  1 MNKPLLWISV LTSLLEAFAH TDLSGKVFVF PRESVTDHVN LITPLEKPLQ
 51 NFTLCFRAYS DLSRAYSLFS YNTQGRDNEL LVYKERVGEY SLYIGRHKVT
101 SKVIEKFPAP VHICVSWESS SGIAEFWING TPLVKKGLRQ GYFVEAQPKI
151 VLGQEQDSYG GKFDRSQSFV GEIGDLYMWD SVLPPENILS AYQGTPLPAN
201 ILDWQALNYE IRGYVIIKPL VWV
```

Start - End	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Sequence
58 - 64	406.1609	810.3072	810.3872	-99	0	R.AYSDLSR.A (Ions score 47)
65 - 76	703.7729	1405.5312	1405.6626	-93	0	R.AYSLFSYNTQGR.D (Ions score 64)
65 - 76	703.7766	1405.5386	1405.6626	-88	0	R.AYSLFSYNTQGR.D (Ions score 16)
77 - 84	497.2186	992.4226	992.5178	-96	0	R.DNELLVYK.E (Ions score 38)
77 - 84	497.2281	992.4416	992.5178	-77	0	R.DNELLVYK.E (Ions score 32)
87 - 96	578.7496	1155.4846	1155.5924	-93	0	R.VGEYSLYIGR.H (Ions score 46)
150 - 162	697.2820	1392.5494	1392.6885	-100	0	K.IVLGQEQDSYGGK.F (Ions score 79)

Figure 4: List of the peptides matching the SAP theoretical sequence determined by Mascot analysis.

MS and MS/MS collected data were confronted to the NCBI non redundant protein database using the Mascot search engine (www.matrixscience.com) (restrictions parameters used were: monoisotopic mass tolerance of 100 ppm, fragment tolerance of 0.3 Da, no fixed modifications, variable modification allows oxydation of methionine, one missed cleavage, mammalians species restriction).

As a control, an identical incubation of SAP and BSA was performed on a surface with-

out Aβ40 (SAM formed with only penta-EG-alkanethiols). The same protocol of incubation, washing steps and digestions of proteins were performed but the proteolytic digestion of this experiment did not lead to the identifiction of any proteins. This result confirmed that the binding capacity of the penta-EG groups SAM is strictly limited to specific interaction. Indeed we did not observe interactions with BSA neither using the β functionalized gold surface nor the non functionalized gold surface.

In conclusion, we have shown that our Aβ functionalized gold surface is suitable for protein-protein interactions studies since only specific interactions were observed. Moreover, the system provides a convenient sensitive range that allows classical proteomics assays such as enzymatic digestions and protein partners identification via MS.

Discussion

If my thesis work had to be summarized in one word I would choose the following one: multidisciplinary. It results from a combination of approaches from scientific fields such as biophysics, organic chemistry, analytical chemistry, proteomics and nanotechnology. Its goal: carry out these techniques to answer biological questions. I do not pretend here to have mastered all these disciplines. On the contrary, I relied on the support of experts of each of these fields. My personal contribution consisted in initiating new research lines at the edge between these fields. I chose to organize this discussion section as following: each article was first placed in its context before being summarized. Next, I focused on some elements that I found especially pertinent to the biological aspects of the work.

Amino acids are the building blocks of proteins. Their side chains have biochemical properties that promote the formation of covalent and non-covalent interactions. Non-covalent interactions are the major forces that drive the folding of proteins and the formation of protein complexes. Crystallography or NMR are usually the methods of choice to study the tertiary and quaternary structure of proteins. Getting the appropriate material (i.e. protein crystals or labeled proteins, respectively) is not straightforward and sometimes represents a technical limitation. Moreover, the analysis of the collected information is usually time consuming due to the inherent complexity of the data and definitely requires the collaboration of a team of specialists.

MS can be used as an alternative approach to get a general idea of the protein architecture and map the residues embedded in structural domains onto the primary sequence. Structural domains, are folded and compact regions of the protein that are stabilized by non-covalent interactions. In **chapter 1**, we describe the development of an approach based on time-resolved limited proteolysis of a protein and the MS analysis of the corresponding resistant fragments. To confirm the positioning of the boundaries of the structural domains, the proteolysis was repeated with proteases having different specific cleavage-sites. The results obtained from these assays were consistent thus validating our MS approach. After proteolysis, the resistant domains were separated by electrophoresis on a denaturating polyacrylamide gel, the different bands were excised and digested. The digestion of the domain with trypsin was performed directly inside the gel. The corresponding peptides were then extracted and analyzed by MS (either MALDI-MS or ESI-MS) for their identification. When the domains contained a cleavage site for trypsin close to the amino terminus, the tryptic digestion produced a short fragment that could not be identified by MS due to its low molecular weight. The identification of the sequence upstream of the cleavage site was performed by Edman degradation on the whole domain.

In short, MS enabled us to get an overview of the tertiary structure of the protein by mapping with precision the residues embedded in structural domains. It may be assumed that the remaining residues are implanted in non-structured or flexible region. It should be mentioned here that the optimization of the amount of protease used for the assay and the time of diges-

tion is essential for the robustness of the study. Proper digestion initiation conditions that avoid complete proteolysis have to be found experimentally.

Point mutations in genes encoding proteins are common between two individuals of the same species or even between different alleles in the genome. The corresponding mutation on the protein remain silent if the amino acid is exchanged for another having equivalent biochemical properties. Occasionally the mutation impairs the function of the protein and is responsible for pathologies. The identification and the characterization of these mutations are central in biomedicine and sometimes represent a first step towards the discovery of a treatment. Over several hundred point mutations in genes encoding the hemoglobin complexes have been described and some of them can lead to severe anemia and reduce life expectancy. In adult humans, the most common hemoglobin type is a tetramer called hemoglobin A, consisting of two alpha and two beta subunits non-covalently bound, each made of approximately 150 residues.

In **chapter 2** we describe the identification and the characterization of a variant of the human hemoglobin beta chain. Hemoglobin complexes were prepared from patient blood and analyzed by ESI under denaturating conditions. The spectrum of the hemolysate showed an additional peak corresponding to a potential beta chain isoform of a slightly higher molecular weight. Reverse-phase HPLC was used to separate wild type beta chain from its supposed isoforms. Both chains were digested and analyzed by LC-MS for the separation and the analysis on line of the peptides. We observed perfect matches between the peptides of the two species with the exception of the N-terminal fragment. MS-MS on this fragment enabled us to identify the amino acid substitution and map exactly the location of the mutation. A consistent result was obtained from the sequencing of the region encompassing the mutation on the genomic DNA of the patient.

Electrophoresis is a classical approach to detect variants in protein complexes and especially in hemoglobin tetramers. This method is valid when the amino acid substitution alters significantly the physicochemical feature of the whole protein. In the aforementioned study, the beta chain variant was electrophoretically silent but could be nevertheless detected by a stepwise MS analysis. The mutation was pointed out and identified without the necessity of systematic sequencing of the corresponding locus on the genomic DNA. This method is particularly useful when DNA sequencing cannot be used. Indeed, post-translational modifications such as acetylation or glycosylation, occurring on proteins, are by definition performed by cellular enzymes after the translation of the target protein. A stress-induced attenuation of the activity of these modifying enzyme would lead to an alteration of the pattern of modifications without leaving any traces in the genome, but would be still detectable by MS analysis.

Natural proteins are made exclusively of twenty different alpha amino acids. These amino

acids share an identical backbone where the amino and the carboxylate groups are attached to the same carbon. As a consequence proteins can only adopt a restricted number of secondary structures due to the unique conformation of their building blocks. Artificial peptides with unusual folding can be designed in the laboratory and produced by organic synthesis. Beta peptides are built with beta amino acids where the amino and the carboxylic groups are linked to adjacent carbons. Due to their original conformation beta peptides are usually resistant to cellular proteases which make them ideal candidates for the design of peptide-based drugs. For instance, they have been used to mimic and substitute natural peptide-antibiotics that are sensitive to proteolytic enzymes. A variety of beta peptides can be engineered by changing parameters such as their length or the nature of their side chains.

In **chapter 3** we chose NMR, TEM and AFM as biophysical approaches to characterize and study the behavior in solution of a new beta peptide. A beta tetrapeptide made of homochiral cyclobutane residues has been synthesized in the laboratory. NMR has shown that it folds in a compact strand-like secondary structure with a predicted length of 2.2 nm. This conformation is stabilized by a network of intramolecular hydrogen bonds involving the three peptide links. Interestingly we observed by TEM the formation of apparently homogeneous fibrils after a 24h-incubation. A sine qua non condition to characterize these micrometer scale structures and investigate the dynamic driving their arrangement was the ability to take snapshots with a significantly higher resolution than possible with TEM. AFM was chosen to obtain a topographic view of the tetrapeptide adsorbed on a mica surface. Images were acquired under intermittent mode to preserve the integrity of the peptides and limit the displacement of potential macromolecular arrangements. The peptide incubated 24h showed a similar tendency to form fibrils on this material. With a resolution in the nm range we could observe a significant disparity in the height of the aggregates. The lowest amplitude was measured at 2.2 nm indicating that the fibers grow first along one axis by self assembly of tetrapeptides. The interaction between adjacent tetrapetides might be stabilized by a redistribution of the intramolecular hydrogen bonds. In absence of experimental evidence the orientation of the peptides along the fiber remains an open question. Larger arrangements are probably built by a complex piling up of these elemental fibers to obtain sheets. It is unlikely that these sheets assemble like a wall that is built by using bricks. Indeed the amplitude observed is never a multiple of 2.2 nm suggesting a more complex organization. Scanning Polarization Force Microscopy (SPFM) is a method to analyze the distribution of charges on a support. A voltage is applied to a conductive cantilever used to scan the surface. Opposite charges between the tip and the surface will bend the lever toward the surface or away from the surface when they both have the same charge. Virtually all peptides have an electrical dipole moment. It results from the individual contribution of the carbonyl groups of each peptide bonds. SPFM experiments are currently performed to analyze

in depth the organization of both the elemental fibers and the sheets.

Water is the natural solvent of all biological system. As a consequence, cellular proteins evolved to be hydrosoluble by displaying hydrophilic residues on their surface and burying hydrophobic ones inside the structure. Some mutations can affect their folding or stimulate unexpected proteolysis. They can lead to the exposure of patches of hydrophobic amino acids. Such proteins tend to aggregate by forming small nuclei that expand over the time. Large particles become toxic when they accumulate in essential organs like the central nervous system. The beta amyloid is produced in human by digestion of a variant of a transmembrane protein called APP. Various isoforms from 39 to 43 amino acids can be released. They have an intrinsic tendency to aggregate and form spreading fibrils which are suspected to participate in the development of Alzheimers disease. Many research lines have been initiated to understand the molecular mechanisms behind the pathology. One of them consist in identifying partners of the beta amyloid. Genetic approaches, like the two-hybrid system, is particularly suitable to characterize the interactome of a soluble targets but inappropriate with proteins that are aggregation-prone.

With these limitations in mind, we describe in **Chapter 4** the nanoassembly of a prototype tool. This tool based on the beta amyloid 40 has been optimized for bioanalytical purposes and validated by AFM and MS analyses. The principle is based on a functionalized gold surface that exhibits the beta amyloid. The preparation of such a chip faced three main issues. Firstly there is the difficulty of the manipulation of an aggregation-prone protein in solution. The second issue is the propensity of protein to adsorb non-specifically onto surfaces, increasing considerably the experimental noise due to potential non-specific binding. The last is the choice of the right strategy to anchor the amyloid peptide onto the surface. The first obstacle was overcome by synthesizing a versatile isoform of the beta amyloid 40. The peptide bond between residue 17 and 18 was modified to stiffen the peptide and prevent its folding. By doing so it remained soluble under acidic pH. A conformational change triggered by a jump in the pH allowed the recovery of the native hairpin structure of the beta amyloid.

Next, we had to treat the gold to ensure the beta amyloid was the only interacting species on the chip. For this purpose we constructed a mixed self-assembled monolayer of two species of PEGylated alkanethiols on the surface, one of which was modified with an amine to interact with a maleimide group that will bind the beta amyloid. They organized themselves as homogenous and dense isolating coat. Finally the soluble form of the beta amyloid was coupled to the maleimide group and the chip was activated by modifying the pH. The distribution of the beta amyloid and the general integrity of the surface were analyzed after assembly by AFM, MS and immunostaining. They all confirmed the promising bioreactive potential of our chip.

Alkanethiols are central pieces for the anchoring of our biomolecules on the gold surface.

The hydrocarbons ensure efficient packing of the monolayer while the terminal group provides a specific binding site for the protein. On the other side of the molecule the sulfur groups interact with the gold surface with a strength comparable to a covalent bond. Gold surfaces can be recycled, but special care is required to remove the coat of alkanethiols. We used a chemical electropolishing system to clean the gold surface prior to performing any new incubation.

SAMDI analyses were performed directly on a gold monocrystal Au(111). This support is significantly thicker than a classical MALDI plate that is calibrated for MS analysis. Typically the manipulation of a non-optimized plate has a negative effect on the quality of the data collected. The uniformity of the gold surface is central for the formation of an homogeneous self assembled monolayer. Due to this limitation, commercial gold surfaces adapted for MALDI-MS are unfortunately not suitable for SAM experiments due to a lack of regularity.

Fishing fanatics could tell you that the two secrets for a fortuitous catch are the choice of the right bait and a lot of patience. These golden rules are also true when the prey is a single molecule. We describe here above the assembly of a chip designed to fish molecular partners of the beta amyloid 40. In **chapter 5** we move a step forward by checking more in depth the bioreactivity and the specificity of the chip. Serum amyloid P (SAP) is a 23 kDa protein known to interact with the beta amyloid. Its affinity for the bait is much lower than the affinity of the antibody used for the immunostaining. Consequently, it reflects more the typical strength observed between two proteic partners and thus constitutes an appropriate prey. Antibodies recognize small epitopes on their targets. These epitopes are short sequences of typically four or five amino acids. A positive signal by immunostaining does necessarily mean that the entire molecule of beta amyloid is correctly folded. By contrast, reversible protein complexes are usually characterized by large contacts between the partners. As expected SAP was found to interact with the chip confirming the native conformation of the beta amyloid used as a bait. Bovine serum albumin (BSA) did not bind to the chip nor displaced SAP-amyloid interactions. In a short we have shown here that our chip has the following feaures:

- The bait dispayed on the surface has a native conformation
- The bait is spread uniformly with proper spacing to allow the binding of macromolecular pray
- The surface apparently does not promote unspecific binding
- The surface does not interfere with the binding of a natural partner of the beta amyloid

The next step of this project will be to use this chip to fish new ligands of the beta amyloid. For this purpose, total protein will be extracted of a dead patient and incubated on the chip.

Conclusion

1. We have been able to identify the folded domain of a protein by coupling limited proteolysis experiments and MS. These results have been confirmed by NMR analysis and fluorescence studies. This approach could be straightforwardly applied to other multidomain proteins and/or multiprotein assemblies.

2. We have identified a new mutation in a variant of human Hemoglobin beta-chain by a complete MS characterization. This mutation was not detectable by usual electrophoretic procedures. This MS analysis could be routinely used for hemoglobin characterization.

3. We have demonstrated the ability of AFM to provide structural informations of a beta-tetrapeptide and its behavior of self-assemble.

4. We have been able to construct a beta-amyloid functionalized surface that shows high compatibility with AFM and SAMDI-MS studies.

5. We have identified and characterized specific protein-protein interaction on a functionalized beta-amyloid surface by using proteomic approaches.

Appendix

This appendix summarizes an ensemble of data that are not intended to be part of the five publications that form the core of the thesis, but that are also related to the general thesis objective.

1 Experiments on gold surface

1.1 Inhibitor Peptide of the beta-amyloid aggregation

Beta-amyloid (Aβ) peptide is a self-agregating protein found in senile plaques of Alzheimers disease and is thought to play a major role in this disease. Here, we present the study of βA specific interaction with one of its inhibitor peptide (sequence LPFFD). This study combined self-assembled monolayers (SAMs) on gold with MALDI-TOF mass spectrometry to detect peptide-peptide/peptide-protein interactions directly on a functionalized surface (inhibitor chip). Such direct on chip analysis avoids protein loss and considerably simplifies sample preparation for mass spectrometry as it allows washing steps and direct MS analysis.

The gold surface was functionalized via thiol-gold chemistry. In the present case, we used an inhibitor peptide sequence where we coupled a cystein aminoacid residue at the N-terminal which enabled the bindind of the peptide to the gold surface using the SAMs methodology. The synthesis of the inhibitor peptide was performed by SPPS and thus the cystein at the N-terminal was easily added to the sequence.

In a first step (see Fig.V.1A), a gold surface Au (111) was incubated with a solution of inhibitor peptide (1μM, H_2O). A SAM monolayer was expected on the gold. Indeed after 2 hours of incubation, AFM analysis reveals the beginning of a SAM formation. The shapes observed on the surface, named ”islands”, are very typical from a SAM formation. Moreover, the heights measured by AFM of these islands were the theoretical size expected of the inhibitor (see Fig.V.2).

After longer incubation, which corresponds to 14h of total incubation time, the same gold surface was analyzed by AFM. The background observed was uniform and without major defect, which indicates a complete and good formation of the SAM (see Fig.V.3).

In a second step (see Fig.V.1B), a solution of synthetic Aβ42 was incubated on a gold surface presenting a monolayer of the inhibitor peptide (1μM in PBS). After 2h of incubation, the surface was washed and MALDI matrix was added directly to the surface. The gold was inserted in a MALDI-TOF instrument and analyzed by MS. The SAMDI mass spectrum clearly indicated the presence of the Aβ42 on the surface and so its interaction with the peptide inhibitor (see FigV.4).

In a last experiment (see Fig.V.1B), the inhibitor SAM was incubated with a crude brain protein extract from an Alzheimer’s patient. Our goal was to isolate Aβ from the protein brain

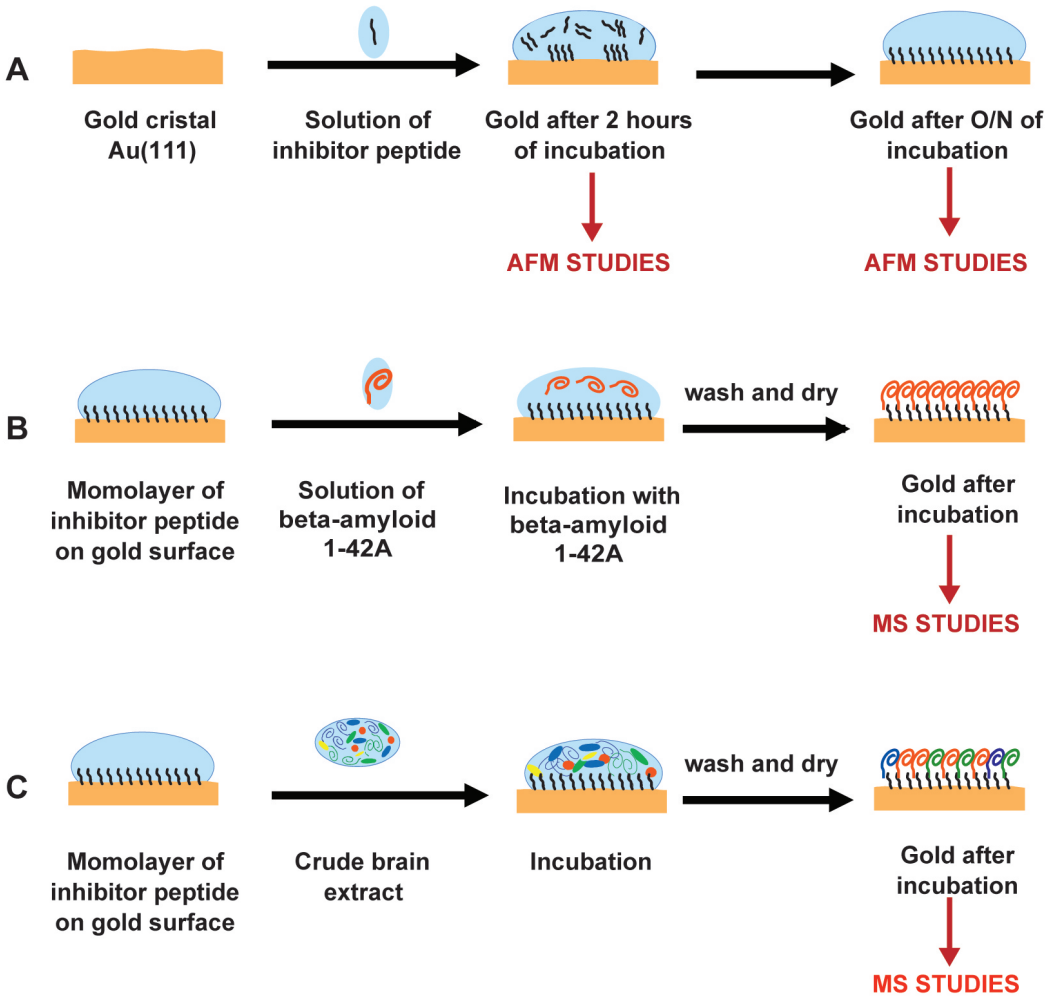


Figure V.1: Schematic representation of the strategy used to study the inhibitor peptide - Aβ42 interaction; (A) formation of the functionalized surface; (B) interaction with Aβ42; (C) interaction with proteins from a crude brain extract.

extract via the interaction with the inhibitor peptide monolayer. After incubation and washing steps, the gold surface was analyzed by MS; the SAMDI mass spectrum showed the presence of two signals, which m/z ratio are very close to the ones of the amyloid peptides (see Fig.V.5). These peaks could be assigned as the Aβ40 (theoretical MW 4300Da) and Aβ45 or Aβ(2-46) (MW 4800Da) peptides.

In this study, we have shown an interaction between the Aβ and its inhibitor LPFFD directly on the peptide functionalized gold surface by mass spectrometry. First, by the incubation of the funcionalized surface with a solution of Aβ42, and second, by the assay of interaction between the inhibitor peptide attached to the gold surface with a complex crude protein brain extract. In both we could characterize molecular weigt signals corresponding to different species of BA peptide by SAMDI-MS thecnology.

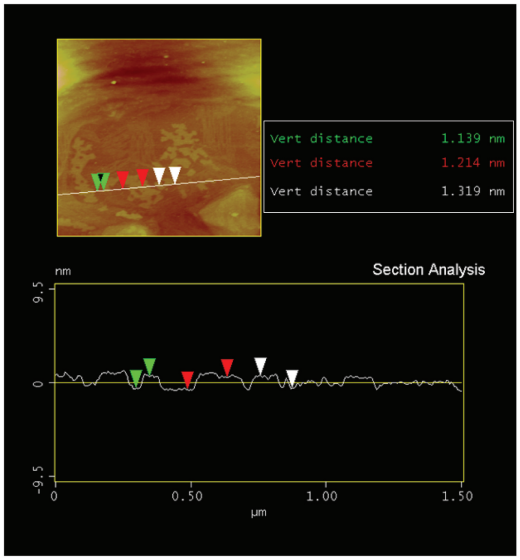


Figure V.2: AFM analysis of inhibitor peptide on gold surface after 2 hours of incubation. Islands correspond to the inhibitor peptide bound to the surface. The size measured 1.2 nm is the size expected of the inhibitor peptide

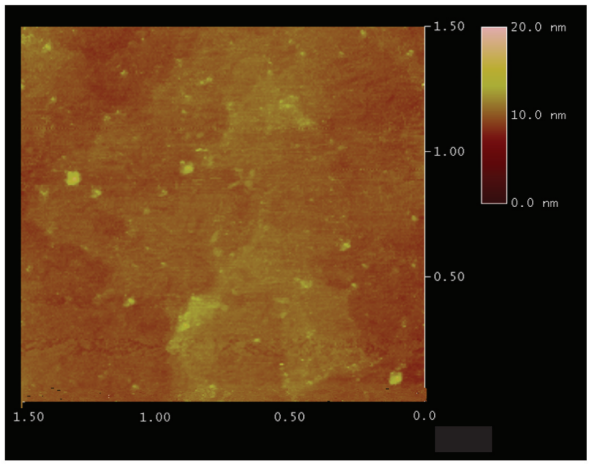


Figure V.3: AFM analysis of the gold surface after 14 hours of incubation. Dense surface corresponds to the presence of a monolayer of the inhibitor peptide on the gold surface.

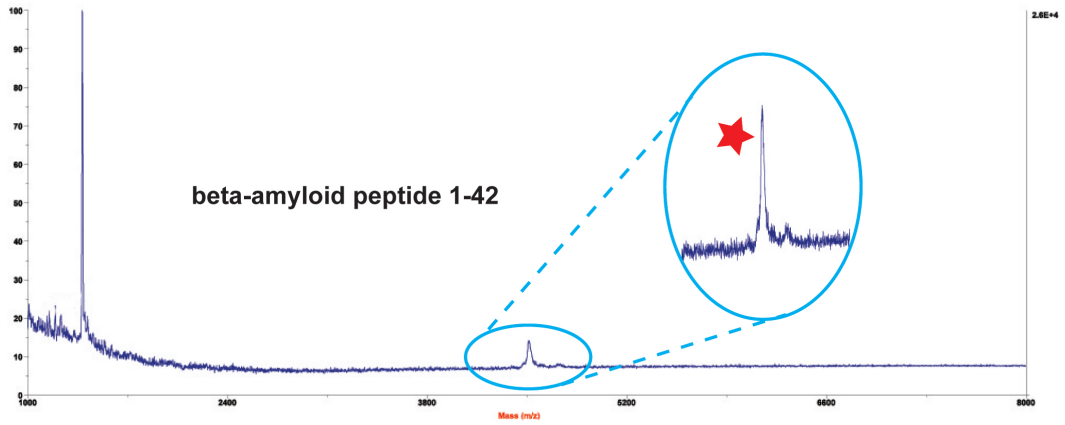


Figure V.4: SAMDI mass spectrum of the inhibitor- Aβ42 interaction on gold surface. MS peak measured corresponds to the exact theoretical average mass of the Aβ42 peptide.

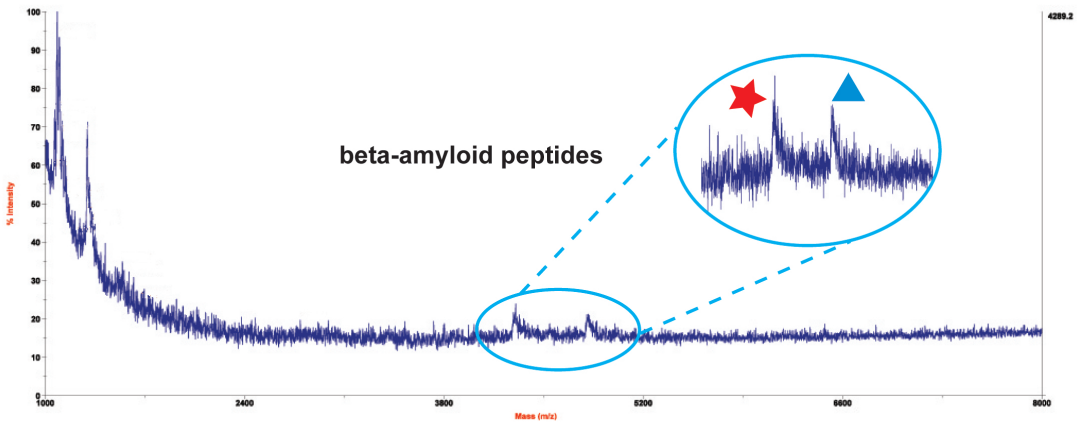


Figure V.5: SAMDI mass spectrum of inhibitor peptide-peptide interaction on gold surface. MS peaks measured could be assigned to Aβ40 (star) and to Aβ(1-45) or Aβ(2-46) (polygon).

1.2 Microcontact printing and MS

In this study, we used microcontact printing technique to print the inhibitor peptide LPFFD on a gold surface. The goal of this study was to isolate proteins from a crude protein brain extract interacting with the inhibitor peptide on the gold surface. After, the molecules involved in such interaction were analyzed by SAMDI-MS and by proteolytic digestion followed by MS analysis (see Fig.V.6).

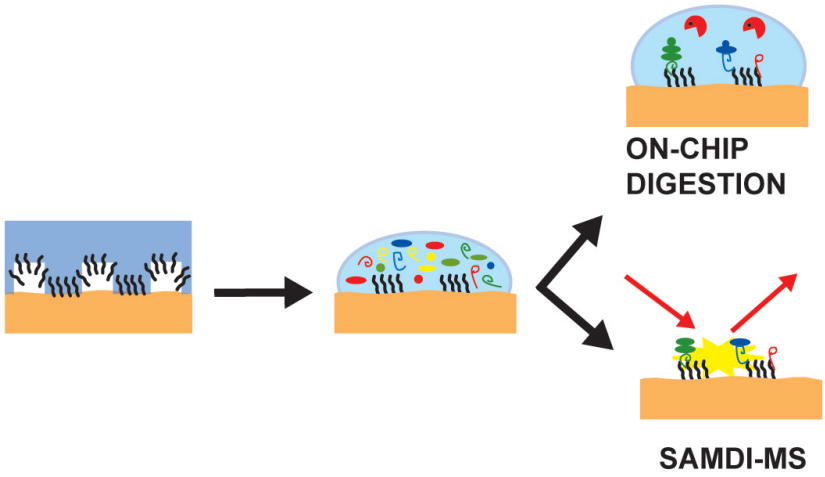


Figure V.6: Schematic representation of the strategy used to study the inhibitor peptide - protein brain extract interaction by microcontact printing ans MS.

The first step of this study was to pattern a gold surface using a poly-dimethyl-siloxane stamp (PDMS-stamp). The PDMS-stamp was incubated 15 minutes with an aqueous solution of the inhibitor peptide (1mM), dried under a blow stream of nitrogen and immediately put in

contact with the bare gold surface. In that case, we used a silicium surface coated with a thin layer of gold, in order to fit the stamp on it. The pressure on the stamp was done manually. Special care must be taken when applying pressure on the stamp in order to not deform the stamp on the surface and keep the pattern on the gold as accurate as possible (Fig.V.8). Once the peptide was printed on the gold, the surface was incubated with the protein brain extract (4℃, 12h).

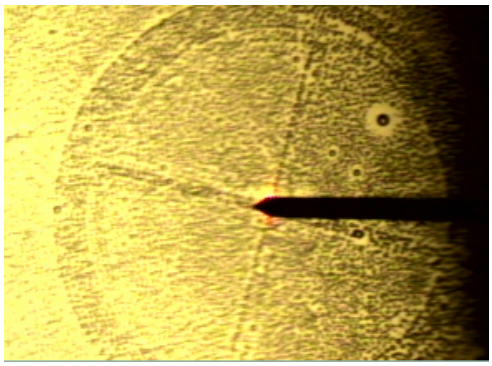


Figure V.7: Picture of the pattern let after microcontact printing on a gold silicium surface (observed by optical microscope, in black we can see the AFM probe).

After incubation, the gold was washed and analyzed by SAMDI-MS or subjected to on-chip proteolytic digestion:

- SAMDI-MS analysis showed some signal from the range of 3000 Da to 20.000 Da, indicating that some proteins were present on the gold surface. Unfortunately, a control experiment done in the same conditions but with another peptide, led to the same SAMDI spectrum. Which indicates that the interaction was not specific.

- After proteolytic digestion on the gold surface (100ng of trypsin, 50mM ammonium bicarbonate buffer, 5h, 37℃), the peptides were extracted (50% acetonitrile in water) and analyzed by MALDI-MS. MALDI-MS spectrum showed typical from contamination by a polymer. We found in the literature that trace contaminants of PDMS-stamp can be left on the surface after printing [158] [159] [159] [160]. We assumed that the origin of the contamination came from the stamp as no other polymer were used during these experiements and as other reagents used in separated experiments did not lead to such MS-MALDI spectrum.

These results gave us the conclusion that:

[158] I Bohm, A Lampert, M Buck, and M Eisert, F andGrunze. *Appl Surf Sci*, 141:237–243, 1999.
[159] D J Graham, D D Price, and B D Ratner. *Langmuir*, 18(1518–1527), 2002.
[160] K Glasmästar, J Gold, A S Andersson, D S Sutherland, and B Kasemo. *Langmuir*, 19(5475–5483), 2003.

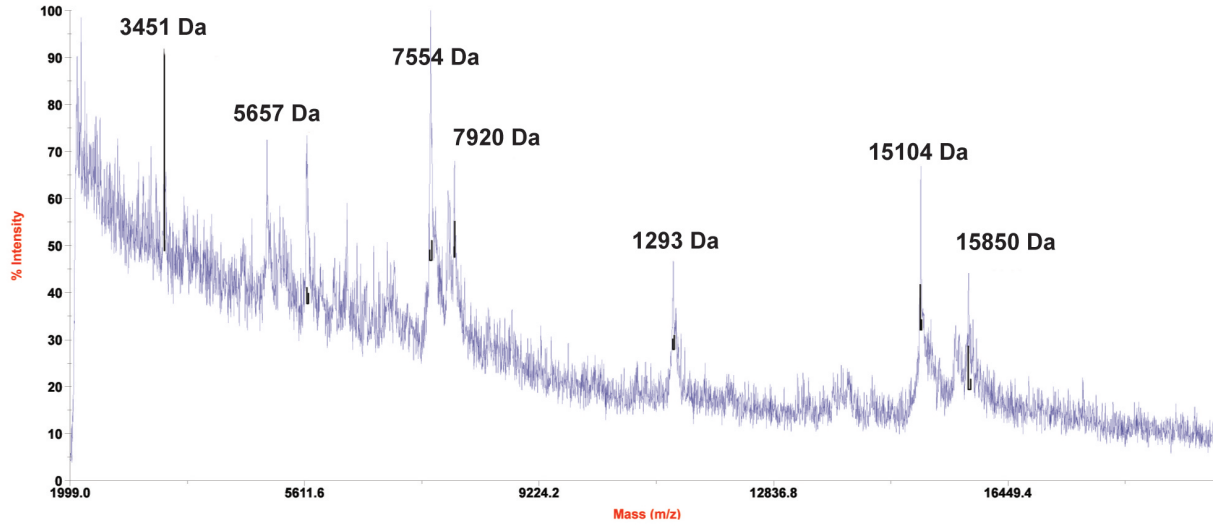


Figure V.8: SAMDI mass spectrum of the functionalyzed gold surface after incubation with protein brain extract.

- the quantity of proteins present on the gold was enough to be detected by SAMDI-MS, and that, SAMDI-MS can be used to detect rapidly proteins on a surface.
- we had to improve on-chip digestion to identify proteins present on the surface, microcontact printing was not the technique of choice as some trace of PDMS can be transfer to the gold and interfere in the MS analysis.
- we had to improve the way to avoid unspecific interaction, specially with the bare gold surface, where any containing cystein proteins could interact with.

1.3 On-chip digestion

Our previous experiments of on-chip protein digestion on gold (see above), led us to develop a new Aβ functionalized gold surface. First, to improve the on-chip digestion and MS analysis, and second, to minimize the unspecific interactions with the surface. This was made by using SAMs of penta-EG-alkanethiols and Lys-penta-EG-alkanthiols (see IV, page 77).

One of our objectives was to use this functionalized surface to fish partners of the Aβ peptide from a crude protein brain extract of an Alzheimer patient.

In a first step on that direction, we incubated proteins on our Aβ peptide functionalized gold surface (2mg/mL, 2h, 4℃) and after washing steps, we performed on-chip digestion (100ng of trypsin, 50mM ammonium bicarbonate buffer, 5h, 37℃). The peptides were extracted three times with 50% acetonitrile and dried prior MS analysis (see Fig.V.9).

Different MS analysis were carried out: LC-ESI-MS-MS and LC-MALDI-MS-MS. In all MS analysis we have been able to identify proteins. Most of the proteins identified by MS analysis,

Figure V.9: Schematic representation of the on-chip study.

are abundant proteins present in the brain and for that reason were not considered as target proteins. Nevertheless, in many MS analysis, and specially LC-MALDI-TOF-TOF analysis, we have detected some proteins that may have a significant interaction with the Aβ peptide [161][162] (see Fig.V.10).

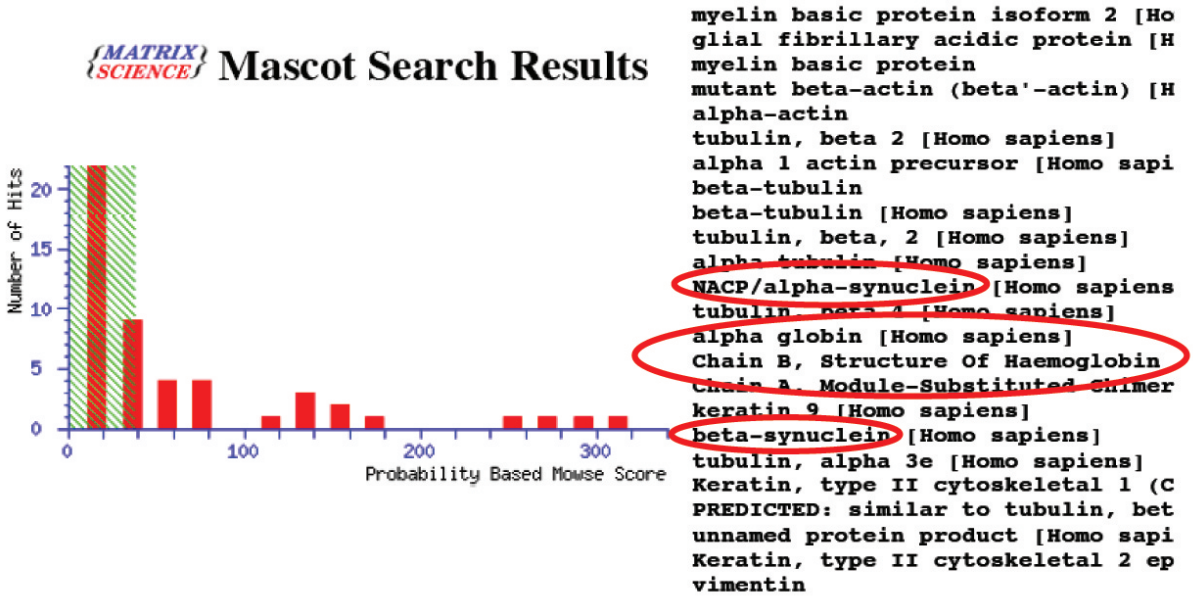


Figure V.10: Result obtained by LC-MALDI-TOF-TOF MS analysis. A large number of proteins were identified, most of them are abundant proteins such as myelin, tubulin or actin, and cannot be considered as target proteins; nevertheless some of the proteins identified (red circles) have been previosly reported in the literature to have a significant role with Aβ peptide.

In order to remove some of these abundant proteins that may prevent the detection and identification of target proteins, we fractionated our brain extract by doing gel filtration (Superdex 75, Vt 25mL, 200μL of proteins brain extract). Aliquots of each peaks were taken and loaded on 12% electrophoretic gel (see Fig.V.11).

Aliquot 3 (lane 3) contained mainly proteins with MW lower than 30kDa. The most abundant

[161] Y Verdier, E Huszar, B Penke, Z Penke, G Woffendin, M Scigelova, L Fulop, M Szucs, K Medzihradszky, and T Janaky. *J Neurochem*, 94(3):617–628, 2005.

[162] R T Perry, D A Gearhart, H W Wiener, L E Harrell, J C Barton, A Kutlar, F Kutlar, O Ozcan, R C P Go, and W D Hill. *Neurobiol Aging*, 29(2):185–193, 2008.

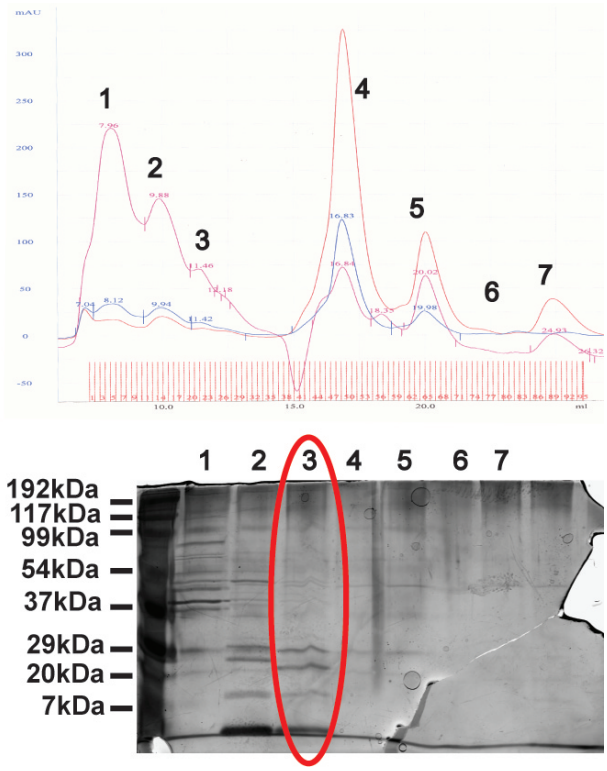


Figure V.11: Fractions obtained by gel filtration separation of brain extract. 7 aliquots were loaded on a SDS-PAGE gel. Aliquot 3 contains proteins with MW lower than 30kDa.

proteins identified, i.e. myelin, tubulin, actin, have all MW around 50kDa, for that reason we decided to incubate proteins from aliquot 3 on our Aβ peptide functionalized gold surface. Unfortunately, after incubation and digestion, MS analysis, again, led to the identification a large number of abundant proteins (mainly myelin, tubulin, actin).

In this experiment we could not identify unambiguously target proteins, our main difficulty came from the high complexity of our brain extract (crude brain extract). Fractionation of our protein extract seems to be the answer for non ambiguous identification of target proteins.

2 Gold Nanoparticles

In a same idea to study Aβ-protein interaction, we decided to functionalyze gold nanoparticles (Au-NPs) with a mixed SAM of penta-PEGylated-alkanthiols and Lysine-penta-pegylated alaknethiols (see IV, page77).

Gold metallic nanoparticles are commonly used in the lab as a tracer, to detect the presence of specific proteins or DNA in a sample, for example. Gold is used for nanoparticle applications because it is unreactive and is not sensitive to air or light. To make sure the particles do not clump together, their surfaces have to be covered with a layer of protective molecules. Sulphur is one of a few elements that gold happily binds with, so sulphur-containing groups are often

used for this protective coating. This may turn the nanoparticles into tracers [163][164][165].

The methodology used was the same than the one we used for the functionalization the gold surface (see Fig.V.12).

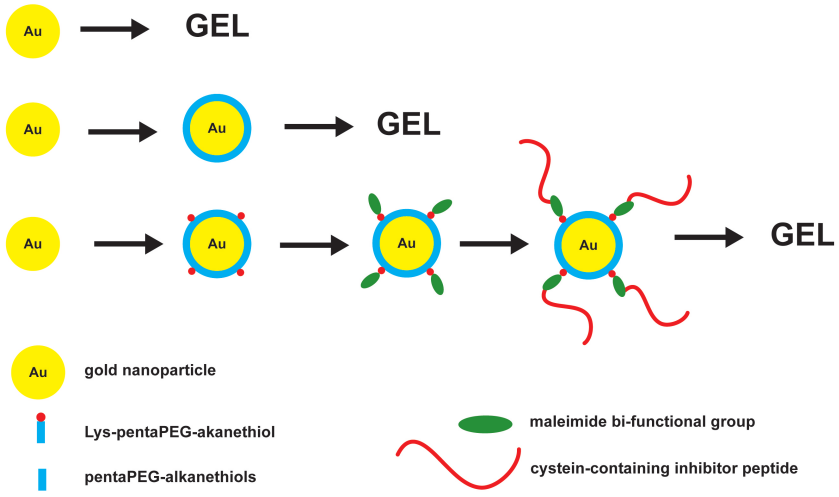


Figure V.12: Schematic representation of the method used to functionalyze gold nanoparticles.

Briefly, to a solution of Au-NPs (1.2mM in citrate buffer) a solution of penta-PEGylated alkanethiols (10μM) or a solution of mixed Lys-penta-PEGylated alkanethiol/penta-PEGylated alkanethiols (35%-65%, v/v, 10μM) was added. After reaction, the wavelengths of Au-NPs, Au-NPs/penta-PEGylated alkanethiol and Au-NPs/mixed alkanethiols solution were measured. A shift of 2nm in the maximum wavelength absorption was observed between functionalized Au-NPs and Au-NPs alone. After a dialysis step, a solution containing a bi-functional maleimide group was added to the Lys-penta-PEGylated alkanethiols functionalized Au-NPs and finally the cystein-containing inhibitor peptide was added. After reactions, a shift of 5 nm in the maximum wavelength absorption was observed between functionalized Au-NPs and Au-NPs alone, indicating clearly a change on the properties of the NPs.

After dialysis, Au-NPs alone, PEGylated Au-NPs and Peptide-Au-Nps were separated on a polyacrylamide gel (16%) and stained with silver nitrate (see Fig.V.13. In the two first lanes, corresponding to Au-NPs without peptide, no coloration was observed while in the third lane, where peptide-functionalized-Au-NPs were loaded, different bands were observed. The presence of those different bands can be explained by a population functionalized-Au-NPs containing different number of bound peptides, and thus different number of Lysine-penta-pegylated alaknethiols. In some lanes (noted INHIBITOR) different concentrations of peptide alone. Such a small peptide can not be retained in the gel, and thus, no coloration was observed. This result

[163] R Shukla, V Bansal, M Chaudhary, A Basu, RR Bhonde, and M Sastry. *Langmuir*, 21(23):10644–10654, 2005.

[164] HK Patra, S Banerjee, U Chaudhuri, P Lahiri, and AK Dasgupta. *Nanomedicine*, 3(2):111–119, 2007.

[165] PD Jadzinsky, G Calero, CJ Ackerson, DA Bushnell, and RD Kornberg. *Science*, 318(5849):430–433, 2007.

confirms that the coloration observed in lane 3 was due to the presence of the peptide attached to the Au-NPs as Au-NPs do not enter into the gel.

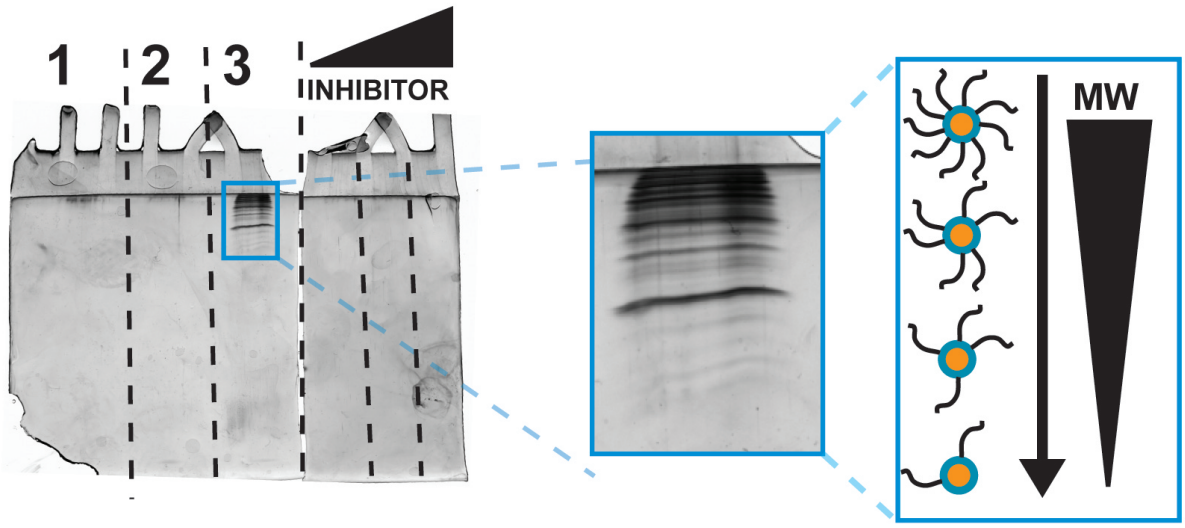


Figure V.13: Polyacrylamide gel obtained after functionalization of gold nanoparticles.

In these experiments, we have been able to functionalyze Au-NP with a peptide via SAMs. The stability and integrity of the SAMs on the Au-NPs surface were not investigated.

3 Agarose experiments

In order to study the Aβ-Serum amyloid P component (SAP) interaction, we decided to use the technology of protein microarray on agarose.

Serum amyloid P component (SAP) is a 23 kDa protein known to interact with Aβ[166][167]. SAP has been localized in neurofibrillar tangles, senile plaques and amyloid angiopathy of the Alzheimer's disease. SAP aggregates have also been observed in other neurodegenerative diseases including Creutzfeldt-Jakob disease, Pick's disease, Parkinson's disease and Lewy body disease [168][169][170]. SAP is also known to promote plaque formation by co-aggregating with Aβ peptide in presence of Ca^{2+}[171].

Microarray on agarose technique enables the immobilization of proteins via its free amino groups and the aldehyde groups of the agarose (Schiff base reaction). After immobilization, the free aldehyde groups are blocked and the slide is incubated with a solution containing a

[166] I Liko, M Mak, E Klement, E Hunyadi-Gulyas, T Pazmany, KF Medzihradszky, and Z Urbanyi. *Neurosci Lett*, 412(1):51–55, 2007.

[167] Z Urbanyi, L Laszlo, TB Tomasi, E Toth, E Mekes, M Sass, and T Pazmany. *Brain Res*, 988(1-2):69–77, 2003.

[168] R N Kalaria and I Grahovac. *Brain Res*, 516(2):349–353, 1990.

[169] R N Kalaria, P G Galloway, and G Perry. *Neuropathol Appl Neurobiol*, 17(3):189–201, 1991.

[170] LS Perlmutter, E Barron, M Myers, D Saperia, and HC Chui. *J Comp Neurol*, 352(1):92–105, 1995.

[171] H Hamazaki. *J Biol Chem*, 270(18):10392–10394, 1995.

target protein. The presence of the target protein on the slide can be verified by classical immuno-detection technology (i.e.incubation with a fluorescent antibody).

In this study we used our synthetic Aβ40-isopeptide (synthesis described in IV, page77).

In a first step, we validated the Aβ - antiAβ antibody interaction on an agarose slide. Briefly, 3 or 4 spots of the Cys-Aβ40 solution were spotted manually on the agarose slide (3μL per spot, solution at 50 ng per μL). As controls, bovin-serum albumin (BSA) and PBS buffer were also spotted. After washing steps and treatment on the slide to unblock other aldehyde groups, the antiAβ antibody was incubated on the whole slide. Finally, a secondary antibody anti-primary antibody was incubated on the slide. The results were read by a fluorescent scanner (see FigV.14).

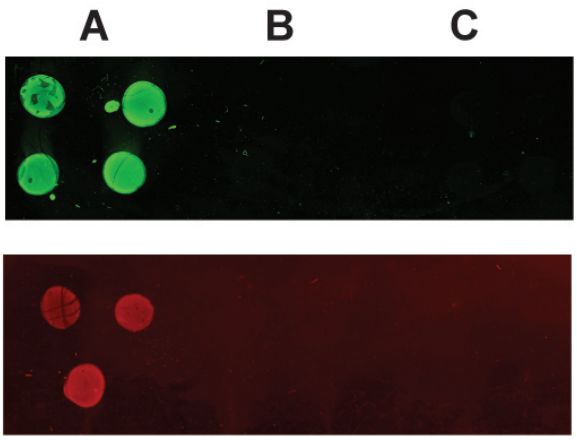


Figure V.14: Visualisation of the agarose slide after incubation with secondary antibody; two kind of fluorescent marked secondary antibodies were used (green and red); (A) signal observed for Cys-Aβ40,. No signals were observed for BSA (B) or PBS (C).

The interaction between CysAβ40 - anti-Aβ antibody is specific, and no unspecific interaction between anti-Aβ-antibody with BSA or PBS were detected.

In a second step, we decided to immobilize the SAP protein in the agarose and follow its interaction with the Aβ (see Fig.V.15). In step A, SAP protein (1), BSA (2), PBS (3) and inhinitor pepitde (4) were spotted on agarose slide (3μL per spot, all solution at 50nL per μL). After washing and blocking steps, a solution of Cys-Aβ40 (same buffer than in V, page 97) was incuated on the slide for 2 hours. Primary and then secondary antibody were incubated (see FigV.15C and D). The slide was read by the scanner. As expected, we obtained signal only with SAP protein and the inhibitor peptide (see Fig.V.15 and Fig.V.16, E1 and E4). Moreover, the Aβ did not interact with BSA as no signal was detected. A control slide, without incubation of Cys-Aβ40 peptide showed no unspecific interactions between SAP, BSA or PBS and antibodies.

We also tested the compatibility of this agarose microarray with MS. To do so, we immobilized BSA protein on a slide and we performed on-chip tryptic digestion (50-100 ng of trypsin

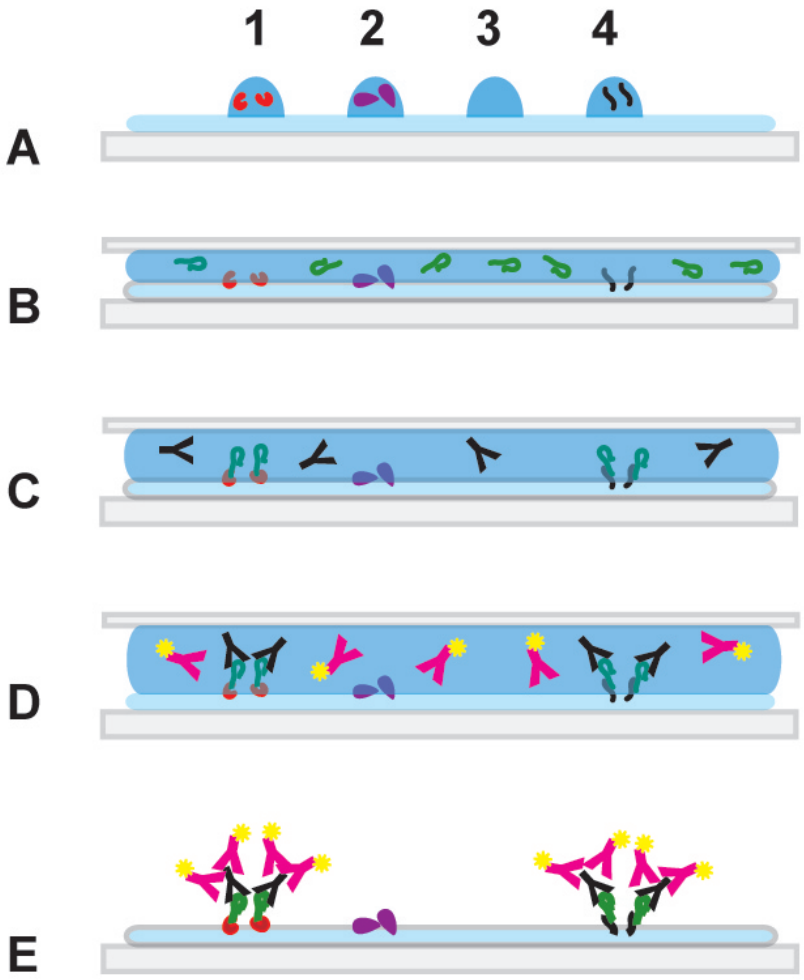


Figure V.15: Strategy used to study the interaction between the SAP protein and the Cys-Aβ40. (A) incubation of SAP (1), BSA (2), PBS (3) and inhibitor peptide (4).; (B), (C) and (D) represente the incubation with Cys-Aβ40, anti-Aβ antibody and secondary antibody respectively.; (E) shows the fluorescent signal of the secondary antibody on specific interaction of the primary antibody.

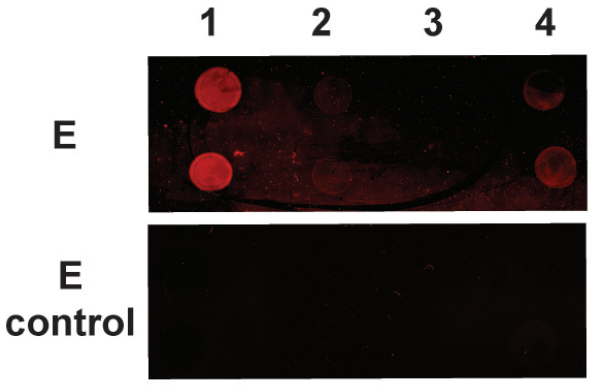


Figure V.16: Agarose slide of the SAP-Aβ interaction.

per spot in ammonium bicarbonate buffer 50mM, 5 hours, 37℃). The slide was kept wet during the digestion by adding ammonium bicarbonate buffer. After digestion, the peptides were extracted with a solution of 50% acetonitrile 50% H_2O and passed trough a micro-column to remove salts and concentrate the peptides. The peptides were eluted by 3μL of matrix (5 mg alpha-cyano-4-hydroxy-cinnamic acid per mL in 50% acetonitrile) directly on a MALDI-plate. We have been able to detect and identify BSA by MS analysis(seef Fig.V.17).

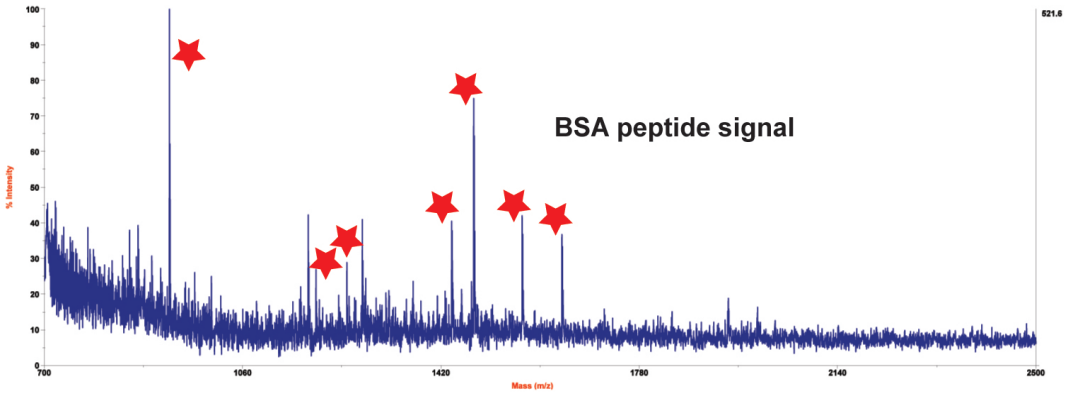


Figure V.17: MALDI-MS spectrum of BSA digested on an agarose slide. The stars indicate peaks tryptic peptides of BSA.

We also wanted to know if we could detect proteins of a complex by MS analysis after digestion of the complex on the agarose slide. The Aβ peptide is a small protei and its theoretical tryptic digestion generates 3 peptides. Because of this, it would be difficult to detect it by MS in a complex with larger protein such as SAP. Thus, we used another model complex formed by two proteins: NHS (15.6 kDa) [172] and YmoA (8 kDa)[173]. The first protein (NHS) was immmobilized on agarose and afterwards, the second protein (YmoA) was incubated on the slide. After washing steps, we performed tryptic digestion on the agarose slide. After extraction steps and elution trough a micro-column, we have been able to detect peaks from both proteins by MS analysis (see Fig.V.18).

We have shown that conventional techniques of protein and complex protein immobilization on agarose microarrays are compatible with on-chip proteolytic digestion for MS analysis. We are currently investigating the specificity of the protein interactions on agarose and the identification by MS of protein complexes formed on agarose after incubation of a mixture of proteins.

[172] C J Dorman. *Nat Rev Microbiol*, 2(5):391–400, 2004.

[173] G R Cornelis, C Sluiters, I Delor, D Geib, K Kaniga, C Lambert de Rouvroit, M P Sory, J C Vanooteghem, and T Michiels. *Mol Microbiol*, 5(5):1023–1034, 1991.

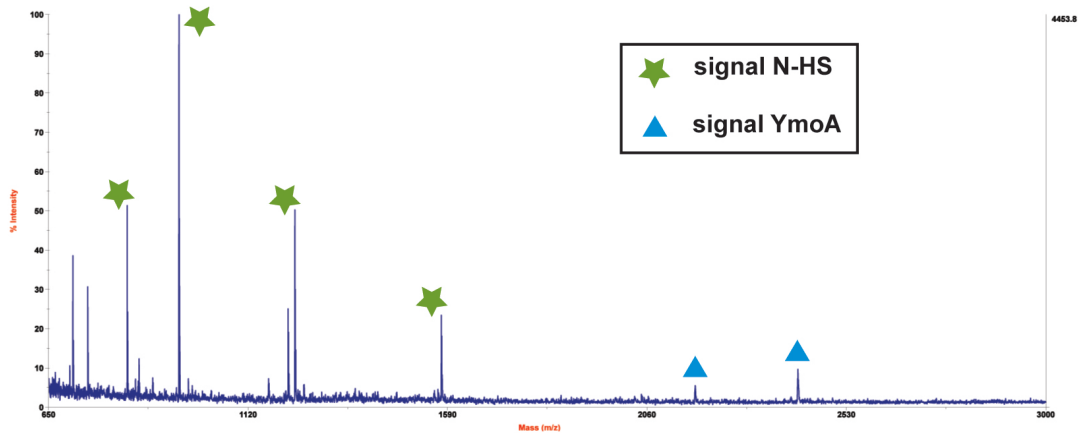


Figure V.18: MALDI-MS spectrum of the complex Ymoa-NHS digested on the agarose slide; the stars indicate peaks of NHS and thepolygons indicate peaks of YmoA.

Resum en catala

Resum de tesi d'Stéphanie Boussert :

« UTILITZACIÓ DE L'ESPECTROMETRIA DE MASSES PER A L'ESTUDI ESTRUCTURAL DE PROTEÏNES I L'ESTUDI DE LES INTERACCIONS PROTEIQUES NO COVALENTS. »

Aquesta tesi es basa en el desenvolupament i la utilització d'aproximacions basades en l'espectrometria de masses per a identificar i caracteritzar proteïnes o complexes no covalents de proteïnes.

En un primer moment hem emprat l'espectrometria de masses com a una aproximació alternativa i complementària a les aproximacions clàssiques de la biologia estructural, com són la cristal·lografia, ressonància magnètica nuclear,... Les interaccions febles no covalents, intra o intermoleculars, juguen un paper dins l'estructura i el funcionament del sistema biològic. És per això que es essencial obtenir informació sobre la identitat dels pèptids implicats en aquestes interaccions. L'estructura tridimensional de la subunitat proteica P62 del factor de transcripció TFIIH no es coneix. És per aquest motiu que hem desenvolupat un mètode d'identificació dels pèptids que estan enllaçats de manera no covalent a la subunitat del factor de transcripció TFIIH mitjançant la tècnica d'espectrometria de masses. Aquesta subunitat es va posar en presència d'enzims de digestió en concentracions creixents. L'anàlisi per espectrometria de masses dels pèptids resistents a la digestió, i prèviament separats mitjançant un gel d'electroforesi unidimensional, ens va permetre identificar els residus organitzats en dominis compactes de la seqüència primària de la proteïna, oferint així una apreciació de la seva organització modular.

L'espectrometria de masses també ens va permetre, mitjançant la cromatografia de líquids i l'espectrometria de masses en tàndem, trobar evidencies de la mutació d'un aminoàcid en un complex multiproteic no covalent. Aquesta mutació puntual de la cadena beta de l'hemoglobina humana, presenta la particularitat de no modificar les propietats físico-químiques de l'hemoglobina, això fa que no sigui detectable amb altres tècniques electroforètiques clàssiques.

En segon terme, ens vam interessar per l'estudi d'estructures de proteïnes i de complexes proteics no covalents sobre una superfície. La microscòpia de forces atòmiques ha estat escol-

lida com la tècnica més adient per a aquest tipus d'estudi. Així, vam estudiar i visualitzar per microscòpia de forces atòmiques, la formació de fibril·les per l'auto assemblatge d'un tetrapèptid.

Aquest primer resultat ens va portar a desenvolupar eines d'anàlisi per a les interaccions proteiques no covalents, compatibles amb les tècniques de nanotecnologia i espectrometria de masses. Així doncs, vam funcionalitzar una superfície amb el pèptid beta amiloide, amb la finalitat de caracteritzar la seva interacció amb proteïnes. Aquesta superfície d'or es funcionalitza mitjançant una cadena alquílica amb un extrem tiol per a assegurar la interacció amb la superfície i amb l'altre extrem un pentaetilenglicol, per a evitar una absorció inespecífica de proteïnes sobre la superfície. Aquestes cadenes modificades s'absorbeixen sobre la superfície per a formar una monocapa homogènia. Algunes d'aquestes cadenes modificades contenien una amina lliure que permetia la introducció d'un grup maleïmida, la qual va permetre la unió amb el pèptid beta-amiloide.

El pèptid beta-amiloide és un pèptid implicat en la malaltia d'Alzheimer. La seva acumulació i la seva agregació condueix a la formació de plaques senils, una de les lesions observades en la malaltia. La tendència natural d'agregació d'aquest pèptid fa que sigui molt difícil treballar en solucions aquoses. Recents treballs de síntesi en fase sòlida han permès la síntesi i la purificació del pèptid beta amiloide. L'estratègia de la síntesi es basa en la utilització d'un aminoàcid O-acil en la seqüència del pèptid en qüestió per a augmentar considerablement la seva solubilitat. Un cop obtingut, aquest O-acil isopèptid es pot convertir fàcilment en un pèptid N-acil (que és el pèptid original) mitjançant una reacció de reorganització intramolecular a pH bàsic. Utilitzant aquest mètode vam obtenir sense dificultat l'isopèptid soluble O-acil beta amiloide. L'originalitat d'aquest treball és basa en la utilització de l'isopèptid soluble O-acil beta amiloide, per a immobilitzar-lo principalment en forma monomèrica sobre la nostra superfície i permetre la seva reconversió en el pèptid original via reorganització molecular directament sobre la superfície. Aquesta superfície es va caracteritzar mitjançant espectrometria de masses i microscòpia de forces atòmiques tal d'assegurar la presència del pèptid beta-amiloide sobre la superfície.

Vam demostrar que la nostra superfície funcionalitzada amb el pèptid beta amiloide era funcional i permetia les interaccions no covalents pèptid-proteïna utilitzant un anticòs anti-pèptid amiloide. Aquesta interacció antigen-anticòs es va observar mitjançant microscòpia de forces atòmiques.

L'objectiu a llarg termini és utilitzar aquesta tècnica per a identificar noves interaccions entre el pèptid beta amiloide i proteïnes extretes de cervells de pacients amb Alzheimer en diferents estats de l'evolució de la malaltia.

El nostre model d'interacció sobre superfície és va validar amb altres proteïnes que inter-

accionen amb el pèptid beta amiloide i vam demostrar la seva compatibilitat amb els mètodes de proteomica actual. Així doncs, vam realitzar digestions enzimàtiques directament sobre la superfície permetent així la identificació de proteïnes mitjançant espectrometria de masses i espectrometria de masses en tàndem. En conclusió, l'espectrometria de masses s'ha establert com a eina potent per a la identificació i caracterització de proteïnes i complexes proteics no covalents. La seva sinergia amb la microscòpia de forces atòmiques ens ha permès el desenvolupament d'una superfície adequada per a l'estudi d'interaccions proteiques no covalents.

Rsum en francais

Résumé de thèse Stéphanie Boussert :

« UTILISATION DE LA SPECTROMÉTRIE DE MASSE POUR L'ÉTUDE STRUCTURALE DE PROTÉINES ET L'ÉTUDE D'INTERACTIONS PROTÉIQUES NON-COVALENTES. »

Les interactions faibles, non-covalentes, intra ou intermoléculaires jouent un rôle essentiel dans la structure et dans le fonctionnement de tous les systèmes biologiques. Il est donc essentiel d'obtenir des informations sur l'identité des peptides impliqués dans ces liaisons. Ce travail de thèse repose sur le développement et l'utilisation d'approches basées sur la spectrométrie de masse afin d'identifier et de caractériser des protéines ou des complexes protéiques non-covalents.

Dans un premier temps, nous avons utilisé la spectrométrie de masse comme une approche alternative et complémentaire aux approches classiques de la biologie structurale (cristallographie, résonance magnétique nucléaire,...) pour apporter des éléments d'information sur la structure d'une protéine dont la structure cristallographique est inconnue. La protéine étudiée, nommée P62, fait partie d'un complexe macromoléculaire, le facteur de transcription TFIIH.

TFIIH est impliqué dans plusieurs processus cellulaires importants : la transcription, la réparation de l'ADN par excision-resynthése de nucléotides et le cycle cellulaire. TFIIH est un facteur multi-protéique composé de 9 sous-unités. Chez l'homme, il est organisé en deux sous-complexes : le core-TFIIH (p34, p44, p52, p62 et XPB) et le CAK (cdk7, cyclin H et Mat). P62 est organisée en 3 domaines : amino-terminal, médian et carboxy-terminal. La structure du domaine N-terminal a été résolue par RMN. Notre étude a donc porté sur l'obtention d'informations structurales sur le domaine médian de P62 (P62M).

Cette sous-unité fut soumise à une protéolyse ménagée avec différentes concentrations d'enzymes de digestion spécifiques en conditions non-dénaturantes. Les sites de coupures ne participant pas la structure tridimensionnelle et situés à la surface de la protéine sont les plus accessibles et donc les premiers clivés, tandis que les sites de coupures impliqués dans l'architecture tridimensionnelle de la protéines sont eux moins accessibles aux enzymes et donc plus résistants. Après la protéolyse ménagée, le milieu de digestion fut séparé sur gel électrophorétique. Une protéolyse ménagée limitée dans le temps nous a permis d'isoler sur gel différents corps résistant selon la quantité d'enzymes présente dans le milieu de digestion. Les peptides non-résistants ne furent pas retenus dans le gel. Ces domaines résistants fut excisés du gel et soumis à une digestion trypsique classique de protéomique. Pour chaque domaine résistant, les peptides trypsiques ainsi générés furent extraits du gel et analysés par MALDI-MS. Les peptides identifiés par masse spectrométrie furent localisés sur la séquence primaire de P62M et nous avons ainsi pu délimiter le domaine commun résistant de la protéine. Ce domaine résistant mis en évidence par protéolyse ménagée

L'utilisation de plusieurs enzymes de restriction lors de la protéolyse ménagée nous a permis d'affiner l'identification des frontières de ce domaine et un séquence d'Edman nous a permis d'identifier les acides aminés de la partie N-terminal de P62M. La spectrométrie de masse, couplée à une protéolyse ménagée, s'est avérée un outil puissant, en absence de données cristallographiques, pour obtenir un aperçu de l'organisation modulaire d'une protéine. Des données, par résonance magnétique nucléaire et par des études de fluorescence, ont par ailleurs confirmé les résultats obtenus par spectrométrie de masse.

La spectrométrie de masse nous a également permis, par des couplages chromatographie liquide et spectrométrie de masse en tandem, de mettre en évidence une mutation d'un acide aminé dans un complexe multiprotéique non-covalent. Cette mutation ponctuelle, de la chaîne bêta de l'hémoglobine humaine, présente la particularité de ne pas modifier les propriétés physico-chimiques de l'hémoglobine, ce qui la rend indétectable au travers des approches électrophorétiques classiques.

L'hémoglobine humaine est un tétramère composé de deux globines alpha et de deux globines bêta liées par des liaisons faibles. Les sous-unités sont structurellement similaires et ont à peu près la même taille. Chaque globine a une masse moléculaire d'environ 16 000 daltons, pour une masse totale d'environ 64 000 daltons. Chaque globine de l'hémoglobine contient un hème, de telle manière que la capacité totale de liaison de l'hémoglobine pour le dioxygène est de quatre molécules. Sa principale fonction est le transport du dioxygène. L'hémoglobine se trouve essentiellement à l'intérieur des globules rouges du sang.

Une différence de 13Da fut mis en évidence par spectrométrie de masse electrospray sur une des chaînes bêta d'un lysat d'hémoglobine. Afin d'identifier la mutation, la chaîne mutée fut séparée des chaînes sauvages par chromatographie liquide et soumise à une digestion enzymatique en solution. Les peptides résultant de cette protéolyse furent séparés et analysés par un couplage chromatographie liquide-spectrométrie de masse electrospray (LC-ESI-MS). Le profil obtenu de la digestion fut comparé avec celui de la protéolyse théorique d'une chaîne bêta non-mutée. La différence de 13 Da fut ainsi rapidement localisée sur un peptide N-terminal de la séquence de la chaîne bêta. La séquence d'acide amines de ce peptide N-terminal laissa supposer que la mutation Thr/Ala serait a l'origine de la différence de 13Da. Pour confirmer cette hypothèse, ce peptide muté fut analysé par spectrométrie de masse en tandem (MALDI-TOF-TOF). Cette technique permet de fractionner les peptides et ainsiséquence d'obtenir la séquence du peptide étudié. La séquence obtenue confirma la mutation de l'acide aminé xx par xx, suspectée après LC-ESI-MS.

L'approche classique pour détecter des mutants dans un complexe de protéines, et spécialement dans l'étude sur les hémoglobines, est une approche par électrophones. Cette méthode est valide quand la mutation implique altère les propriétés physico-chimiques de la protéines. Dans notre étude, la mutation présente dans la chaîne bêta n'a pas été détectée par électrophorèseséquence, et est donc considérée comme une mutation silencieuse. Néanmoins, la différence de masse résultant de cette mutation, a pu être mise en évidence par spectrométrie de masse. Cette mutation fut donc caractérisée et identifiée sans avoir recours à un séquencage de l'ADN. La spectrométrie de masse est particulièrement utile lors de la recherche de mutations post-translationelles (acetylation, glycosylation) de protéines. En effet ces mutations

ont lieu après l'expression de la protéine et sont donc absentes dans le génome.

Dans un deuxième temps, nous nous sommes intéressés à l'étude de structure de protéines et de complexes protéiques non-covalents sur une surface. Les récents progrès et développements en microscopie à force atomique ont permis une nouvelle approche dans l'étude structurale de protéines et complexes de protéines sur support. La microscopie à force atomique est une technique permettant d'analyser le relief à l'échelle atomique.

Doté d'une pointe placée sur un levier flexible, le microscope à force atomique enregistre les interactions entre les atomes de la pointe et ceux de la surface à analyser. Il se produit soit une attraction, appelée force de Van des Waals, soit une répulsion (à très faible distance). Ces forces provoquent des déplacements de la pointe, entraînant des déviations du levier qui sont enregistrées et traitées par ordinateur pour donner le relief.

La technique de microscopie à force atomique nous a ainsi permis d'étudier le comportement en solution d'un bêta-tetrapeptide synthétique. Les acides aminés sont à la base de la constitution des protéines et autres peptides, même s'ils n'en sont pas les uniques constituants (par exemple l'hème, groupement prosthétique de l'hémoglobine). L'atome de carbone sur lequel est fixé le groupement amine -NH2 et le groupement acide carboxylique -COOH est appelé par convention carbone alpha. Ces acides aminés sont appelés acides aminés alpha. Un peptide bêta est par définition un peptide dont le groupement amine est porté non pas sur le carbone alpha mais sur le carbone bêta. Par conséquent la chaîne peptidique des peptides bêta est sensiblement plus longue que la chaîne peptique d'un peptide alpha, entraînant une structure secondaire différente de celle rencontrée avec les acides amines alpha. Cette conformation confère aux peptides bêta une résistance particulière aux dégradations de protéases. Cette résistance fait des peptides bêta des candidats idéaux pour la conception de médicaments.

Dans notre étude nous avons utilisé les techniques de résonance magnétique nucléaire, de microscopie à transmission électronique et la microscopie à force atomique comme approches biophysiques afin de caractériser et étudier le comportement en solution d'un bêta tétrapeptide

synthétique.

Les études par résonance magnétique nucléaires ont montré que ce tetrapeptide bêta avec une longueur théorique de 2.2 nanomètres. Les études par microscopie a transmission électronique ont révélé la formation de fibrilles homogènes après 24 heures d'incubation en solution. Finalement, une solution fraîchement préparée de bêta-tetrapeptide fut adsorbée sur une surface de mica et analysée par microscopie à force atomique. Nous avons ainsi pu visualiser des molécules de bêta-tetrapeptide isolées sur la surface, la hauteur mesurée de ces molécules correspondait a la mesure théorique calculée par résonance magnétique nucléaire. Une solution de tétrapeptide bêta, incubée pendant 24 heures, fut également analysée par microscopie à force atomique. Les informations obtenues, nous ont permis de visualiser de des fibrilles similaires à ceux observée par microscopie à transmission électronique. Une certaine disparité put être observée dans la hauteur mesurée des fibrilles. La taille mesurée pour les fibrilles de basse amplitudes correspond à la taille théorique calculée pour le tetrapeptide bêta indiquant que les fibrilles se forment par auto-assemblage d'unités de tetrapeptide bêta. Cet auto-assemblage en fibrilles est probablement conditionné par une redistribution des liaisons hydrogènes intramoléculaires. Une plus grande complexité dans l'arrangement observé des fibrilles de plus hautes amplitudes, ne nous a pas permis proposer un modèle de structure. En effet, les amplitudes des fibrilles mesurées ne sont pas multiples de 2.2, suggérant une plus grande complexité dans l'arrangement de plusieurs fibrilles. L'orientation du tetrapeptide bêta le long du fibrille n'a pas été élucidée. Une étude par Scanning Polarization Force Microscopy permettant une analyse de distribution des charges sur la surface, devrait pouvoir apporter des éléments de réponse concernant l 'organisation de cette architecture intermoléculaire.

Ainsi par microscopie à force atomique, nous avons confirmé la propriété de notre tetrapeptide bêta à former par auto-assemblage des structures en solution. La microscopie à force atomique s'est révélée particulièrement bien adaptée a cette étude sur surface.

Ce premier résultat sur surface, nous a incité à développer un outil d'analyse des interactions non-covalentes protéine/protéine compatible avec les techniques de nanotechnologie et de spectrométrie de masse. La microscopie à force atomique a cette unique capacité à pouvoir

explorer la surface d'un échantillons biologique dans des solutions physiologiques, permettant de visualiser aussi bien des structures biologiques complexes que des molécules uniques dans leur état fonctionnel.

Notre projet fut donc de construire une surface fonctionnalisée avec le peptide amyloïde afin de pouvoir étudier sur la surface des interactions spécifiques peptide-protéine / protéine-protéine par microscopie à force atomique et par spectrométrie de masse. Cette étude repose sur trois paramètres : la fonctionnalisation adéquate d'une surface afin d'éviter toute adsorption non spécifiques de protéines sur la surface, l'obtention du peptide amyloïde soluble et une compatibilité d'étude par microscopie à force atomique et spectrométrie de masse.
Les surfaces d'or présentent l'avantage d'être facilement fonctionalisables, principalement par la formation de monocouches auto-assemblées (self-assembled monolayer). Cette technique se base sur la forte adsorption de molécules thiol-alcane sur la surface en or. Ces molécules thiol-alcane s'assemblent sur la surface avec un haut degré d'organisation sans intervention externe. Le thiol s'adsorbe très fortement avec la surface en or (force de liaison comparable à d'une force de liaison covalente) tandis que la chaîne alcane aide au compactage de la monocouche et peut être modifiée avec un groupement fonctionnel afin de modifier les propriétés de la surface. L'ajout de groupements éthylène glycol sur ces alcanes contribue à minimiser les interaction non-spécifiques de protéines sur la surface. Dans notre étude nous avons synthétisé sur phase solide deux types de thiol-alcane-éthylène-glycol molécules, l'une d'entre elles fut modifiée, lors de la synthèse, avec un groupement amine attaché après les groupement éthylène glycols. Une solution contenant ces deux molécules fut ensuite incubée sur une surface en or, permettant ainsi la formation d'une monocouche auto-assemblée. Cette monocouche présente ponctuellement à la surface un groupement amine appartenant aux thiol-alcane-éthylène-glycol modifiés. Ce groupement amine a permis de lier de façon covalente une molécule bi-fonctionelle jouant le rôle de lieur entre la surface et le peptide à immobiliser. Une fois le lieur attaché sur la surface, le peptide amyloïde fut immobilisé sur la surface.
Le peptide bêta-amyloïde est un peptide impliqué dans la maladie d'Alzheimer. Son accumulation et son agrégation conduisent à la formation de plaques séniles, une des lésions observée dans la maladie. Du fait de sa tendance naturelle à s'agréger il est très difficile de travailler

avec le peptide bêta-amyloïde en solutions aqueuses, et son obtention par voies synthétiques classiques est très difficile. De récents travaux en synthèse de peptides en phase solide ont permis de synthétiser et purifier le bêta-amyloïde peptide. Cette synthèse est basée sur l'utilisation d'un acide aminé "O-Acyl" dans la séquence d'un peptide, augmentant ainsi considérablement sa solubilité. Une fois obtenu, cet O-Acyl isopeptide peut être converti facilement en N-Acyl-peptide (peptide natif) dans des conditions de pH basiques par une réaction dite de réarrangement intramoléculaire de groupement acyle d'O vers N. Utilisant cette méthode nous avons obtenu sans difficulté le O-Acyl- bêta-amyloïde isopeptide soluble. La synthèse en phase solide nous a permis d'ajouter facilement un résidu cystéine du cote N-terminal de la séquence. Cette cystéine va permettre l'ancrage du peptide bêta-amyloïde sur le lieur déjà présent sur la surface. L'originalité de ce travail repose sur l'utilisation de cet O-Acyl-bêta-amyloïde isopeptide soluble en milieu acide pour l'immobiliser principalement sous forme monomérique sur notre surface. Sa reconversion en peptide natif via le réarrangement intramoléculaire s'est faite après son immobilisation et directement sur la surface. Avec cette stratégie nous avons pu minimiser les risques d'agrégation du peptide bêta-amyloïde en solution et permettre sa fixation sur la surface sous forme principalement monomérique.

La présence du peptide bêta-amyloïde sur la surface fut vérifiée spectrométrie de masse et microscopie à force atomique. L'analyse par spectrométrie de masse fut faite directement sur la surface. Cette technique est appelée SAMDI-MS, pour "self-assembled monolayer desorption ionization mass spectrometry". Le signal en masse obtenu confirma la présence du peptide bêta-amyloïde et son intégrité sur la surface. La taille du peptide mesurée par microscopie à force atomique nous a permis de confirmer son repliement en épingle à cheveux, et donc que le réarrangement intramoléculaire sur la surface ait bien eu lieu.

Une fois le bêta-amyloïde immobilisé sur la surface, nous avons testé sa capacité d'interaction avec un anticorps anti bêta-amyloïde. Cette interaction fut caractérisée par microscopie à force atomique et immunofluorescence. Après incubation avec un anticorps anti bêta-amyloïde, la surface fut analysée par microscopie à force atomique. Nous avons ainsi pu visualiser le complexe bêta-amyloïde peptide - anticorps anti bêta-amyloïde peptide sur la surface. La taille mesurée de ce complexe est conforme à la taille attendue de ce complexe. De plus, après in-

cubation avec un anticorps secondaire, la présence de l'anticorps anti bêta-amyloïde, et donc du peptide bêta-amyloïde, fut mise en évidence par immunofluorescence. Nous avons ainsi montré que notre surface fonctionnalisée avec le peptide bêta-amyloïde était fonctionnelle et permettait les interactions non-covalentes peptide/protéine.

L'objectif à long terme est d'utiliser cette technique pour identifier de nouvelles interactions entre le peptide bêta-amyloïde et des protéines extraites de cerveaux de patients à différents stades de l'évolution de la maladie. Nous avons donc validé notre modèle d'interaction sur surface avec la protéine sérum amyloid P component (SAP), connue pour interagir avec le peptide bêta-amyloïde. Dans cette dernières étude, nous avons incubé la protéine SAP, puis après lavages, procédé à une digestion trypsique directement sur la surface. Les peptides résultant de la digestion furent extraits et analysés par spectrométrie de masse. L'analyse par spectrométrie de masse nous a permis d'identifier la protéine SAP, et donc de confirmer sa présence et son interaction avec le peptide bêta-amyloïde. Nous avons montré que notre surface était compatible avec les méthodes de protéomique actuelles.

En conclusion, la spectrométrie de masse s'est avérée un outil puissant dans l'identification et la caractérisation de protéines et complexes protéiques non covalents. Son couplage avec la microscopie à force atomique nous a également permis la mise au point d'une surface adéquate à l'étude d'interactions protéiques non-covalentes.