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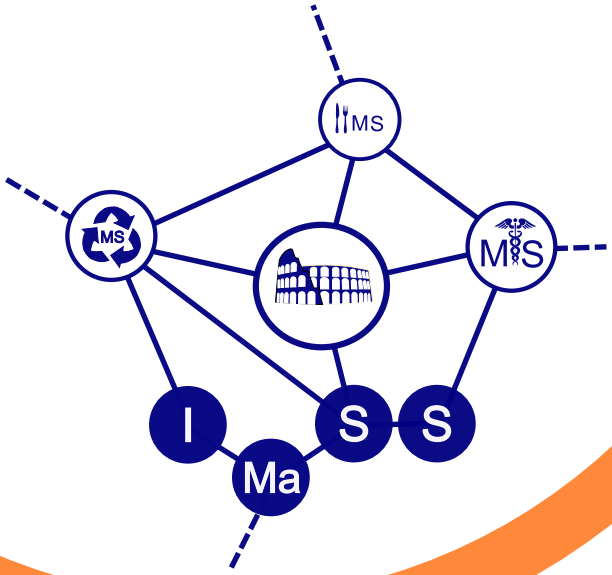

Italian Mass Spectrometry Society

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1st IMaSS NetWork

Innovation, Food Analysis & Healthcare Research

Roma, 26-27 Maggio 2014
Teatro Centrale Carlsberg

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BRUKER

Monday, May 26th

14.00 - 15.00 *Registration*

Opening ceremonies

15.00 - 15.10 Welcome
Dr. Silvia Catinella - IMaSS President
(Chiesi Farmaceutici S.p.A., Parma, IT)

15.10 - 15.30 IMaSS presentation
Dr. Enrico Davoli - IMaSS Vice-president
(IRCCS Istituto Mario Negri, Milano, IT)

Innovation on MS instrumentation
Chair: Dr Mahmoud Hamdan

15.30 - 16.00 MALDI on tissue: Pitfalls on the road to hope
Prof. Daniel Lafitte
University Aix-Marseille (Marseille, FR)
Sponsored by Shimadzu Italia

16.00 - 16.30 Glycoprotein Analysis for Biomarker Discovery Using a Benchtop Orbitrap Mass Spectrometer
Dr. Marco Gaspari
Università "Magna Graecia" (Catanzaro, IT)
Sponsored by Thermo

16.30 - 17.00 SWATH Proteomics: Sample Preparation and Applications
Dr. Floriana Capuano
University of Cambridge (Cambridge, UK)
Sponsored by ABSciex

17.00 - 17.20 Open discussion

17.20 - 17.40 *Coffee Break*

Food Analysis

Chair: Prof. Arnaldo Dossena
Università degli Studi di Parma (Parma, IT)

17.40 - 18.10 The increasing role of mass spectrometry in understanding and preventing food allergies
Prof. Stefano Sforza
Università degli Studi di Parma (Parma, IT)

18.10 - 18.40 Mass Spectrometry and Food Analysis Nowadays. How heavy is an electron?
Dr. Emiliano De Dominicis
Chelab-Silliker, Mérieux NutriSciences Company (Resana (TV), IT)

18.40 - 19.00 Open discussion

19.00 - 19.40 **Nanomechanical systems for single-particle sensitivity and neutral particle Mass Spectrometry**
Dr. Sebastien Hentz
CEA - LETI (Grenoble, FR)

19.40 - 20.30 Cocktail

Tuesday, May 27th

Clinical Laboratory

Chair: Dr. Antonello Nonnato
A.O.U. Citta della Salute e della Scienza di Torino (Torino, IT)

9.00 - 9.40 **The pivotal role of mass spectrometry for the "omics" in Personalized Medicine**
Prof. Maurizio Simmaco
Università "La Sapienza" (Roma, IT)

9.40 - 10.10 Mass spectrometry imaging for tissue mapping of low molecular weight compounds: facts and expectations
Dr. Maurizio Ronci
Mawson Institute, University of South Australia (Adelaide, AU)

10.10 - 10.40 ^{13}C-Phenyl lipid metabolic analysis in Zebrafish embryos by LC-HRMS
Dr. Federica Dal Bello, Dr. Chiara Martano
Università di Torino (Torino, IT)

10.40 - 11.00 Open discussion

11.00 - 11.20 *Coffee Break*

Proteomics

Chair: Dr. Simona Scarpella
Nerviano Medical Sciences (Nerviano, IT)

11.20 - 11.50 Quantitative mass spectrometry in biomedical research
Dr. Angela Bachi
IFOM (Milano, IT)

11.50 - 12.20 Use of mass spectrometry for vaccines discovery
Dr. Nathalie Norais
Novartis Vaccines & Diagnostics (Siena, IT)

12.20 - 13.00 Round table
Dr. Silvia Catinella - Chiesi Farmaceutici
Dr. Enrico Davoli - Istituto Mario Negri
Dr. Pietro Franceschi - FEM

13.00 - 14.30 Lunch

Any Other Business MS

Chair: Dr. Barbara Pioselli
Chiesi Farmaceutici S.p.A. (Parma, IT)

14.30 - 15.00 Working in a bioanalytical facility: addressing ADME and OMICS for many different projects. And results by yesterday, please…
Dr. Andrea Armirotti
Istituto Italiano di Tecnologia Genova (Genova, IT)

15.00 - 15.30 Label Free Absolute Quantitation of Human Milk Proteins using Multiple Reaction Monitoring
Dr. Rocchina Sabia
Università "La Sapienza" (Roma, IT)

15.30 - 16.00 Hair analysis for the study of alcohol, caffeine and nicotine abuse in adolescents
Dr. Fabio Vaiano
Università degli Studi di Firenze (Firenze, IT)

16.00 - 16.40 **Advanced analytical technologies for Cultural Heritage**
Dr. Vincenzo Palleschi
ICCOM, CNR U.O.S. di Pisa (Pisa, IT)

Closing ceremony

16.40 - 17.00 Final remarks
Dr. Pietro Franceschi
Fondazione E. Mach
(S. Michele all'Adige, Trento, IT)

Contents

Innovation on MS instrumentation

MALDI on Tissue: Pitfalls on the Road to Hope

Daniel Lafitte

Aix Marseille Universit, PIT2 group, School of Pharmacy, Marseille (France)

MS-based imaging technologies are label-free techniques using various ionization modes such as MALDI, desorption electrospray ionization (DESI) or SIMS. As conventional imaging methods, Label-free measurements allow detection of many molecules simultaneously. Pharmaceuticals, proteins, lipids, sugars and nucleic acids, as well as pollutants such as metals, are easily located. Small molecule localization has primarily been investigated using radiolabeled probes. MSI provides complementary features that overcome many labeling imaging pitfalls such as the inability to distinguish between the parent compound and metabolites, long exposure time or labeling decay. MSI technologies combine the molecular specificity of MS for separation between parent drugs/metabolites and the spatial distribution information of radiolabeling techniques. Identification of molecules on tissue coupled to MALDI imaging is challenging. We present here various strategies to obtain from small to big molecule identification without the need of complex purification. We present also the advantages and limits and the future improvement for direct in situ analysis of molecules.

Glycoprotein Analysis for Biomarker Discovery Using a Benchtop Orbitrap Mass Spectrometer

Marco Gaspari

Proteolab@UMG, Department of Experimental and Clinical Medicine, Magna Graecia University of Catanzaro, Catanzaro, Italy

Protein glycosylation is a key post-translational modification present in the majority of cellular proteins expressed on the cell surface and in the extracellular space. Glycoproteins play many biological roles such as signal transduction, cell adhesion, and cell migration. Furthermore, many studies have associated abnormal expression of N-linked glycoproteins to various diseases, such as cancer, suggesting that glycoproteins may be used as biomarkers for cancer diagnosis.
This work presents data on glycoprotein analysis in the context of colorectal cancer (CRC). N-linked glycoproteins in CRC tissue samples were compared to adjacent healthy colorectal tissue in 19 patients undergoing CRC surgery. Tissue proteins were extracted and digested by trypsin; then, glycopeptides were isolated using solid phase extraction of glycopeptides (SPEG); finally, the enriched peptides were deglycosylated by PNGase F. Peptides obtained from the same patient were mixed in pairs (healthy vs. diseased) after ^{18}O stable isotope labeling of the tumor-derived peptides, in order to achieve relative quantification. The mixtures of formerly N-glycosyltated peptides were analysed by nanoLC coupled to a benchtop Orbitrap-based mass spectrometer (Q-Exactive) operating in data-dependent mode. Over 50 glycoproteins were found to be up-regulated in CRC tissue, many of which are known to be of extracellular or membrane origin.
Strategies for the on-going validation of candidate markers in serum will be also presented.

SWATH Proteomics: Sample Preparation and Applications

Floriana Capuano

University of Cambridge

Mass spectrometry experiments enable the identification of a high number of proteins per single experiment. This technique effectively complements the classic techniques for protein analysis such as western blotting or enzyme-linked immunosorbent assays (ELISA). In recent years, the advent of second generation mass spectrometers has favoured the shift in focus of mass spectrometry experiments from protein identification towards protein quantification. Further, an increasing interest has been raised for label-free quantification which has several advantages over methods based on the use of labelled standards, such as lower experimental costs and allowing for the comparative analysis of multiple samples. In particular in label-free quantitative mass spectrometry, sample preparation can substantially affect protein analysis. It is therefore crucial that strategies are implemented to compensate for protein loss during sample preparation. In our laboratory we have evaluated the performances of several sample preparation workflows including a selection of in gel, on filter, and in solution digestion procedures, in combination with a state of the art mass spectrometry technique known as SWATH (Selected Window Acquisition of All Theoretical Precursors) to identify the optimal sample preparation protocols for protein analyses. We have scored the protocols on the basis of several factors, including the number of proteins detected and precisely quantified, using both data dependent and data independent acquisition modes, the proteome coverage and finally, the costs. We concluded that as the different protocols cover different set of proteins and cellular compartments, the decision for selecting the optimal protocol has to be made on the basis of the biological question and the nature of the samples.

Furthermore, we have applied SWATH-MS to the analysis of a diverse nature of samples to address several biological questions, spanning from characterizing the cellular response to oxidative stress in yeast to understanding the molecular mechanisms responsible for sex hormone-driven behavioural plasticity and sexual dimorphism in singing birds.

The role of polyamines in cell cycle regulation and stress response Survival during stress conditions requires rapid cellular adaptations mediated by the stress response machinery. By exposing the non essential knock out deletion collection to H_2O_2 a yeast mutant was isolated that showed increased resistance to this oxidant. This mutant also showed to re-enter the cell cycle faster than the wild type following oxidant exposure. The sequencing analysis showed that the mutant lacked the polyamine membrane transporter TPO1. The molecular function of polyamines is still under debate, however evidence shows that exposure to these compounds prolongs the life span of several organisms and reduces the damages caused by various oxidants. The measurement of intra- and extracellular polyamines showed in our study that the mutant accumulated polyamines, while the wild type and the strain

over-expressing the transporter had a decreased polyamine content. As polyamines influence translation, the proteome of cells was analysed during the oxidative stress response. By SWATH-MS 404 proteins were robustly identified and quantified for the wild type and the mutants. The proteome profiles recorded at different time points after exposure to H_2O_2 and analysed by similarity clustering showed an increased expression of stress related proteins such as Hsp70, Hsp90, Hsp104 and Sod1. Moreover, their expression occurred faster in the TPO knockout mutant than in the wild type. Meanwhile, in the sensitive TPO1 over-expressing strain their induction was delayed. Co-clustering analysis further identified 18 antioxidant enzymes, ribosomal components, chaperones and nucleotide synthesis factors (Pnc1) that followed the same pattern as Hsp104. To analyse this data a targeted approach of spectral data extraction for representative peptides was used in addition to the non targeted approach. After normalisation of peak intensities using a reference protein (Tdh1), the expression profiles of Hsp70, Hsp90 and Hsp104 confirmed previous results from the non targeted approach, showing a faster accumulation of these proteins over time for the TPO1 deletion mutant as compared to the wild type. Meanwhile, no up-regulation of these proteins was observed in the over-expressing strain. We therefore conclude that TPO1 is involved in controlling the level of polyamines during oxidative stress and coordinates two central responses: induction of stress related proteins and extension of H2O2-induced cell cycle arrest. Our work also highlights the innovative principle of controlled export of metabolites in the dynamics of the cellular stress response.

The Serinus canaria genome project: How does the canary sing? Canaries are used as a model to understand the molecular mechanism of speech learning, memory formation, sex hormone-driven behavioural plasticity and sexual dimorphism. The Serinus canaria genome project led by Professor Manfred Gahr (Max Planck Institute for Ornithology, Seewiesen) has achieved a high-quality sequence of the entire 1.2 Gbp canary genome. This project's aim is to establish the mechanisms responsible for the hormone-dependent circuit recruitment in the canary that can explain the species-specific and the hormone-dependent singing behaviour. SWATH-MS, performed on one of the major hormone-sensitive song-control brain regions (HVC) as well as on the non-hormone-sensitive, non-vocal brain area (the entopallium, ENT) identified 13 proteins of interest, mainly involved in cellular signalling. Further, 10 out of the 13 proteins had a strong positive correlation with the corresponding mRNA. For example, the endophilin SH3GL2 had a substantially higher protein expression level (as predicted from its RNA level), whereas the calcium-transporting ATPase (ATP2B1) levels were lower (as its mRNA expression would indicate). This data therefore suggests that for some of these proteins, the post-translational control of gene expression is crucial.

Food Analysis

The increasing role of mass spectrometry in understanding and preventing food allergies

Stefano Sforza

Department of Food Science, University of Parma, Italy

Food allergies are a public health issue of primary concern, affecting an increasing number of subjects, with a high variability among countries and for the type of allergen involved. The prevalence of food allergies has been estimated to be around 1-3% in adults and 4-6% in children. Globally, 220-250 million people are estimated suffer from food allergy.

When studying food allergy, information at the molecular level about the allergenic proteins involved are of paramount importance. Exact determination of primary sequences, epitope identifications, isoform characterization, allergen purification, resistance to gastrointestinal digestion, localization in the food material, modifications after technological treatment, are all needed in order to assess the impact of the allergens. Moreover, detection of allergens in foods is also essential in order to ensure the protection of the allergic consumers. Mass spectrometry can give fundamental contributions in all these aspects, often faster and more reliably than traditional techniques.

In this presentation several examples will be presented in which mass spectrometry played a fundamental role in dealing with food allergens. The exact sequence of peach Lipid Transfer Protein (LTP), one of the major allergen of the Mediterranean area, could be assessed by low and high resolution mass spectrometry. MALDI imaging could also provide the exact localization of LTP in different food materials, such as peach and tomato. The resistance of various LTP (peach and plum) to simulated gastrointestinal digestion was verified by analyzing the digested mixtures by LC/MS, which allowed both to quantify the remaining intact protein and to identify the major species formed upon digestion. One of the major wheat allergens, the CM3 protein, was identified by using LC/MS, and its content determined by digesting the wheat protein extracts and quantifying a specific marker peptide, allowing to conclude that the amount of this allergen in wheat is strictly related to the place of cultivation. Finally, the peptides generated upon gastrointestinal digestion by gluten, which are responsible for the celiac disease, were identified by LC/MS and LC/MS/MS in different wheat varieties, demonstrating that the toxicity of wheat for celiac patients is dependent upon genetic determinants, and that less toxic varieties might be accessible. Marker peptides for gluten can also be used for LC/MS/MS quantification of gluten in different food products, usually more reliably than other analytical techniques such ELISA.

These results demonstrate how mass spectrometry, with its unique ability to identify compounds in complex mixtures, is becoming an indispensable tool in food allergy studies.

Mass Spectrometry and Food Analysis Nowadays. How heavy is an electron?

Emiliano De Dominicis, Gianluigi Boschello, Marialuisa Piva, Alberto Stocco

R&D Department Chelab-Silliker, a Mrieux NutriSciences Company, Resana, Italy

In Food Analysis field, mass spectrometry has become by now the most preferred technique, especially in relation to target determination of residues of contaminants, additives, toxins, pesticides, antibiotics and hormones. In the first decade of 2000s, mass spectrometry, in our company, as well as in all analytic field, established itself as triple quadrupole sensible and selective analyzer, both for multiresidual determinations and for single molecules or specific chemical classes analyses. Some examples and applications, especially in milk and dairy products analyses, will show this already well-known and mature approach both at national and world wide level.

Yet, the use of mass spectrometry is increasingly being completed by screening approaches generally based on TOF/HRMS technology able to characterize, compare and therefore identify and observe intentional adulteration (fraud) and/or unintentional adulteration (environmental and/or of process) and able to study in details aspects linked to contaminants metabolism not yet considered by regulation, but potentially source of danger at toxicity level. Also at authenticity and food integrity level, such technologies, with the assistance and the support of chemiometry, are able to face/solve complex and uneasy analytical problems. Some examples and applications will show how mass spectrometry is entering this Food Analysis field.

The presentation will be completed by a curious and interesting technical occurrence linked to a real study performed for industry through which, by comparison between significantly different expected data and experimentally obtained data, we were authors/spectators of an absolutely to share and discuss experience: weigh an electron.

Neutral Particle Mass Spectrometry

Nanomechanical Systems For Single-Particle Sensitivity and Neutral Particle Mass Spectrometry

E. Sage[1], A. Brenac[2], T. Alava[1], R. Morel[2], M. S. Hanay[3], S. Kelber[3], M. L. Roukes[3], L. Duraffourg[1] and S.Hentz[1]

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[2] *INAC/SP2M and Universit Joseph Fourier, CEA Grenoble, France*
[3] *Kavli Nanoscience Institute and Departments of Physics, Applied Physics, and Bioengineering, California Institute of Technology, CA, USA*

Nano Electro Mechanical Systems (NEMS)-based Mass Spectrometry (MS) holds great promise for biological research as well as biomedical applications, with unique attributes like unprecedented resolution and speed [1]. While bottom-up devices have recently demonstrated ygrange limit of detection [2], single-protein NEMSMS [3] has recently been reported with scalable topdown silicon devices. These devices operate in a high mass range where particles display a broad mass distribution. The comparison of NEMS-MS with a reference measurement like conventional ion-based MS is crucial for future applications as there is no mass standard in this range.

We describe here how a NEMS resonator was introduced in a setup allowing mass sensitivity calibration, NEMS-MS operation as well as Time of Flight (TOF) MS.

Fig. 1 shows the resonator used here. It consists in a single-crystal silicon doubly-clamped beam, 160nm thick, 300nm wide and 10m long. It has been designed for simultaneous tracking of the two first modes for both particle mass and position measurement [3]. It is electrostatically actuated and transduced thanks to piezoresistive nanogauges; electrical characterization uses a downmixing scheme [4]. The fabrication process is a VLSI 200mm CMOS-compatible process starting with a p-doped SOI wafer [4].

The mass deposition is performed in the deposition chamber of a sputtering-gas aggregation setup able to produce metallic clusters with tunable deposition rate and diameter (ranging from 4 to 10 nm in this study). The cluster mass distribution can be measured by both TOF-MS and NEMS-MS (see Fig. 2). The cluster growth is performed by sputtering a metallic target with a magnetron head inserted in a liquid nitrogen cooled chamber. By collisions with the cold argon gas, the sputtered atoms aggregate in the growth zone before being expelled into the high vacuum deposition chamber.

The first step consists in deducing the NEMS mass sensitivity by measuring its frequency shift rate during cluster deposition at high flux rate. Different mass deposition rates are obtained by tuning the parameters of the cluster source and are then measured by the QCM (while the NEMS is moved away from the cluster beam). Typical sensitivities are 12 Hz/ag and 30 Hz/ag for modes 1 and 2 respectively. These figures agree well with expected values.

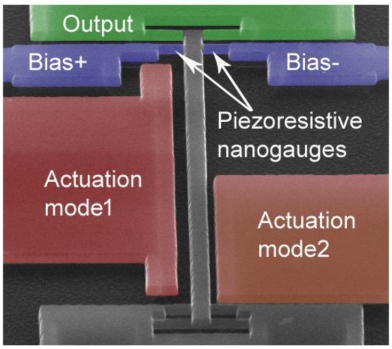

Figure 1: Typical doubly clamped resonator used for NEMS- MS allowing for two-mode operation

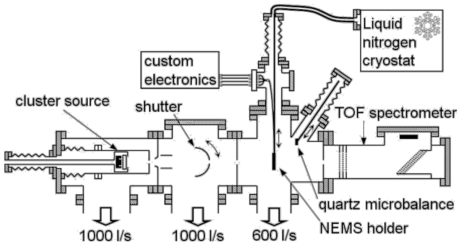

Figure 2: Schematic of the sputtering gas aggregation setup (see [5] for a full description) de operation

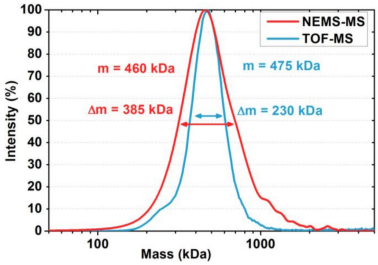

Figure 3: Comparison of mass spectra from TOF-MS and NEMS-MS of tantalum clusters. The mass is displayed in kilodalton. 1 dalton = 1.66 10^{-27} kg

In a second step, tantalum clusters were deposited at lower flux rates so all individual particle landings could be detected thanks to a fast Phase Lock Loop. Each jump is translated into a mass event with its associated uncertainty [3]. The latter is deduced from prior measurements of both mass sensitivity and frequency stability: Allan deviations of both modes are on the order of 10-7, yielding a mass resolution of a few 10kDa, very close to the TOF spectrometer resolution in this high mass range.

Fig. 3 shows the first comparison between TOF-MS and NEMS-MS spectra obtained with clusters of about 500kDa. The central mass is reproduced with fidelity by NEMS-MS. On the other hand, the Full Width at Half Maximum (FWHM) is slightly larger with the NEMS spectrum. This is not the case anymore at higher masses ($>$ MDa): as opposed to TOF-MS, the NEMS displays a constant mass resolution over the whole measurement range: it is ideally suited for ultra-high mass species ($>$ 10 MDa), a range inaccessible today for conventional ion-based MS. The ability of NEMS-MS to measure neutral species will be demonstrated. This ability makes it a technology ideally suited for Native MS experiments to study, for example, intact macromolecular complexes, whole viruses or therapeutic antibodies.

Acknowledgements
This work has been partially funded by the project Carnot NEMS-MS.

References

1. A. Boisen, Mass spec goes nanomechanical, Nature nanotechnology, vol. 4, pp. 404-405, 2009.
2. J. Chaste et al, A nanomechanical mass sensor with yoctogram resolution, Nature Nanotechnology, vol. 7, pp. 301-304, 2012.

3. M. S. Hanay et al, Towards single-molecule nanomechanical mass spectrometry, Nature Nanotechnology, vol. 7, pp. 602-608, 2012.
4. E. Mile et al, In-plane nanoelectromechanical resonators based on silicon nanowire piezoresistive detection, Nanotechnology, vol. 21, pp. 165504, 2010.
5. R. Morel et al, Growth and properties of cobalt clusters made by sputtering gas-aggregation, Eur. Phys. J. D, 24, pp. 287-290, 2003.

Clinical Laboratory

The pivotal role of mass spectrometry for the "omics" in Personalized Medicine

L. Lionetto[2], G. Gentile[1,2], M. Borro[1,2], and M. Simmaco[1,2]

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The one size fits all approach of medicine is clearly inadequate to face the societal challenges of the next future. In the last decade, the exponential growth of scientific knowledge about the molecular features of pathophysiology and of drug metabolism and action, joined to the availability of robust and sensitive technologies for high-throughput analysis of the individual genomic profile, made closer the clinical set up of Personalized Medicine. That is, the "generic aspect" of the classically meant systems of diagnosis and care will be replaced by personalized diagnosis, based on evaluation of the individual genomic profile and metabolic competence, followed by tailored and integrated therapeutic strategies. Personalized Medicine will have an outstanding impact upon key issues of the health systems management, as rationalization of funding resources and improvement of patients care.

At present, the availability of sustainable and cost-effective technologies, characterized by medium-to-high-throughput, high performance and user-friendly interfaces, allows the rapid translation into clinical practice of advanced diagnostics tests aimed to treatment tailoring. Among them, mass spectrometry represents one of the most robust, cost-effective and flexible techniques. It represents the core technology employed by the Advanced Molecular Diagnostics (DiMA) Lab in the SantAndrea Hospital of Rome, providing patients and clinicians an efficient but sustainable service of treatment personalization, ranging from genomic medicine and pharmacogenomics to therapeutic drug monitoring (TDM) and metabolomics.

The Sequenom MassARRAY is a tool combining the benefits of a simple, reproducible primer extension reaction chemistry with state of the art MALDI-TOF mass spectrometry, to quickly and cost effectively characterize genotypes with the highest levels of accuracy. It use a 96 or 384 microplate/chip formats and allows to set-up custom-made analysis panels including up to 36 different SNPs in a single well. Thus, the Sequenom MassARRAY technology joins the key features (easy-to-use platform, low-cost, high performance) required to speed the development of high impact diagnostics tests and, most striking, to deliver these tests to a large numbers of patients. At present, the DiMA Lab offers a large number of SNPs analysis panels (available at www.simep.it/dima) to be used in a wide range of therapeutic tailoring schemes, from oncology to psychiatry.

The DiMA Lab approach to TDM and metabolomics employs a LC/MS-MS system (Agilent, ABSciex), which conjugates high sensitivity/specificity with easy sample preparation, good processivity, low cost per analysis. LC-MS/MS technology

allows detection of multiple drugs in the same run and adaptation of previously developed methods to include analysis of additional drugs. This approach is useful for poly-therapies and add-on therapies. In contrast with other methods, LC-MS/MS allows accurate quantization of both the drug and its metabolites. We established an on-demand service to develop and translate into clinical practice specific TDM assays (including antiepileptics, antipsychotics, antimanics, antiarrythmics, antibiotics, antivirals, antiretrovirals; complete list available at www.simep.it/dima).

Mass Spectrometry Imaging for Tissue Mapping of Low Molecular Weight Compounds: Facts and Expectations

Maurizio Ronci[1,2], Andrea Urbani[2] and Nicolas H. Voelcker[1]

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[2] *University G. DAnnunzio of Chieti, Italy*

Mass Spectrometry Imaging (MSI) is becoming a popular tool inside the scientific community, mainly as a routine complement of proteomics workflows but also as a source of precious information for drug development, metabolomics and pharmacokinetics studies. In particular, small molecules mass imaging can dramatically speed up the drug development phase for novel drugs, through the ability to collect, rapidly and efficiently, valuable information about the distribution of target drugs and their metabolites in many body districts. However, standard MALDI-MSI in the low mass range (less than 1kDa) is challenging due to the presence of matrix signals, which mask the signals of relatively less abundant target compounds. Desorption/ionization on porous silicon (DIOS) is a technique well suited for the analysis of small molecules since it does not require the use of conventional MALDI matrices for desorption/ionization to occur. DIOS-MS employs a porous semiconductor layer as an effective medium for trapping and desorbing analytes. Chemical modifiers can be covalently bonded to the pSi surface, thus enhancing or modulating the affinity for target compounds, in analogy with conventional solid-liquid extraction. Yanes et al. introduced in 2009 a matrix-free MSI approach, using functionalized porous silicon (Nanostructured Initiator Mass Specrometry - NIMS) on extremely thin mouse brain sections (2 to 4 μm thick), necessary to obtain good mass spectra because ionization occurs only after the tissue has been etched with several laser shots. It is worth noting that histologically different regions may also behave differently when irradiated with laser light, potentially leading to virtual image artifacts. We recently reported an implementation of Mass Spectrometry Imaging using DIOS (DIOS MSI) with a novel protocol for tissue preparation, and we demonstrated the potential of this technique to study the distribution of biologically active low molecular weight metabolites. In particular, we have studied the localization of biologically active brominated compounds in the hypobranchial gland of the marine whelk Dicathais orbita showing some interesting advantages over conventional MSI. In particular we showed the possibility of using 30 to 50 μm thick sections that facilities the sample preparation (no specific sectioning skill is required), that good mass spectra can be recorded with just a few laser shots (typically less than 50 per pixel) and that the absence of MALDI matrix allows to record clean spectra in the low mass range and extend the ion source cleaning intervals. We believe that being a matrix-free technique DIOS-MSI has the potential to bring about a change in the achievable lateral resolution which is a major drawback in conventional MALDI-MSI. More studies to assess the reproducibility of the tissue removal and the tunability of the interaction surface-analytes are being performed. Nonetheless, the possibility to selectively extract different classes of compounds by changing the surface chemistry, together with fast

sample preparation, high sensitivity, no needs of sectioning skills and longer source cleaning intervals, makes DIOS-MSI an attractive alternative for metabolomics and drug discovery.

^{13}C-Phenyl Lipid Metabolic Analysis in Zebrafish Embryos by LC-HRMS. From biotech to mass spec.

Federica Dal Bello, Chiara Martano

Molecular Biotechnology and Health Sciences Dept., Universit degli Studi di Torino

The lipids characterization has become an emergent and fundamental issue for many omics sciences, and it plays an important role especially in metabolomics. Against this background, liquid chromatography coupled with an atmospheric pressure source to a mass spectrometer analyzer represents a key instrumentation in order to understand biochemical pathways. The partnership between our mass spec and the biotech labs was established few years ago, to investigate the UBIAD1 enzyme function during zebrafish embryos development. The point was how to demonstrate the activity of the non-mitochondrial enzyme Ubiad1 in the biosynthesis of the Coenzyme Q10 (ubiquinone or CoQ10) by using isotopomers. CoQ10 is synthesized from 4-hydroxy-benzoate and trans-decaprenyl-pyrophosphate, through the intermediate 4-hydroxy--3-polyprenyl benzoate (PPHB). CoQ10 is an important prenyl lipid-soluble antioxidant, available in the membranes of Golgi, which has been demonstrated to play a critical role in protecting the cardiovascular system from oxidative stress. The LC-HRMS method developed was based on the quantitation of the13C-isotope-labeled CoQ10 in zebrafish (Danio rerio) embryos after isotopomeric 4-hydroxy-benzoate administration. The study involved wild-type animals and mutants (called barolo, a null allele of zebrafish Ubiad1), used as metabolomics analysis model. Extraction and liquid chromatography separation protocols were examined and studied. A liquid-liquid extraction of 13C-labeled CoQ10 from zebrafish embryos using n-heptane was selected, followed by a liquid chromatography separation performed with a reverse phase C4 column and finally a mass spectrometry analysis carried out with a LTQ-Orbitrap mass analyzer equipped with an ESI source. This protocol was developed and optimized to analyze the CoQ10, in zebrafish tissue, but it is adaptable to the investigation of many others metabolic lipids classes, such as sterols, fatty acids, triglycerides and so on. Moreover this procedure represents an innovative in vivo fluxomics-suited approach to study and understand the metabolic pathways of several lipids classes involved in physiological pathways and metabolic dysfunctions.

Proteomics

Quantitative Mass Spectrometry in Biomedical Research

Angela Bachi

IFOM- FIRC Institute of Molecular Oncology, Milano, Italy

In the recent years, thanks to technological innovations in mass spectrometry, proteomics has evolved from the identification of thousands of proteins that compose a biological sample into the comprehensive qualitative and quantitative detection, measurement and mapping of complex molecular systems.

In our lab we apply quantitative proteomics to address biomedical issues. In particular, I will present examples of SILAC-based quantitative proteomics to study i) protein-protein interaction dynamics in order to elucidate how a point mutation, responsible for the onset of a monogenic autoimmune disease, can affect the functional protein interaction network and ii) to elucidate the microvescicles-mediated crosstalk between neural precursor stem cells and the recipient cells.

I will also discuss quantitative label free proteomic approaches, based on high resolution and high mass accuracy mass spectrometry, to identify new biomarkers for Chronic Lymphocytic Leukemia (CLL) and for salt-sensitive and salt resistant hypertensive patients.

Mass Spectrometry for Vaccine Discovery and Development

Nathalie Norais

Novartis Vaccines, Siena, Italy

Mass spectrometry (MS) has rapidly developed from being a research tool used primarily in physical chemistry to a point, where biochemical and medical research applications not only account for a high proportion of its usage but direct much of the effort for development. Continuous improvements from the mass spectrometer designers to increase instrument performances, to make the instruments available at a quite low price and friendly to manipulate by scientists without a deep knowledge in MS continue to expand the scope of its application in medical research. A simple search in PubMed (`http://www.ncbi.nlm.nih.gov/pubmed/`), with the keyword Mass Spectrometry and Vaccine, shows that the number of publications increased by 100 over the last 20 years.

MS has become an indispensable tool for vaccine discovery intervening in all stages of the long path bringing an antigen to a vaccine, from its initial selection to its final characterization. Modern vaccines are those based on subunit vaccines, either glycoconjugates or protein subunit vaccines. They present the advantage to be highly pathogen-specific and to meet the extremely high standard of safety and chemical-physical properties requested by regulatory authorities. Glycoconjugate vaccines could not be developed for all pathogens and protein-subunit vaccines are then the alternative. Protein vaccine candidates are selected from a process based on 4 main steps: (i) antigen selection; (ii) cloning/expression of the selected genes and purification of the recombinant forms of the antigens; (iii) in vitro and in vivo assays to define protection and toxicity; and (iv) when an antigen demonstrates protection in animal model and no toxicity, structural, functional, epidemiological and immunological characterizations are undertaken to define if the antigen could be considered as a promising vaccine candidate. It will not be possible to overview all of the applications of MS invaccinology field in the frame of this presentation, and only few aspects have been selected to be reported.

Proteomics for Bacterial Vaccine Discovery

Ideally, good vaccine candidates, besides being exposed on the bacterial surface should also be abundant, conserved among a large panel of strains, and expressed during infection. Although bioinformatic tools can predict the cellular location of bacterial proteins their abundance, post translational modifications and surface protein-protein interactions are relevant information for vaccine design that cannot be assessed by bioinformatics. In the last decade, development of mass spectrometers and publication of bacterial genomes have allowed the emergence of the proteomic field. Application of proteomic approaches to bacterial vaccine development has been established and often consists of the identification of the surface proteins, refereed as surfaceome or surfome. In the first attempt, the methodology

was based on protein separation by bi-dimensional electrophoresis, and identification of the separated proteins by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF)-MS. Although gel-free methods have then been developed to overcome many of the problems associated with bi-dimensional electrophoresis, the major challenges in using proteomics for identifying vaccine candidates remains the difficulty in isolating the cellular compartment of interest, free of contamination from the other cellular fractions. Because of the different cellular organization of Gram-positive and Gram-negative bacteria, different protocols have been developed to purify either the Gram-positive cell wall or Gram-negative outer membrane fractions. The resistance of Gram-positive bacteria to lysis has permitted the development of an attractive approach for the identification of surface exposed proteins. The procedure is based on the use of a proteolytic enzyme to shave the surface-exposed proteins of live bacteria in conditions that avoid bacterial lysis. The released peptides are then identified by LC-MS/MS. Unfortunately, the approach failed when applied to the Gram-negative bacteria. The thinner cell walls of Gram-negative render them more susceptible to lysis during digestion of surface-exposed proteins. A wide variety of Gram-negative bacteria constitutively secrete Outer Membrane Vesicles (OMVs) during growth. Although, the function of these OMVs is not well clarified, they represent a material of choice to identify surface antigens since they are mainly composed of outer membrane proteins and periplasmic proteins encapsulated during vesicles formation but not of cytoplasmic and inner membrane proteins. The identification of several vaccine candidates from these two approaches was reported demonstrating the power of proteomics in this field.

Hydrogen/Deuterium exchange for epitope mapping

Mapping of epitopes recognized by functional monoclonal antibodies (mAbs) is essential for understanding the nature of immune responses and designing improved vaccines. In recent years, identification of B-cell epitopes targeted by neutralizing antibodies has facilitated the design of peptide-based vaccines against highly variable pathogens. Here, we characterized the interaction between the 12C1 mAb against factor H binding protein (fHbp), a key virulence factor of Neisseria meningitidis, included in the Bexsero vaccine. This mAb displayed the interesting property of exhibiting bactericidal activity. Identification of the fHbp epitope targeted by 12C1 was attempted in various ways. Our results showed that different approaches give different pictures of the same epitope and that hydrogen/deuterium exchange (HDX)-MS was the most effective method to rapidly supply near-complete information about epitope structure. However, only X-ray crystallography provided the fine molecular details of the antigenantibody interaction. This multidisciplinary study allowed an extensive comparison of various epitope mapping approaches and highlighted the reliability of HDX-MS and X-ray crystallography for epitope mapping.

Native Mass Spectrometry to assign protein function

Understanding the interactions of small molecules with proteins is a challenging

task but extremely important in various aspects of life science, e.g. to assess side effects of drug candidates or to identify proteins within signaling pathways. In a context of vaccinology, identification of the ligand associated to a hypothetical protein, the GNA1030 included in the Bexsero vaccine allowed the identification of its biological function. Native MS has been used to demonstrate that GNA1030 is a homodimer non-covalently associated to the ubiquinone-8, indicating that the protein might be involved in the response to oxidative stress or to transport and storage of ubiquinone necessary to a correct function of the respiratory chain.

Any Other Business MS

Working in a Bioanalytical Facility: Addressing ADME and *-omics* Issues for Many Different Projects. And Results by Yesterday, please

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As any other bioanalytical facility in the world, the bioanalytical lab of the Drug Discovery and Development (D3) Department of Istituto Italiano di Tecnologia in Genova is constantly under pressure. Many different research projects, both internal to D3 or in collaboration with other research institutions worldwide, need to have reliable data generated on a daily basis. Bioanalytical activities are ranging from the evaluation of ADME properties of newly synthesized compounds to the investigation of changes in the metabolome, proteome and lipidome. Every day, the same analyst might be required to jump from a pharmacokinetic study early in the morning to a stability assay after lunchtime, to a peptide mapping issue in the evening. Furthermore, all these activities need to go together with very strict (sometimes impossible) deadlines that are required for a timely progression of projects. A few representative examples of these activities will be showed in the presentation, with the aim to illustrate how such a big diversity in the bioanalytical data requests can be efficiently addressed by a small team operating in a scientifically stimulating environment. All this is feasible thanks to top-end instrumentations, a good provision of flexibility, a lot of self-driven initiative and a good amount of improvisation. The presentation will illustrate an example of puzzling ADME properties, the setup and validation of a targeted method for lipidome profiling, the bottom-up investigation of covalent drug-target interactions and some intact protein analysis, also by means of ion mobility acquisitions.

Label Free Absolute Quantitation of Human Milk Proteins using Multiple Reaction Monitoring

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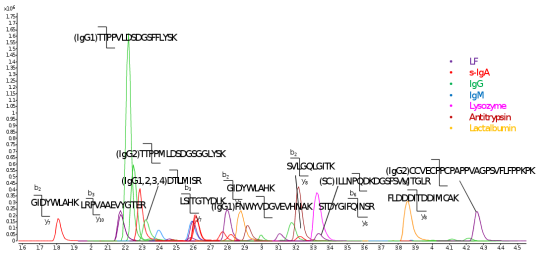
An important goal in proteomic is to quantify the profile changes of protein abundances in biological systems. Countless reports on absolute quantification of target molecules by external or internal standards have been developed nowadays, using different isotope composition and incorporation strategies [1]. Here we report an absolute quantitation method to measure human milk proteins in biological fluids (milk, feces and urine) using multiple reaction monitoring (MRM) [2], including the selection of proteotryptic peptides and the optimization and validation of transitions. MRM transitions were developed for each peptide from the fragmentation patterns obtained with the LC-Q-TOF-MS/MS. At least two MRM transitions were selected to quantify and identify each structure in the same run. The absolute amount of the proteins was determined using peptide calibration curves created by seven commercial protein standards.

References

1. Bantscheff, M., Schirle, M., Sweetman, G., Kuster, B. Anal Bioanal Chem (2007) 389:1017103.
2. Hong, Q., Lebrilla, C.B., Miyamoto, S. and Ruhaak, R. L. Anal Chem. 2013 17;85(18):8585-93.



Hair Analysis for the Study of Alcohol, Caffeine and Nicotine Abuse in Adolescents

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The research project ToxiCap have been funded and supported by the Department of Antidrugs Policies of the Italian Governments Presidency Council has been undertaken by the Forensic Toxicology Division, University of Florence, in order to identify the age of onset of abuse of substances that are not scheduled drugs, but that can likewise lead to health risks, especially in the young. These drugs are alcohol, nicotine and caffeine. In fact, the excessive drinking patterns of young people (binge drinking), a return to the habit of cigarette smoking at early age, and the excessive use of energy drinks, represent a serious health risk and the knowledge about this phenomenon by means of scientific (not only by questionnaire) evidence is the only basis for prevention strategies.

This study recruited a large number of students (n = 874) aged between 13 and 16. Students, as volunteers, and with written consent by their guardians, donated a small lock of their hair. A questionnaire on the drinking and smoking habits was administered. The population has been divided by the gender and into two age groups: 13-14 and 15-16. The toxicological researches were carried out on 100 mg of hair (3-4 cm proximal) in order to quantify the following substances: fatty acids ethyl esters (FAEE) and ethyl glucuronide (EtG), as marker for alcohol intake; nicotine and cotinine; caffeine. The cut-offs proposed by the Society of Hair Testing have been used for alcohol markers. A control population of 50 smokers and 50 coffee consumers (at known concentration and/or total amount for both substances) has been used to obtain cut-off values for nicotine and caffeine. The analyses for FAEE was performed by means of SPE (solid phase extraction) columns and gas chromatography mass spectrometry (GC-MS) analysis in single ion monitoring (SIM) mode. EtG, nicotine, cotinine and caffeine were determined with a LLE (liquid-liquid extraction) and a triple quadrupole liquid chromatography tandem mass spectrometer (LC-MS/MS) instrumentation in multiple reaction monitoring (MRM) mode. Both the procedures are fully validated and routinely used in our laboratory. The toxicological main results showed that 17.6% drinks alcohol (3% is a heavy drinkers), 43.5% ingests more than an espresso/day (21.4% more than 2 espresso/day) and 24.3% is an active smokers (17.1% of heavy smokers).

Spectroscopic Techniques for Cultural Heritage and Archaeology

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The analysis of materials related to Cultural Heritage and Archaeology brings, together with the difficulty usually related to the quantification of materials composition, the additional challenges of having to deal with object often inhomogeneous, degraded by the environment in which they have laid, sometimes for hundreds or thousands of years, in most of the cases unique and, as such, impossible to deal with destructive techniques. The unique history of cultural heritage and archaeological materials also makes difficult the application of methods based on the comparison with standards, as all the techniques based on the use of calibration curves impose.

In this talk, I will focus the attention on the application of non-destructive spectroscopic techniques for the analysis of such materials, and in particular on in situ analysis, which is mandatory whenever the sampling of material from the objects is not advisable and the conditions or the cultural and intrinsic value of the objects does not allow for their analysis in an external laboratory.

The range of spectroscopic techniques that can be used non-destructively in situ is extremely wide. For giving an example of the activity developed since many years at my lab, I will limit the discussion to a few examples, that should nevertheless give the idea of the difficulties, but also of the advantages of such an approach.

A great part of our activity is based on the application of optical spectroscopy methods; optical spectroscopy has the definite advantage of working at a distance from the surface of the object to be analysed. This is particularly useful in the case of large object such as paintings on canvas, panels and frescoes. In that respect Multi-spectral Imaging, a technique which is often assimilated to classical photography for object documentation has, on the contrary, interesting analytical capabilities that can be exploited even more usefully when coupled with other non-destructive techniques. The recent development of a photogrammetric 3D multispectral imaging system has opened new perspectives for this kind of analysis. An example will be presented on the use of multispectral imaging and 3D reconstruction in the study of the mural paintings of the Tomb of the Monkey, an Etruscan tomb in Chiusi (Siena). In that case, the results of multispectral imaging helped to interpret the in situ measurements performed with a portable X-Ray Fluorescence (XRF) instrument.

X-Ray Fluorescence is an extremely powerful technique, which owes its great popularity for Cultural Heritage and Archaeological analysis to the possibility of obtaining precise quantitative information on the materials composition in short time and in a completely non-destructive way.

On the other hand, XRF has also some drawback, related to its strong dependence on the surface degradation of the materials to be analysed, its poor sensitivity

(especially to low Z elements) and its need to operate in close proximity of the objects to be studied.

In my presentation, I will show examples on the capability of portable XRF instrumentation for Cultural Heritage and Archaeology, and will stress the complementarity of this technique with another technique, called Laser-Induced Breakdown Spectroscopy (LIBS).

LIBS is technique based on the laser ablation of an extremely small quantity of material from the samples surface (in a way similar to the sampling stage of Laser-Ablation ICP-MS); however, in LIBS the laser itself is used for ionizing and exciting the resulting plasma and the detection stage is associated to the optical emission of the plasma itself.

Although the LIBS technique is micro-destructive, in some cases the in-depth information that can be gathered through it justifies its use on Cultural Heritage and Archaeological objects.

A dedicated mobile instrument has been developed at ALS lab, allowing for in situ application of this technique.

In my talk, I will present several examples of the joint application of the spectroscopic methods here described, pointing out the complementarity of information that can be obtained using these techniques, operating in the field, with the one that can be obtained in the laboratory.


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