Microarray-based mass spectrometry for mammalian cell culture monitoring in biotechnological production processes

A thesis submitted to attain the degree of DOCTOR OF SCIENCES of ETH ZURICH (Dr. sc. ETH Zurich) presented by

Robert Friedrich Steinhoff
M.Sc. in Chemistry, Technical University Munich (TUM)

born on 06.05.1985
citizen of Lindau (Bodensee), Germany

accepted on the recommendation of
Prof. Dr. Renato Zenobi, examiner
Prof. Dr. Massimo Morbidelli, co-examiner

2016
Acknowledgements

Dear reader,

The present work on microarray MALDI mass spectrometry has been accomplished in the years 2012 to 2016 at ETH Zurich in the lab of Prof. Zenobi. The excellent infrastructure within ETH Zurich has been the fruitful foundation for this thesis.

I am thankful to Prof. Renato Zenobi, who accepted me to work in his lab, firstly as a master student and later as PhD-student within the European Marie Curie initial training network ISOLATE.

I am deeply grateful to my parents, my brother, my grandmas, my aunts – hereof especially aunt Gabriela Konietzko who always encouraged and supported me - my uncles, my cousins and their families for their endless understanding and support throughout my education.

This work would not have been possible without the great support of many individuals. I want to thank Dr. Martin Pabst for his patient and constant supervision, and Jasmin Krismer for our great PhD-student time.

I would like to thank my colleagues from the first year in the Zenobi group: Carolin Blum, Lothar Opilik, Christian Berchtold, Simon Weidmann, Stephan Fagerer, Pavel Sagulenko, Lukas Meier, Stefan Schmid, Kostya Barylyuk for the great time we spent together and all the help they offered me in my early days at ETH. Thank you to the Zenobi- and the Morbidelli-group members, above all Guido Zeegers, Lukas Bregy, Daniel Karst, Fabian Steinebach, Vania Bertrand, Miroslav Soos. I would like to thank Marie Kopp for her outstanding work on metabolite identification in the framework of her Master’s thesis.

I would like to thank the entire initial training network ISOLATE for great meetings in Gothenborg, Odense, Prague, Oxford, and Zurich. A special thanks to Prof. Andreas Hierlemann, Alexander Stettler, and Gregor Schmid from the Department of Biosystems Science and Engineering (D-BSSE,
ETH Zurich) and Simon Bacheler for their support in the development of new MAMS chips. Thank you to Rolf Brönnimann from the EMPA Dübendorf for his great and comprehensive work on laser ablation fabricated MAMS. Thank you to Louis Bertschi and Rolf Häfliger from the LOC MS-Service for fruitful discussions and the great support. Thank you very much Felix Kurth and Klaus Eyer, for your continuous and consistent work at the cell bench! I would like to thank Brigit Bräm, and her team for the excellent administrative work and the ETH-LOC workshop team, including Christoph Bärtschi and Christian Marro for their support in many different projects.

I would like to thank my friends and colleagues from Lindau and the Belvoir Rowing club for balancing work life in such a great way. Thank you for the wonderful time at ETH Zurich and on the lakes and rivers of Europe to: Robert and Nora Schreiber, Moritz, Manuel, Marie, Jossi, Annika, Raban, Jan-Georg, Marco, Madlaina, Corinne, Camille, Jean-Philippe D., Leo, Carla, Felix K., Jonathan, Pipo, David, Theresa, Chris, Albert, Johannes.

Please enjoy reading. Zurich, fall 2016
INTRODUCTION ............................................................................. 1
  1.1 MONITORING BIOPROCESSES .................................................. 2
  1.2 ANIMAL CELLS: BASIC CONCEPTS ........................................ 2
    1.2.1 Animal cell structure ...................................................... 2
    1.2.2 Cell growth ................................................................. 3
    1.2.3 Cell culture ................................................................. 4
    1.2.4 Cell metabolism ......................................................... 6
  1.3 BIOCHEMICAL ENGINEERING .............................................. 7
    1.3.1 IgG structure ............................................................... 7
    1.3.2 Cell culture processes ............................................... 8
  1.4 ANALYTICAL TOOLS FOR METABOLITE MONITORING ............ 9
    1.4.1 Enzymatic methods ..................................................... 10
    1.4.2 Spectroscopic methods .............................................. 10
    1.4.3 Mass spectrometric methods ..................................... 11
  1.5 MOTIVATION ........................................................................... 13

METHODS .................................................................................... 15
  2.1 MASS SPECTROMETRY .......................................................... 16
  2.2 MATRIX-ASSISTED LASER DESORPTION IONIZATION .............. 16
  2.3 TIME–OF-FLIGHT MASS ANALYSIS ........................................ 22
    2.3.1 Working principle ....................................................... 22
    2.3.2 Detector types in TOF analysis .................................... 24
  2.4 ION CYCLOTRON BASED MASS ANALYSIS ................................ 26
    2.4.1 Working principle ....................................................... 26
  2.5 MALDI MECHANISMS ............................................................ 27
    2.5.1 The incorporation controversy .................................... 27
    2.5.2 Physics of ablation ...................................................... 27
    2.5.3 Ion formation models ................................................ 28
    2.5.4 Refined lucky survivor .............................................. 28
    2.5.5 The gas-phase protonation model ............................... 29
    2.5.6 Excited electronic state models .................................... 29
  2.6 SAMPLE PREPARATION ......................................................... 30
  2.7 MICROARRAY FOR MASS SPECTROMETRY .............................. 32
    2.7.1 Working principle ........................................................ 32
    2.7.2 Fabrication ................................................................. 34
  2.8 SEPARATION SCIENCES .......................................................... 37
    2.8.1 Size-Exclusion Chromatography .................................. 37
    2.8.2 High-performance liquid chromatography ..................... 37
RAPID ESTIMATION OF THE ENERGY CHARGE ................. 39
3.1 OVERVIEW ................................................................................ 40
3.2 INTRODUCTION ........................................................................ 40
3.3 EXPERIMENTAL ....................................................................... 43
   3.3.1 Metabolites ......................................................................... 43
   3.3.2 Matrices ............................................................................. 44
   3.3.3 Sample preparation ........................................................... 44
   3.3.4 Instrumentation ................................................................. 45
3.4 RESULTS AND DISCUSSION ....................................................... 46
   3.4.1 In-source decay vs. post source decay ............................... 46
   3.4.2 Evaluating the ISD products from selected nucleotides .. 46
   3.4.3 Comparison of ISD in an FT-ICR-MS and a TOF-MS ... 49
   3.4.4 Influence of the laser power .............................................. 50
   3.4.5 Influence of the extraction pulse delay .............................. 51
   3.4.6 In-source decay and analyte quantification ..................... 52
   3.4.7 Measuring cell batches and cell extracts ......................... 53
   3.4.8 Estimating the energy charge in cell lysates .................... 55
5. CONCLUSIONS ............................................................................ 56

HIGH-THROUGHPUT NUCLEOSIDE PHOSPHATE
MONITORING .................................................................................... 59
4.1 OVERVIEW ................................................................................ 60
4.2 INTRODUCTION ........................................................................ 61
4.3 MATERIALS AND METHODS ...................................................... 64
   4.3.1 Chemicals and solvents ..................................................... 64
   4.3.2 Metabolite standards, stable isotope internal standard .. 64
   4.3.3 MALDI matrix ................................................................. 64
   4.3.4 Cell culture ........................................................................ 65
   4.3.5 Fed-batch cultivation process ........................................... 65
   4.3.6 Sample preparation ........................................................... 66
   4.3.7 Sample aliquoting ............................................................. 67
   4.3.8 MALDI-TOF-MS ............................................................... 67
   4.3.9 Monitoring strategy ........................................................... 68
4.4 RESULTS AND DISCUSSION ...................................................... 69
   4.4.1 Viable cell concentration ................................................... 69
   4.4.2 Analysis of intracellular metabolites using MALDI-MS . 71
   4.4.3 Different metabolite patterns described by PCA .......... 75
4.5 CONCLUSIONS .......................................................................... 77
4.6 SUPPLEMENTARY INFORMATION ............................................ 78
Abstract

Today, mass spectrometry (MS) enables the detection of molecules over a broad mass range, from small molecules such as metabolites to big biomolecules such as antibodies or even viral assemblies. MALDI-MS is a soft ionization technique and enables the detection and quantitation of intact biomolecules and metabolites with very high sensitivity. However, a drawback of MALDI-MS is its poor reproducibility caused by a high spot-to-spot heterogeneity. This problem has been partially overcome by the development of a new microarray for mass spectrometry (MAMS) platform in the lab of Prof. Zenobi in 2011.

The present work describes the development of a microarray-based MALDI-MS method for the investigation and monitoring of bioprocesses on the metabolite and product level. The first part of the work focuses on the detection of intracellular phosphorylated nucleotides from a mammalian cell culture. The second part summarizes the metabolite analysis of 4 different fed-batch culture conditions, including hypothermia and varying osmolality. The metabolite data sets were analyzed using multivariate statistics and were found to cluster according to time as well as to reactor conditions in the inter reactor comparison. In the third part, a method has been applied to monitor a continuous cell culturing process. In the last part, the influences of different fed-batch feeding strategies on the N-glycosylation of the antibody and the metabolite profile have been investigated.
Zusammenfassung

Chapter 1

Introduction

This chapter introduces basic mammalian cell concepts. Relevant biotechnological mammalian cell lines and process types are discussed and the motivation to monitor these on the metabolic level is given. Finally, state-of-the-art analytics are presented. The role and impact of mass spectrometry in metabolite research is addressed in detail throughout the entire chapter.
Introduction

1.1 Monitoring bioprocesses

Industrial sustainability is globally seen as one of the most important and influential topics in our societies. Traditional biotechnological processes (e.g. microbial production of enzymes, antibiotics or antibodies) changed the chemical industry fundamentally towards higher sustainability standards. Biotechnology impacts all sectors of the chemical industry, including basic chemicals, specialty chemicals, consumer care and life science products. Its impact on healthcare, food production and processing, agriculture, production of new materials, etc. is already substantial today. The biotechnological landscape itself is constantly developing in all four engineering disciplines, namely genetic, protein, metabolic and bioprocess engineering. In order to effectively support these developments, new analytical tools are required.

In 1992, Ryll and Wagner suggested for the first time bioprocess monitoring based on metabolic data. In their study, Ryll and Wagner connected intracellular ribonucleotide pools to the cell physiological state. Today, many process characteristics such as productivity, viability, and product forming can be determined from the intracellular metabolic network.

1.2 Animal Cells: Basic concepts

1.2.1 Animal cell structure

Mammalian cells have two distinct compartments, the nucleus and the cytoplasm. The cytoplasm, which is surrounded by the plasma membrane, refers to the cytosol and any organelles or other inclusions. The plasma membrane is a lipid bilayer barrier that separates the intra- from the extracellular environment. The bilayer acts in the
Introduction

first place as an impermeable barrier, which is selectively modulated by membrane proteins to allow the active and passive transport of important molecules and ions.\textsuperscript{6} Secondly, the membrane actively contributes to structural stability of a cell by maintaining its characteristic architecture even at varying external pH and ionic strength conditions.\textsuperscript{6,7} Although cells consist of approximately 70% water by weight, most of the cytoplasmic volume is made of macromolecules.\textsuperscript{6} Organelles such as the mitochondria, Golgi complex, lysosome, and endoplasmic reticulum are immersed in the cytosol and are responsible for high metabolic activity. In particular, mitochondria are enzyme-rich structures that oxidize the organic nutrients by oxygen molecules in order to produce chemical energy. The chemical energy is used to generate ATP, which is an important energy transporter in biological systems.\textsuperscript{7}

1.2.2 Cell growth

Cellular reproduction is a process that requires sources of energy and carbon. Cell duplication follows a strict sequence of events, which are represented in the cell cycle. The average doubling time ($t_d$) for mammalian cells is 24 hours. $t_d$ can vary depending on environmental conditions and individual cell characteristics.\textsuperscript{8} The error-free duplication of chromosomal DNA (deoxyribonucleic acid) and the subsequent segregation of the copies is controlled and regulate by complex signaling machineries. Growth factors and hormones both present in the intra- and extracellular environment trigger the start for a successful division. The cell cycle passes several checkpoints that are triggered by a protein family known as cyclin-dependent kinases (CDK). In the case of incorrect DNA duplication the protein p53 is activated to interrupt cell cycle progression and induce cell death or senescence mechanisms. Necrosis and apoptosis are two distinct cell death
mechanisms. Necrosis is characterized by membrane and organelle rupture, whereas apoptosis is stimulated by an intracellular enzyme cascade and leads to DNA fragmentation and specific changes in the chromatin.

1.2.3 Cell culture

Cell culturing forms the basis of many biotechnological processes and has been subject of many journal articles, reviews and book chapters since its modest origins towards the end of the nineteenth century. 9,10 An established cell line has to be maintained once it was thawed from the cell bench stock. Cell maintenance includes a periodic change of the culture medium since cells are depleting medium nutrients and produce metabolic waste products. Mammalian cells, which are growing in suspension, are often maintained by dilution with fresh medium in a so-called repeated batch mode. Consequently, repetitive units of the in sigmoidal growth profile figure 1-1 shown can be achieved.
The growth profile can be classified as four segments, namely the lag, exponential, stationary and death phase. The following two equations describe growth rate and nutrient depletion in batch operation, where X is the cell concentration (10^6 cells/mL), μ the specific growth rate (day^-1), k_d the specific death rate (day^-1), S the substrate concentration (g/mL), q_s the specific substrate consumption (g/10^6 cells/day) and t the time (day).

\[
\frac{dX}{dt} = (\mu - k_d) \times X
\]

**eq. 1**

\[
\frac{dS}{dt} = -q_sX
\]

**eq. 2**

**Figure 1-1** Sigmoidal growth profile of a batch cell culture process. μ is the specific growth rate. The dots represent measured cell densities.
Introduction

Cell proliferation in cell culture depends on many different variables such as nutrient availability, gas concentrations, temperature, pH and osmolality. Many mammalian cells have optimal growth temperatures of 35-37°C. The temperature influences the solubility of different media components, including the solubility of CO₂ and O₂. Another key factor for achieving high cell proliferation is the pH. Most mammalian cells proliferate optimally at pH 7.4. The optimal osmolality range for mammalian cells lies between 260 and 320 mOsm/kg. Osmolality variations are minimized in cell culture by saturation of the culture environment humidity.

1.2.4 Cell metabolism

The metabolome is referred to as the entirety of small, non-genetically encoded molecules associated with the highly complex cellular network. Metabolites interact on all functional levels of the cell. Our understanding of the metabolome is under steady renewal. While in 1955 a compilation of the discovered metabolic pathways included 20 metabolic pathways, today's knowledge about model organisms such as yeast *Saccharomyces cerevisiae*, comprises about 800 metabolites and 1200 enzymatic reactions. The human metabolome database (hmdb.ca) lists approximately 41,993 metabolites among which 4860 metabolites are represented with analytical spectra. These numbers are continually increasing. Most biotechnologically used cell lines have to satisfy an unnatural demand of growth and production and have therefore an altered metabolism. As a result these cell lines have high glucose and glutamine intake rates. This phenomenon is known as the Warburg effect and was discovered the first time in highly proliferating cancer cells.
Introduction

Warburg suggested the uptake rates to be abnormally high (i) as a consequence of malfunctioning mitochondria and (ii) as a survival strategy of cancer cells.\textsuperscript{13-15} Glucose is actively transported in the cell by glucose transporters and subsequently converted by two main pathways: glycolysis and the pentose phosphate cycle (PPC).\textsuperscript{7} The net ATP production in glycolysis is two ATP molecules per glucose molecule. In the presence of \( \text{O}_2 \) the glycolysis product pyruvate is shuffled to the tricarboxylic acid cycle (TCA) wherein up to 38 ATP molecules per glucose molecule are generated.\textsuperscript{16} In hypoxia conditions the pyruvate is converted to lactate and secreted from the cytosol without further ATP production. Most mammalian cells in vivo are deregulated and unable to fully transport pyruvate to the TCA but rather produce high amounts of lactate.\textsuperscript{5} The second pathway, the PPC, converts glucose to NADPH and ribose 5-phosphate for nucleoside biosynthesis.

1.3 Biochemical Engineering

1.3.1 IgG structure

Immunoglobulins are a family of Y-shaped proteins that play a key role in the human immune response. Within the Immunoglobulin family there are five antibody isotypes, namely IgA, IgD, IgE, IgG and IgM. The basic structural units of an IgG antibody are two paired heavy and light chains. Each light chain consists of two 110 amino acid domains (CL and VL), and the heavy chain consists of four of these domains (CH1, CH2, CH3, VH).\textsuperscript{17} IgG consists of an antigen binding fragment (fab) and the constant fragment (Fc) (figure 1-2). Three hyper variable complementarity-determining regions (CDRs) are forming the antigen-binding site.
1.3.2 Cell culture processes

Industrial-scale production of biopharmaceutics dates back to the first penicillin deep tank fermentation in the 1940s by Kane and coworkers from Pfizer. Significant improvements in the cultivation processes have increased the yield and efficiency. Among the developed process types are the fed-batch and the perfusion cell culture.

1.3.2.1 Fed batch

A fed batch is defined as a technique in which one or more nutrients are supplied to the microbe containing bioreactor during cultivation and no product is removed until the end of the run.\(^\text{18}\) This process type has the advantages of being very flexible for various products and relatively easy to scale up. A major drawback of fed batch processes in industrial
applications is the occurrence of gradients during the runs and accumulation of toxic metabolites. Generally fed batch processes are subdivided into non-feedback and feedback controlled processes.

1.3.2.2 Continuous cell culturing

In a continuous culture one or several feeding streams are fed continuously into the reactor and product and waste products are constantly removed, which allows for the flexible adjustment of culture conditions. Continuous processes require cell retention devices in order to achieve high cell densities. Two retention systems, the tangential flow filtration (TFF) and the alternating tangential flow filtration (ATF), have been investigated by Karst et al. In this work, using these filtration retention devices cell densities up to 60x10^6 cells/mL were achieved. The ATF has been found superior as higher antibody yields were found compared to the TFF. Continuous culturing processes can generally achieve higher cell densities and consequently higher product yields than fed-batch processes.

1.4 Analytical tools for metabolite monitoring

Metabolomics is the science of quantifying and identifying the entire collection of extra- and intracellular metabolites. Metabolic profiling is the identification and quantitation of a set of metabolites in a selected metabolic pathway. Generally, monitoring methods are categorized according to their implementation as off-line, at-line, online or in-line. For off-line analysis, either the cell culture or the cell-free supernatant can be sampled. For successful mammalian cell cultivation it is inevitable to monitor process parameters including temperature, oxygen concentration, cell density and
Introduction

viability. However, the intra- and extracellular metabolite levels enable critical insights into cell cultivation processes.\textsuperscript{1,22-25} In the following, enzymatic, spectroscopic and spectrometric metabolite monitoring methods are described.

1.4.1 Enzymatic methods

The relevant nutrients and metabolic byproducts for cell cultivation are often monitored off-line using enzymatic and amperometric methods. Enzymatic tests take various forms, but one common underlying principle is redox chemistry. For example, in a first step, a specific enzyme oxidizes an analyte. Secondly, the reduced enzyme gets oxidized and the corresponding oxidizing agent (often $\text{H}_2\text{O}_2$) is detected electrochemically.

1.4.2 Spectroscopic methods

Optical spectroscopy such as near, mid and far infrared and Raman spectroscopy have a long history in process monitoring. Their measurement principle – the energy absorption of material matter after exposure to electromagnetic radiation allows for in-line analytics. The near infrared (13,000 – 4,000 cm\(^{-1}\)) is predominantly probing hydrogen atom containing functional groups such as O-H, C-H, or N-H. In bioprocess control NIR spectroscopy is often used to determine cell density and selected metabolite concentrations.\textsuperscript{26,27} NIR spectrometers applicable to stainless steel bioreactors have been commercialized. Mid-Infrared spectroscopy (MIRS) is used to probe lactate and glutamate concentrations, number of viable cells, relative cell viability, and antibody amount in the bioreactor.\textsuperscript{28} A big drawback of MIRS is the strong interference from water, which masks important information in the spectra.
1.4.3 Mass spectrometric methods

Mass spectrometry based metabolite analysis is possible on three major platforms, namely (i) separation free, (ii) desorption, and (iii) separation based platforms.\textsuperscript{29}

Common ionization sources for metabolite analysis are summarized in table 1-1. Matrix-assisted laser desorption ionization (MALDI), electrospray ionization (ESI) and electron impact (EI) have very prominent roles in metabolite research. MALDI and ESI are soft ionization methods that enable the analysis of intact metabolites with very high throughput. In contrast, EI is a hard ionization technique that is often coupled to gas chromatography (GC).

Gas chromatography mass spectrometry (GC-MS) has developed into a widely accepted method for metabolite profiling. The use of stable isotope labeled internal standards as well as technical improvements such as the use of glass-based capillary columns provides exceptionally high precision and accuracy for quantitation.\textsuperscript{30} The major limitation in GC is the necessity of derivatization agents in order to ensure volatility of the analytes.

Liquid chromatography-mass spectrometry (LC-MS) enables the analysis of polar and thermally unstable metabolites. Due to the complexity of metabolite samples, LC run reproducibility is a big challenge. Besides GC-MS and LC-MS, capillary electrophoresis (CE-MS) is a powerful approach.\textsuperscript{21,31-35} The separation based platforms have been optimized to decrease analysis time and improve throughput. Recent publications on UPLC-MS and GC-MS state separation runtimes of <4min.\textsuperscript{36} The general goal of the separation based platforms is to minimize simultaneous analyte arrival at the MS detector and to reduce biological background.

The second platforms used for metabolite analysis are separation-free analysis principles. In these approaches, a higher throughput can be achieved, however the metabolite
Introduction

coverage is dramatically reduced. The direct sample introduction into the ionization source is either done by direct infusion/injection (DI) or flow injection/infusion (FIE). The FIE allows for real-time metabolome analysis, whereas the DI is limited to static samples.\textsuperscript{37,38} Real-time metabolome profiling based on a flow-injection time-of-flight (TOF) mass spectrometry has enabled the detection of 419 deprotonated metabolites.\textsuperscript{39}

The third platform comprises techniques that are desorption based.\textsuperscript{29} Desorption ionization techniques for metabolite analysis take various different forms such as desorption electrospray ionization (DESI)\textsuperscript{40,41} and direct analysis in real-time (DART) that are based on electrospray or plasma desorption, respectively. In the 1960s laser desorption methods have been developed and due their principle (nanosecond pulsed ion extractions) those methods provide extremely high throughput.\textsuperscript{29} Among the laser ablation systems are, laser ablation electrospray ionization (LA-ESI),\textsuperscript{42} laser desorption ionization (LDI),\textsuperscript{43} and secondary ion MS (SIMS).\textsuperscript{44} Surface-assisted laser desorption/ionization (SALDI) approaches as desorption/ionization on silicon (DIOS), and nanostructure-initiator MS (NIMS) are more recent developments.\textsuperscript{45} The most widely used ionization technology among the desorption techniques is matrix-assisted laser-desorption/ionization (MALDI). MALDI has been used to study metabolite profiles in various cell types including cancer cells and plant cells with throughputs in the second range per sample.\textsuperscript{4,46-48} Throughput in the third platform depends mainly on sample size and scan rates. More desorption based ionization techniques are reviewed by Dorrestein et al.\textsuperscript{49}
Introduction

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Name</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>separation free platforms</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DI</td>
<td>direct infusion</td>
<td>50</td>
</tr>
<tr>
<td>FIE</td>
<td>flow injection electrospray</td>
<td>39</td>
</tr>
<tr>
<td><strong>desorption platforms</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDI</td>
<td>laser desorption/ionization</td>
<td>43</td>
</tr>
<tr>
<td>SIMS</td>
<td>secondary ion ms</td>
<td>44,51</td>
</tr>
<tr>
<td>LAESI</td>
<td>laserablation electrospray ionization</td>
<td>42</td>
</tr>
<tr>
<td>NIMS</td>
<td>nanostructure-initiator ms</td>
<td>45,52</td>
</tr>
<tr>
<td>DESI</td>
<td>desorption electrospray ionization</td>
<td>53</td>
</tr>
<tr>
<td>DESI</td>
<td>desorption/ionization on silicon</td>
<td>54,55</td>
</tr>
<tr>
<td>DART</td>
<td>direct analysis in real-time</td>
<td>56</td>
</tr>
<tr>
<td>MALDI</td>
<td>matrix-assisted laser desorption/ionization</td>
<td>51,57,58</td>
</tr>
<tr>
<td><strong>separation based platforms</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ESI</td>
<td>electrospray ionization</td>
<td>29,32,59</td>
</tr>
<tr>
<td>APCI</td>
<td>atmospheric pressure chemical ionization</td>
<td>60-62</td>
</tr>
<tr>
<td>APPI</td>
<td>atmospheric pressure photoionization</td>
<td>63</td>
</tr>
<tr>
<td>EI</td>
<td>electron impact</td>
<td>33,62</td>
</tr>
<tr>
<td>CI</td>
<td>chemical ionization</td>
<td>64</td>
</tr>
</tbody>
</table>

Table 1-1 Current ionization techniques used in metabolite research.

1.5 **Motivation**

MALDI-MS has been developed to analyze large biomolecules. Matrix molecules and clusters in the low mass region (<1000 m/z) have hindered the analysis of small molecules by MALDI-MS in the early days of MALDI-MS. Instrumental developments such as reflectron ion path, and ion delayed extraction have increased resolution of commercial mass spectrometers and thus helped to overcome this limitation. Today, high-resolution instruments such as FT-ion cyclotron resonance MS (FT-ICR-MS) allow for clear differentiation between analyte and matrix molecules. Besides this
instrumental hurdle MALDI-MS is known to provide poor reproducibility. The individually crystallized spots in MALDI-MS often suffer from heterogeneities such as crystallization times, crystal sizes and diameter/heights of the spots. In 2011, Pabst has launched a series of investigations in the Zenobi lab to improve MALDI-MS reproducibility and to lift the limitations of MALDI-based quantitation. The basis of these investigations is a microarray for mass spectrometry (Diss. ETH No. 18976, Dr. Andrea Amantonico and Diss. ETH No 21684, Dr. Stephan Fagerer, Patent 2011 US9211542 B2). In the present thesis, the development and a novel fabrication process of a microarray for mass spectrometry (MAMS) for quantitative MALDI-MS is presented (chapter 2 - methods). We investigated the nucleotide phosphate metabolism of a mammalian cell line (THP-1 monocytes, chapter 3) using MALDI-MS. Nucleotide phosphate monitoring using MALDI-MS works in negative ion mode with excellent reproducibility. The occurrence of in-source fragmentation has been investigated in negative ion mode using 9-aminoacridine as a matrix in chapter 3. Moreover, the metabolism of a biotechnologically exploited hybridoma cell line has been investigated using microarray-based MALDI-MS in negative ion mode (chapter 4). Chapter 5 describes a microarray-based MALDI-MS method to monitor IgG1 directly from the cell supernatant as an alternative to common size exclusion techniques. The methods robustness is investigated in chapter 6 in a monitoring experiment of 22 microscale bioreactors.
Chapter 2

Methods

The main focus of this chapter lies on the description of the MALDI-TOF mass spectrometer and the custom-designed developed microarray for mass spectrometry (MAMS). Moreover, two widely accepted MALDI mechanism models, namely the refined lucky survivor model and the gas phase protonation model are briefly introduced.

And yet in a funny way our lack of success led to our breakthrough; because, since we could not get a cell line off the shelf doing what we wanted, we were forced to construct it. And the original experiment ... developed into a method for the production of hybridomas ... [which] was of more importance than our original purpose.

César Milstein, Nobel Lecture in Physiology and Medicine (8.Dec 1984)
Methods

2.1 Mass spectrometry

A mass spectrometer consists of an ion source, a mass analyzer, and a detector. The ion source creates gas phase atomic or molecular ions. The mass analyzer separates those ions according to their mass-to-charge ratio (m/z). The detector amplifies and detects the ion abundances. The detected electrical signals are converted to respective m/z values. Finally, a mass spectrum allows extracting exact masses and relative signal abundances.

Today, various ionization sources are available for metabolite analysis. A list of ionization sources used in metabolite analysis is given in table 1-1 (chapter one). In the following, MALDI-MS is presented and discussed in further detail. From the various available mass analyzer systems, we will focus on two main analyzer types that are used with MALDI-MS, the time-of-flight (TOF) and the ion cyclotron resonance spectrometer (FTMS, FT-ICR-MS). Two detector types for TOF instruments, namely the multichannel plate (MCP) and the ion conversion dynode detector will be presented.

2.2 Matrix-assisted Laser desorption Ionization

MALDI-MS is a soft ionization technique that has been developed from laser mass spectrometry. In 1985, Michael Karas and Franz Hillenkamp have investigated wavelength dependent ionization of small organic molecules in UV-LDI-MS and discovered that the ionization of the amino acid L-alanine showed enhanced ionization efficiency in the presence of the UV-light absorbing molecule L-tryptophan. This led Karas and Hillenkamp to the conclusion: “[...] the use of strongly absorbing matrix at a fixed laser wavelength offers a more controllable energy deposition and thus “soft” ionization [...]” Already in 1983, Hillenkamp et al. developed a commercial LDI TOF instrument, the LAMMA 1000, which
was later used in MALDI experiments. MALDI-MS was born. Since these days, various matrix molecules have been investigated and found suitable for MALDI analysis. Typical matrix molecules are relatively low molecular weight organic compounds with strong optical absorption in the UV range (examples are given in figure 2-1). The choice of a proper matrix is essential for a successful MALDI experiment, since the matrix itself can be involved in chemical ionization processes (protonation/deprotonation) in the liquid- or the gas-phase.

2,5-Dihydroxybenzoic acid (DHB)  
Sinapinic acid (SA)  

2,4,6-trihydroxyacetophenone (THAP)  
9-aminoacridine (9-AA)  

Figure 2-1 Common MALDI matrices for positive (upper panel) and negative (lower panel) ion mode.
Methods

In positive ion mode, the acidity of the matrix is believed to play a major role. Lee et al. have studied different dihydroxybenzoic acid (DHB) isomers and found significant differences in the ionization efficiency of polyethylene glycol samples. They found that the analyte ion yield correlates with the acidity of the exchangeable proton in the different DHB isomers. In negative ion formation the matrix basicity is believed to be essential. Vermillion-Salsbury et al. addressed analyte deprotonation for 9-AA. However the mechanistic investigation on positive and negative ion formation in MALDI are not complete.

Another important matrix property is its ability to cause fragmentation of the analytes during MALDI. Vertes et al. have classified CHCA, SA, and DHB according to their internal energies and fragmentation abilities as cold, intermediate and hot, respectively. In-source or post source fragmentation is used in MALDI peptide analysis, but can be a challenge in trace analysis and quantitation experiments. Post-source decay occurs in the μs timescale (0.1-10 μs) and can be distinguished from in source decay by comparing linear and reflectron mode experiments. Post-source generated fragments appear in a reflectron mode spectrum as broad peaks. In-source decay happens on ns timescale (<100 ns) and is very difficult to distinguish from any other type of sample transformation (such as solution phase hydrolysis). A typical ion-source setup of an ABsciex 4800 MALDI-TOF/TOF is schematically shown in figure 2-2. Latest ion source instrumental developments have introduced galvanometer mirrors in the laser light path to rapidly and precisely move the laser light in x and y direction across the moving sample surface. This technological invention added another order of analysis speed and finds its application in imaging problems.
MALDI sources can indeed be coupled to a variety of mass spectrometers, including axial TOF, quadrupole TOF (qTOF), quadrupole ion trap TOF (QIT-TOF), iontrap, FT ICRs and orbitrap mass analyzers. All of these combinations have individual advantages and disadvantages (summarized for TOF, Orbitrap and FT-MS in Table 2-1).

However, the lack of pre-ionization separation techniques in MALDI increases the importance of mass accuracy and mass resolution. The drawback in axial MALDI TOF instruments regarding poor resolution and mass accuracy can be partially balanced by MS/MS capabilities. For the identification of near isobaric analytes MS/MS or MS^n modes are required.

**Figure 2-2** Schematic representation of an ABSciex 4800 MALDI ion source setup with typical voltage conditions.
Methods

Measurement accuracy is directly connected to sample thickness and homogeneity. A typical MALDI source is equipped with a sample plate at > 10 kV and the plate is positioned in a distance of ~5mm from the grounded extraction optics. This results in an electric field of ~2V/µm. Now, considering a 10 µm desorption difference due to thick matrix application or a bended sample plate, this would result in a 0.2 % difference in kinetic energy and consequently a variation in the arrival time at the detector. 75

Among groundbreaking applications of MALDI-MS are the detection of an intact monoclonal antibody (IgG1) and its drug conjugates in 1991, the first MALDI-based imaging in 1995 as well as in 2005 the first metabolomics analysis.76-78
### Methods

<table>
<thead>
<tr>
<th>Analyzer</th>
<th>TOF-TOF</th>
<th>Orbitrap</th>
<th>FT-ICR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mass accuracy (ppm)</td>
<td>&lt;5</td>
<td>&lt;3</td>
<td>&lt;3</td>
</tr>
<tr>
<td>Mass resolution</td>
<td>40,000</td>
<td>&gt; 100,000</td>
<td>&gt;100,000</td>
</tr>
<tr>
<td>Mass range (Da)</td>
<td>50-1x10^6</td>
<td>50-4000</td>
<td>100-10,000</td>
</tr>
<tr>
<td>Laser rep. rate (Hz)</td>
<td>up to 10,000</td>
<td>60</td>
<td>2000</td>
</tr>
</tbody>
</table>

#### Strengths
- Acquisition speed, mass range, sensitivity
- Resolution and mass accuracy, Ms^n possible MS/MS tools

#### Weaknesses
- MS/MS selectivity, poor timed-ion-selector window, resolution and mass accuracy depends on sample preparation
- Acquisition speed, analysis, high costs per sample

**Table 2-1** Performance characteristics of different MALDI-MS analyzers. Mass resolution is given according to the FWHM definition. [adapted from 74]
Methods

2.3 Time–of-flight mass analysis

2.3.1 Working principle

The basic TOF mass spectrometer consists of a source region and a drift region. The length of the source regions is usually kept very short, whereas the length of the drift region is maximized. In the source region, a defined ensemble of ions is accelerated by an electrical field, which is defined by the accelerating voltage \( E = V/s \).\(^{79}\) The resulting travelling velocity of the ions is mass-dependent (eq. 3, with \( V \) the accelerating voltage, \( q \) the ion charge).

\[
v = \sqrt{\frac{2Vq}{m}}
\]

eq. 3

The travel time for the ion to first approximation is:

\[
t = \left(\frac{m}{2eV}\right)^{1/2} D
\]

eq. 4

where \( D \) is the drift distance. However eq. 4 does not consider the residence time of the ions in the source region. Since ions are staying for a finite time in the source region and travel a first distance resulting in a initial spatial distribution \( \Delta s \), a position correction has to be applied to eq. 4 in the form of \( s = s_0 + \Delta s \) (see eq. 5).\(^{79}\)

\[
t = \left(\frac{m}{2eEs}\right)^{1/2} (2s + D)
\]

eq. 5

As a consequence the flight time is distributed which has an unfavorable effect on the mass resolution \( m/\Delta m = t/2\Delta t \).\(^{80}\) Additionally, the ions have an initial kinetic energy distribution. Initial velocity focusing is achieved using the
Methods

delayed extraction, which was introduced by Wiley and McLaren as time-lag focusing. Delayed extraction pulses narrow the initial kinetic energy distribution in axial TOF instruments. The optimum time lag for velocity focusing is depending on the m/z, the initial kinetic energy, the amplitude of the extraction pulse, as well as source geometry parameters.\textsuperscript{80-82} High acceleration voltage increases the spatial distribution but minimizes initial kinetic energy distributions (since eEs \gg kinetic energy U_0). Mamyrin et al. introduced a non-magnetic mass reflector based on reflecting electrodes. This invention improved the mass resolution in TOF instruments significantly and allowed the construction of shorter TOF tubes. A reflector is an electric field at the end of the time-of-flight tube that slows down and ultimately reverses the direction of ions. Considering a reflectron mass analyzer with a first drift length L_1, an average ion penetration depth in the reflectron d and a second travel path L_2 eq. 4 can be modulated according to:

\[ t = \left( \frac{m}{2eV} \right)^{1/2} [L_1 + L_2 + 4d] \]

eq. 6

The reflectron leads to a refocusing of ions of different initial kinetic energies and velocities. Both principles, the delayed extraction pulses and the reflectron have improved MALDI MS resolution significantly.\textsuperscript{82,83} Compound identification in MALDI TOF experiments generally requires MS/MS experiments. Ion selection for MS/MS experiments is often based on electrical ion gates. The principle of timed-ion selector (TIS) for mass selection has been invented by Bradbury and Nielson in 1936.\textsuperscript{84}
2.3.2 Detector types in TOF analysis

Two detector systems are used in MALDI-TOF mass analyzers, the microchannel plate and, much less widespread, the ion conversion dynode. Micro- or multichannel plates consist of conducting channels in high density. The channels are typically 10 µm in diameter and spaced 15 µm. Each of the channels works as a continuous dynode electron multiplier. An impacting ion on one of the channels starts a cascade of electrons. The cascade propagates through the channel and is detected at the opposite side of the channel, often by simple anodes. After one cascade, the detector has to recover for a certain time interval. This principle allows the amplification of the original signal by several orders of magnitude.

Figure 2-3 Ion conversion dynode detector principle.
Ion conversion dynode detectors (figure 2-3) are used for the detection of high mass ions. High mass ions have a very low ion momentum when reaching the detector. This ion momentum is too low to release electrons from the surface of a multichannel plate, which yields in poor sensitivity.

Figure 2-4 MCP and Ion conversion dynode detector working ranges and selected measurement examples. ATP has been detected using 9-AA in the negative ion mode. Polystyrene (18kDa and 1MDa) has been detected using DCTB as a matrix in positive ion mode. The PS 18 kDa has been recorded with an MCP (black) and an ion conversion dynode detector (red). The PS spectra have been recorded for a collaboration project with the Bundesamt für Materialforschung Berlin for work on PS cationization with Steffen Weidner, Stephan Gabriel and Dominique Houstek.
Methods

In an ion conversion dynode detector, primary ions are converted into secondary ions. (see figure 2-3) The secondary ions are re-accelerated and detected on an electron multiplier, where the distance between the conversion dynode and the front of the electron multiplier is kept minimized. Both detector types have their respective advantages. The ion conversion dynode loses resolution due to the detection principle resolution (figure 2-4), however increases sensitivity for higher mass ions significantly, such that analytes can be detected even in the MDa range (figure 2-4, polystyrene (PS) 1.8 MDa).

2.4 Ion cyclotron based mass analysis

2.4.1 Working principle

Another mass analysis system is Fourier-transform ion cyclotron resonance mass spectrometry (FT-ICR-MS or FT-MS). The ICR signal results from the induction of oscillating ion ensembles on two conductive parallel electrodes. The digitized time-domain ICR signal is transformed by Fourier transformation into a frequency domain spectrum. Ultimately, the frequency domain spectrum is converted to a mass-domain spectrum.\(^8\)

\[
\omega_c = \frac{qB_0}{m}
\]

eq. 7

eq. 7 describes the unperturbed cyclotron equation, with \(\omega_c\) being the unperturbed ion cyclotron frequency, \(B_0\) the magnetic field strength, \(m\) the ionic mass and \(q\) the charge. FT-ICR analyzers have orders of magnitude higher resolution than TOF instruments.
2.5 MALDI mechanisms

Since its introduction in the 1980s MALDI has been the subject of many investigations concerning the mechanism of ablation and ionization. The following section will try to emphasize the major contributions in this complex field of investigations. Excellent comprehensive reviews of the subject have been written by Zenobi & Knochenmuss (1999)\textsuperscript{86}, Karas & Krüger (2003)\textsuperscript{87}, Hillenkamp (2000)\textsuperscript{65}, Knochenmuss (2006)\textsuperscript{88}, Zenobi (2010)\textsuperscript{89} and Bae & Kim (2015)\textsuperscript{90}.

2.5.1 The incorporation controversy

It was hypothesized that successful MALDI depends on a successful incorporation of the analyte into the matrix crystals. This phenomenon has been investigated for different matrices and analytes including sinapinic acid.\textsuperscript{91} However this hypothesis has been proven to be incomplete since new sample preparation methods such as the surface preparation method, the dry matrix application, or the use of ionic liquid matrices showed ionization without incorporation.\textsuperscript{92,93,94} Consequently, this hypothesis has not been pursued. However, the crystal morphology is believed to influence signal intensity and resolution.\textsuperscript{95}

2.5.2 Physics of ablation

Laser fluences ranges for MALDI experiments – depending on the setup - are between 20-6500 Jm\textsuperscript{-2}.\textsuperscript{96-98} A laser fluence of 100 Jm\textsuperscript{-2} results in the theoretical absorption of 0.7 photons per matrix molecule.\textsuperscript{99} This high excitation energy density leads ultimately to an explosive ablation, whereas the quantity of removed material has a sigmoidal dependence on the laser fluence.\textsuperscript{96,98} The ablation in MALDI goes from molecular desorption at very low laser fluence to volume
Methods

ablation at elevated fluences.\textsuperscript{97} At the time point of ablation a broad initial ion velocity distribution is present in the ion plume for each combination of laser energy and matrix molecules (reviewed by Karas et al.).\textsuperscript{87} MALDI ablation simulations are very complex, since the ablation volume and time scale are very large, on the order of few picograms and in the nanosecond regime.\textsuperscript{100} Hillenkamp et al. recorded high-speed photographs of the plume expansion and showed a plume expansion to happen on the order of several tens of micrometers for the first 100 ns.\textsuperscript{99}

2.5.3 Ion formation models

Several different ion formation models have been proposed in the last decades. The chemical environment in a MALDI plume (high pressure, temperature) allows for secondary ionization process such as gas-phase proton transfer, electron transfer and more. Whereas there is consensus about the necessity of protonated ([M+H]\(^+\)) and deprotonated matrix ([M-H]\(^-\)) in the analyte ionization process, there is some debate on the formation of [M+H]\(^+\) and [M-H]\(^-\).\textsuperscript{88} Two conflicting theories have been established: the refined lucky survivor model and the gas-phase protonation model. In 2011, Jaskolla and Karas have proposed the co-existence of the two models as a unified MALDI analyte protonation mechanism. Nevertheless, even after 25 years of intense research the MALDI mechanism is not fully understood.

2.5.4 Refined lucky survivor

Karas and colleagues have proposed that analytes are incorporated in the matrix with a respective solution charge state.\textsuperscript{101} In order to facilitate the following descriptions, protons with matrix origin are specified as D, and protons
that originate from the analyte are H. The matrix clusters formed carry a charge \([\text{M+D}]^+\) or \([\text{M-D}]^-\) due to charge separation upon the disruption of the matrix-analyte solid during the laser irradiation. Counterion neutralization, initiated by these matrix ions, leads to protonated analytes. (see eq. 8) Negatively charged analytes are formed by deprotonation. Ultimately, the charged analytes can be detected as so-called lucky survivors in case they are not neutralized by absorption of photoelectrons or electrons from the metallic target.\(^{102}\)

\[
\begin{align*}
[A+H_n]^n^+ + [nX]^n^- +^{[M+D]^+} &\rightarrow [A+H]^+ + [X + D] + (n - 1)[X + H] \\
\text{eq. 8}
\end{align*}
\]

2.5.5 The gas-phase protonation model

The gas-phase protonation model starts with neutral analytes in the gas phase. Gas-phase collisions of these neutral species with matrix ions lead to proton transfer. As a result protonated or deprotonated analyt ions can be detected (see eq. 9).

\[
\begin{align*}
[A]^{+[M+D]^+} &\rightarrow [A + D]^+ \\
\text{eq. 9}
\end{align*}
\]

2.5.6 Excited electronic state models

Excited electronic state models, such as multiphoton ionization, exciton pooling, and excited state proton transfer have been reviewed by Knochenmuss et al. (2006).\(^ {88}\) More recently, the thermal ionization model has been reconsidered.\(^ {90}\) Nonetheless, a globally valid model has not been suggested yet.
Methods

2.6 Sample preparation

Samples that originate from biological backgrounds such as metabolite extracts or protein supernatants need particular attention during sample preparation. The options for sample preparation include desalting, filtration, solid-phase extraction or liquid-liquid extraction. In the analysis of nucleotides and nucleotide sugars, an extraction based on 60% MeOH (aq.) has been found to facilitate the analysis. The MeOH extraction separates the aqueous metabolites from lipids and many proteins. Dietmaier et al. developed metabolite quenching and extraction protocols for mammalian cells and found MeOH superior or equal suitable than EtOH or MeCN. A crucial step for axial MALDI TOF experiments is the crystallization of the matrix together with the analyte. A homogenous crystallization has a positive impact on reproducibility and appearance of the MALDI spectra. Moreover, the matrix-sample crystal morphology significantly influences sensitivity, resolution and mass accuracy of the analysis. Consequently, the matrix choice is essential for a successful experiment. Generally, the matrix application serves two functions: firstly, the usually organic matrix solvent helps to extract analytes from the sample (e.g. tissue) and often leads to pre-separation of salts and analytes. Secondly, the matrix molecules absorb the impacting laser energy and softly dissipate the energy to desorb and ionize the analyte from the surface. Studies on solvent choice, sample cleanup strategies, and matrix-sample application including e.g. multilayering, pneumatic or electrospray-assisted matrix deposition, sublimation or “thin-film” deposition underscore the complexity of successful MALDI experiments. Each class of analytes has its particular best sample preparation and matrix application, whereby in MALDI method development many steps are simply empirically driven.
Methods

Thin-film deposition results in small and homogenous matrix crystals. However in biological samples the analyte distribution within a thin film spot can vary significantly as demonstrated by Garden et al. in a MALDI imaging experiment with 50 µm spatial resolution. Matrix sublimation provides homogenous matrix coverage with small matrix crystals as well and allows for imaging experiments with a spatial resolutions down to 1-2µm. The dried-droplet technique is prone to on-plate analyte segmentation also known as the “sweet spot phenomenon”. Nishikaze et al. investigated matrix sweet spot phenomena in glycopeptide samples using Raman imaging and MALDI-MS. Thereby, dimorphic forms of 2,5-DHBA have been discovered and good quality mass spectra could only be taken from one crystal form (sweet spots). Sinapinic acid is a well known matrix for protein analysis in positive ion mode. This matrix has been demonstrated to be very suitable for the analysis of intact proteins, such as monoclonal antibodies. 9-aminoacridine (9-AA) is very suitable for metabolite analysis in negative ion mode since the matrix produces few background peaks above m/z 200. The matrix is very sensitive for the detection of phosphorylated nucleotides. In 2002, the nobel prize in chemistry was awarded by the Royal Swedish Academy of Sciences to John Fenn and Koichi Tanaka. Along with Fenn’s electrospray ionization system, the matrix assisted laser desorption approach by Koichi Tanaka has been recognized. Tanaka et al. used ultrafine metal powder to form a matrix with glycerol. However, today, the use of organic molecules as demonstrated by Hillenkamp and Karas is almost exclusively applied in MALDI.
2.7 Microarray for mass spectrometry

2.7.1 Working principle

Microarrays for mass spectrometry (MAMS) have been developed by Dr. Urban in the lab of Prof. Zenobi at ETH Zurich in order to isolate single cells for mass spectrometric analysis. The first MAMS generation was fabricated from polysilazane-coated metal and ITO slides. The coating was structured using laser ablation in order to create hydrophilic spot cavities in a hydrophobic surrounding. These spots enabled the retention of small cell-containing liquid aliquots. A more detailed introduction to these MALDI target plates is given by Urban et al.\textsuperscript{107,108} Dr. Martin Pabst has exploited MAMS for quantitative MALDI measurements and created an entirely new field of application for MAMS. Figure 2-5 shows the working principle that Pabst et al. developed for the new MAMS generation.\textsuperscript{109} The analytes are spotted into a 5 µL MAMS reservoir.

\textbf{Figure 2-5} Schematic representation of the MAMS working principle by Pabst et al. [adapted from \textsuperscript{109}]
Methods

Subsequently, virtually identical homogenous matrix–analyte spots are created, by dragging the 5µL volume along the sample plate. Each nanoliter aliquot rapidly crystallizes to homogenous crystals and leads to highly reproducible MALDI mass spectra.\textsuperscript{109} Compared to sequential spotting using pipettes, the MAMS aliquoting creates rapidly a high number (n>10) of technical replicates. Summarized, the MAMS provides the following key advantages: (i) rapid generation of technical replicates, (ii) rapid and homogenous crystallization (thin film) of the matrix analyte mixture due to nanoliter reservoirs, (iii) analysis of the entire spot during one MALDI experiment with spectra addition or averaging possible.

The analysis of technical replicates (n) has a positive effect on the standard error of the mean (SEM) with \((n-1)^{1/2} :\)

\[
\text{standard deviation} = \sqrt{\frac{\sum(x_i - \bar{x})^2}{n - 1}}
\]

eq. 10

With \(x_i\) being an independent sample, and \(\bar{x}\) the sample mean.

A more recent MAMS generation enables aliquoting the 5 µL into 50 cavities per lane. This high replicate number allows for different MS and MS-MS quantification experiments of complex analyte mixtures. As a result the signal distribution in microarray-based MALDI-MS is narrowed compared to standard stainless steel plates. Figure 2-6 shows the signal distribution in MALDI MS of two different sample platforms and laser search pattern. From the comparison of Figure 2-6A to 2-6B (both the signal distribution of \(^{13}\text{C}^{15}\text{N}-\text{ATP}\) in negative ion mode on a commercial stainless steel plate) it becomes obvious that the use of a laser search pattern during MALDI is superior to the random walk. Figure 2-6C depicts the MS/MS signal of the metabolite ecgonine methyl ester-D3 \((m/z 85.069)\) analyzed using a Si-wafer based MAMS platform. The MAMS chip yields in relative standard deviations smaller <10%. 
Methods

Figure 2-6 Signal distribution of $^{13}\text{C}^{15}\text{N}$-ATP (9-AA, 10 mg/ml, acetone) in MALDI-MS experiments on a stainless steel plate using A a random laser walk, B a search pattern. C Ecgonine methyl ester –D3 (2ug/mL MeCN, 5mg/mL CHCHA, 60/40/0.2, MeCN/H$_2$O/TFA) measured on a N/As doped Si-MAMS (resistivity < 0.005 Ohm-cm, thickness 525 µm) using a search pattern.

2.7.2 Fabrication

Originally MAMS have been produced using laser ablation processing methods, laser scanning and laser projection have both been investigated. For laser scanning, a Nd:YAG-laser (SuperRapid from Lumera Laser, Kaiserslautern, Germany) with ~10 ps pulses at 355 nm with a repetition rate of 50 kHz and an average power of 100 mW has been used. The laser light is focused ~10µm diameter with a telecentric lens onto the surface and the surface is scanned with (150 mm s$^{-1}$). In this approach, the spot rims are defined by the focus of the ablation laser light.

Laser projection leads to very defined and well-resolved microstructures. The projection system allows 20 ns pulses of
Methods

248 nm wavelength laser light with a 50 Hz repetition rate to illuminate an area of 16x16 mm$^2$ on a mask. After passing through the mask, the light is focused with a field lens demagnification factor of 5 onto the polysilazane substrate. An average of 500 mJ cm$^{-2}$ laser fluence on the substrate leads to ablation of polysilazane.$^{110}$

Due to the need of a mask, the projection laser system is less flexible with respect to changes in the design of the MAMS compared to the scanning system. However, the structures are better resolved compared to the scanning approach.

**Figure 2-7** Photolithography protocol. The spot examples were recorded with a Nikon Ti microscope (20x magnification) brightfield.
A major concern in the laser ablation-based fabrication is the conservation of the underlying ITO layer. The ITO readily absorbs in the UV-range and thus it is very difficult to selectively remove only polysilazane. In both laser ablation approaches ITO ablation can be observed by microscopy. A new generation of MAMS has been developed in the framework of this thesis in collaboration with Prof. Andreas Hierlemann and Gregor Schmid (both BSSE Basel). Schmid et al. have developed an alternative production process based on photolithography. This approach overcomes the problem of ITO co-ablation that compromises laser ablation based MAMS fabrication processes. In this approach, a photoresist is spincoated on top of the polysilazane coating and subsequently the resist is exposed to UV via a transparency mask. The polysilazane gets removed in the exposed areas by reactive ion etching (see figure 2-7). Finally, the photoresist is washed off. This approach allowed for the first time the production of gold and n- or p-doped silica wafer based MAMS. These MAMS can find their application e.g. in SPR MALDI imaging or in affinity-based MALDI and will open new fields of applications.\textsuperscript{111,112}
Methods

2.8 Separation sciences

Chromatography is a golden standard in protein and metabolite analysis. In the presented work size exclusion chromatography (SEC) and high-performance liquid chromatography (HPLC) has been used to cross validate the developed MALDI-MS methods.

2.8.1 Size-Exclusion Chromatography

In SEC the separation is based on classification of molecular size instead of direct interaction with retention phase material. SEC columns are packed with microporous particles, and their pore diameters determine which molecule sizes are entering the particle and thus have different retention times. An excellent review about SEC has been written by Hong et al.\textsuperscript{113} Hyphenation of SEC to mass spectrometry has been realized to analyze weights of intact antibodies and antibody drug conjugates, however has not been part of this thesis.\textsuperscript{114,115} The SEC separation results in this work have been detected with a UV-detector at 280 nm.

2.8.2 High-performance liquid chromatography

Protein A chromatography has been used in this thesis to validate monoclonal antibody concentration in the fed-batch and perfusion cell culture supernatants. The antibody trace was detected at 280 nm. Ion-pair reversed-phase chromatography has been used to analyze nucleotide sugars and phosphorylated nucleosides. Tetrabutylammonium sulfate (TBAS) has been used as ionpairing agent. The used protocols are summarized in \textsuperscript{22,116}. 
Rapid estimation of the energy charge from cell lysate using MALDI-MS: role of in-source fragmentation

A MALDI-MS based method for the detection of phosphorylated nucleotides in organic cell extracts is presented. The impact of ‘cold’ and ‘hot’ matrices is demonstrated using 9-aminoacridine (9-AA) and 2,4,6-trihydroxyacetophenone (THAP). Analyte fragmentation by insource fragmentation is demonstrated and cross-validated using ESI-MS.


©2013 Elsevier Inc.
Rapid estimation of the energy charge

3.1 Overview

Nucleotides are key players in the central energy metabolism of cells. Here we show how to estimate the energy charge from cell lysates by direct negative ion MALDI mass spectrometry using 9-aminoacridine as matrix. We found a high level of in-source decay of all the phosphorylated nucleotides, with some of them producing considerable amounts of ADP fragment ions. We investigated the behavior of adenosine-5’mono-, di, and triphosphate (AMP, ADP, ATP), as well as the cofactors coenzyme A and acetyl-coenzyme A (CoA, AcCoA), and nicotinamide adenine dinucleotides (NAD+, NADH) in detail. In-source decay of these compounds depends strongly on the applied laser power and on the extraction pulse delay. At standard instrument settings, the 9-aminoacridine matrix resulted in a much higher in-source decay compared to 2,4,6-trihydroxyacetophenone. By adding $^{13}$C labelled ATP to a cell lysate, we were able to determine the degree of in-source decay during an experiment. Analysing a cell extract of the monocytic cell line THP-1 with $^{13}$C-ATP as internal standard, we were able to obtain values for the energy charge that were similar to those determined by a reference LC-ESI-MS method.

3.2 Introduction

Typically, metabolites are low molecular weight compounds (<1000 Da), whose detection and characterization is challenging due to structural similarities and their propensity to degrade during sample preparation and the analytical measurement process. Specifically, the class of nucleotides are prone to degradation. Nucleotides, which play a key role in cellular metabolism, are able to transport reaction energy in living organisms using the energy-rich phosphate bonds. In the early 1960s, Atkinson described for the first time the key role of nucleotides and
expected these to have regulatory functions at all branching points between anabolism and catabolism. This discovery, which included the equilibrium of the three nucleotides adenosine-5’-triphosphate (ATP), adenosine-5’-diphosphate (ADP) and adenosine-5’-monophosphate (AMP), is known today as the “energy charge hypothesis” ([ATP]+0.5*[ADP]/[ATP]+[ADP]+[AMP]).

Investigating the organization and regulation of the nucleotide metabolism, in particular in the central metabolic pathway, provides insight into both catabolic and anabolic regulatory functions in a biological system. If a measurement distorts the ratio of the three phosphorylated nucleosides ATP, ADP and AMP (adenosine-5’x-phosphate), an erroneous value for the energy charge will result, and their regulatory function might be misinterpreted. Commonly, adenosine-5’x-phosphates are analysed optically by immunohistochemical tagging, chemical staining, or alternatively by radiolabeling. However, most optical methods are currently limited in sensitivity, selectivity for the reactive compounds, and their ability to simultaneously detect several target compounds. Investigations of the metabolome require the simultaneous detection of a wide range of metabolites in a quantitative manner. Consequently, improvement of existing as well as development of new analytical technologies and methodologies are thought to be an important subject in the context of metabolomics. State-of-the-art methods for metabolite analysis include liquid chromatography coupled to mass spectrometry (LC-ESI-MS), gas chromatography in combination with mass spectrometry (GC/MS), enzymatic assays, nuclear magnetic resonance (NMR), and on the single-cell level, “live single-cell mass spectrometry”. The list of techniques is extended by biological sensors. Sensors enable online detection, but they are generally limited in their chemical
versatility. Furthermore, matrix-assisted laser desorption mass spectrometry (MALDI-MS) has been shown to be a powerful tool for the investigation of metabolites in biological systems. MALDI-MS as a tool for the analysis of small molecules has gained more and more interest in the last decade. The high sensitivity, selectivity and throughput of MALDI-MS allow experiments even at the single-cell level. A high-density microarray for mass spectrometry that enables high-throughput experiments was recently designed by Urban et al. Negative ion mode MALDI-MS has been demonstrated to be a suitable approach for selective and sensitive detection of phosphorylated analytes in a complex biological matrix. Especially the use of 9-aminoacridine (9-AA), a matrix with low chemical background in the low mass region, enables the analysis of phosphorylated nucleosides with outstanding sensitivity. However, compared to other analytical approaches MALDI-MS has a poor quantification performance. Some strategies, including the use of isotopic labelled standards have been developed to improve the quantification capabilities of MALDI-MS.

A well-documented property of MALDI-MS analysis is the fragmentation of fragile analytes, either via in-source or post-source decay. Hillenkamp et al. categorized possible fragmentations occurring in MALDI-MS according to their time scale into four categories: prompt, fast, early metastable, and metastable. The most prominent decays are the prompt and fast ones, which take place in the source. These decays are a result of three individual processes: Firstly, the direct absorption of the laser energy by the analyte, which results in thermally excited molecules; secondly, collisional activation by the acceleration through the matrix plume; and thirdly exoergic chemical processes such as proton transfer. In 1995, Brown et al. described in-source-decay (ISD) for the first time as promptly formed fragment ions upon ion
Rapid estimation of the energy charge

generation.\textsuperscript{140} In-source decay (ISD) and post-source decay (PSD) are both used, e.g., in top-down sequencing of intact proteins and are powerful tools in the identification of small- and moderately sized peptides.\textsuperscript{138,142} However ISD and PSD can be hurdles in the quantification of phosphorylated nucleosides, since the fragmentation can prevent their correct identification in MALDI-MS analyse of living organisms. Thus, the aim of this study is to characterize fragmentation, and to minimize it by optimizing the conditions while maintaining the high sensitivity of our experiments. In the present study, we are investigating the in-source fragmentation behaviour of selected adenosine containing nucleotides and cofactors in MALDI-MS experiments using 9-AA as matrix. Validation of the metabolite standards is performed with electrospray ionization and an additional MALDI source from a different supplier. Potential fragmentation sources affecting the quantitation performance of our approach were identified and evaluated in view of estimating the energy charge of cells. Finally, the MALDI matrix 9-AA is compared with 2,4,6- THAP, another commonly used matrix in negative ion mode. By adding $^{13}$C-ATP as internal standard to cell lysates, we could estimate the degree of in-source fragmentation during analysis, and perform a correction of the determined ratios. The direct MALDI-MS analysis delivered finally similar energy charge values as a standard reference LC-ESI-MS method.

3.3 Experimental

3.3.1 Metabolites

Adenosine-5’-triphosphate (ATP), adenosine-5’-diphosphate (ADP), adenosine-5’-monophosphate (AMP), flavin adenine dinucleotide (FAD), and nicotinamide adenine dinucleotide phosphate (NADPH) were purchased from Sigma Aldrich (Buchs, Switzerland). Beta-nicotinamin adenine dinucleotide
Rapid estimation of the energy charge

oxidized form (NAD\(^+\)), and beta-nicotinamin adenine dinucleotide oxidized form (NADH) disodium hydrate were purchased from ACROS Chemicals (Belgium). Acetyl-coenzyme A (ACoA) and coenzyme A (CoA) were purchased from AppliChem (Germany). All metabolites were used without further purification and were diluted to a concentration of 100 fmol/\(\mu\)L in 50% MeCN. To avoid measuring hydrolysis artefacts of the standards, all standards were freshly prepared directly before each experiment and validated by direct infusion to ESI-MS.

3.3.2 Matrices

9-Aminoacridine (9-AA) hydrochloride monohydrate 99% was purchased from ABCR (Germany). 10 mg/mL 9-AA were dissolved in 90% ethanol. 2’,4’,6’-Trihydroxyacetophenone monohydrate (THAP) was purchased from Acros Organics. 10 mg/mL of THAP were dissolved in 50% aq. acetonitrile. Dibasic NH\(_4\)citrate (50 mg/mL, aq) was added to the matrix solution at a ratio of 9:1. Experiments with 2’,3’,4’-tri hydroxyacetophenone in MeCN:H\(_2\)O 1:1 (v/v) with dibasic NH\(_4\) citrate, were also conducted, but did not yield good results.

3.3.3 Sample preparation

Prior to analysis, a 2 \(\mu\)L aliquot of the matrix solution (as described above) was mixed with 2 \(\mu\)L of the analyte solution. An aliquot of 0.5 \(\mu\)L of the mix was spotted onto a MALDI plate (384 spots, AB Sciex, stainless steel). Nucleotide extracts were obtained as described elsewhere. 35 Briefly, 1 mL of cells (1*10\(^6\) cells/mL) were washed with cold water. Nucleotides were extracted with 350 \(\mu\)L of cold 80% MeCN and subsequently with 150 \(\mu\)L of cold 50% MeCN. 2 \(\mu\)L of the
combined cell extract were mixed with 1 µL of a 50 µmolar $^{13}$C-ATP standard solution and further with 3 µL matrix. 0.5 µL of this analyte-matrix mix were spotted onto a standard stainless steel MALDI plate. The cell extract was vacuum dried and redissolved in 200 µL 30 mM NH$_4$CO$_3$ for LC-MS analysis.

3.3.4 Instrumentation

A MALDI-TOF-MS instrument (AB Sciex 4800) equipped with an Nd:YAG laser was used in negative ion reflector mode for data acquisition. The Nd:YAG laser operates at 355 nm and has a repetition rate of 200 Hz. A laser attenuator is installed to vary the intensity of the laser beam reaching the sample. As a standard laser energy setting, approximately 5.5 x10$^7$ W/cm$^2$ were used. The optimum extraction time delay was found to be 400 ns. For experiments on the in-plume acceleration, the extraction pulse delay was varied from 100 ns to 400 ns. Ten shots per subspectrum were used and 40 subspectra were recorded. A Bruker Solarix FT-ICR MS equipped with a 9.4 Tesla cryomagnet and an ESI and MALDI source was used for complementary fragmentation and sample validation experiments. The MALDI source was equipped with a "smart beam laser II" and operated with a laser power of 27.00% [arb. units], 150 laser shots. The ESI capillary voltage was operated at 4500 V, drying gas flow was set to 3.7 l/min, 1.3 bar, 200°C. The FT ICR measured in broadband mode, with a source accumulation time of 1ms, an ion accumulation of 20 ms, and an ion cooling time of 1 ms.
3.4 Results and Discussion

3.4.1 In-source decay vs. post source decay

ATP was measured by MALDI-MS using 9-AA (90% EtOH) and, using standard instrument conditions generated around 40% ADP and 5% AMP. Since reference measurements of the ATP standard by LC-MS revealed no hydrolysis products, we were confident that ATP was fragmented to a large extent during the MALDI-MS analysis. By repeating the measurement on the same spot in negative linear mode, a similar ratio between ATP, ADP and AMP was acquired. We thus conclude that the decay process mainly happens in the source. The analyte can release the internal energy collected in-source, by undergoing fragmentation.\textsuperscript{143} In the following experiments, we focus on parameters that potentially influence the degree of in-source decay of our analytes in the reflectron mode.

3.4.2 Evaluating the in-source fragmentation products from selected nucleotides

As seen in figure 3-1A, ATP can show an overall in-source decay rate of as much as 60% using 9-AA. In addition to the deprotonated molecule ATP (505.95 m/z), the MALDI spectrum in fig. 3-1A also shows a variety of different signals: 426.00 m/z is assigned to ADP and 407.99 m/z to ADP-H\textsubscript{2}O. Minor fragmentation to AMP (346.02 m/z) and AMP-H\textsubscript{2}O (328.02 m/z) can also be observed (summarized in figure 3-1E). The asterisks highlight matrix peaks in the spectrum. A 99% purity of the standard was confirmed by LC-MS and ESI-MS measurements. As reported in the literature, we found THAP to be suitable for nucleotide analysis, with less fragmentation of the nucleotide analytes even at high laser powers.\textsuperscript{144} These matrix-specific differences are well known in MALDI mass spectrometry.\textsuperscript{86} Hardly any fragmentation of ATP to ADP was found using THAP (shown in fig. 3-1C).
Tuning of the laser intensity and the extraction pulse delay reduced the in-source decay for 9-AA to less than 10% (as seen in Fig. 3-1B). However this reduced laser intensity also reduces the sensitivity of the method by 40%. The detailed description of the MALDI-MS parameter optimization is discussed in the following sections. To estimate the fragmentation yield in the presence of ADP, a $^{13}$C labelled ATP standard ($^{13}$C$_{10}$-ATP) can be used. The fragmentation yield is expressed as the presence of $^{13}$C-ADP relative to the $^{13}$C-ATP. It must be noted that not only ATP, but also adenosine-phosphate containing cofactors like acetyl-coenzyme A (ACoA) or NAD$^+$/NADH and NADP$^+$/NADPH (nicotinamide adenine dinucleotide), can generate ADP fragments. This might lead to a massive overrepresentation of ADP in the case of a high degree of in-source decay, compromising the relative quantification of ATP, ADP, and AMP. The observed fragmentation rate at instrumental conditions optimized for minimum fragmentation for adenosine-phosphate containing metabolites is shown in fig. 3-2. Metabolites that carry a 5'-adenosine-diphosphate moiety readily fragment to adenosine-diphosphate and adenosine-monophosphate. In case of NAD$^+$, we mainly detected the fragments at 540.05 m/z, which are the result of a ring opening at the adenine and a partial elimination of the nicotinamide ring. Thus the fragmentation to ADP ions here is calculated relative to the major fragment 540.05 m/z. Furthermore, collision-induced dissociation (CID) of ATP (see fig. 3-3), outlines the fragmentation pathway. CID was found to result in the same fragments as in-source decay. Two main neutral losses are observed: [PO$_3$H] and [PO$_3$H+H$_2$O]. The in-source fragments of ATP are ADP and ADP-H$_2$O. In the CID experiment the formation of ADP-H$_2$O is more prominent than in the ISD spectrum. Almost no collision-induced dissociation to AMP is observed.
Rapid estimation of the energy charge

Figure 3-1 (A, B) MALDI mass spectrum of ATP using 9-AA (90% ethanol) with a matrix:analyte ratio of 1:1. A is recorded with standard instrument conditions; B is recorded at optimized instrument conditions. The in-source decay changes with the extraction time delay and laser intensity. Observed fragments of ATP are 426.0 m/z and 408.01 m/z. The loss of 98.00 mass units is assigned to the fragment [PO₃H⁺H₂O]. The loss of H₂O can occur in the phosphate backbone or in the ribose. (C, D) MALDI mass spectrum of ATP using 2,4,6-THAP (20mM NH₄ citrate, 50% MeCN) with a matrix:analyte ratio of 1:1. Varying the laser intensity and extraction pulse delay affects the sensitivity but not the in-source decay to the same extent as in the case of 9-AA. (E) Molecular structure of ATP with the most prominent fragmentation losses.
3.4.3 Comparison of in-source decay in an FT-ICR-MS and a TOF-MS

Reference measurements with a commercial FT-ICR mass spectrometer with MALDI and ESI source (SolariX, Bruker) showed clearly that the degree of analyte fragmentation in ESI differs from in-source fragmentation in MALDI-MS. In the ESI analysis with 50% MeCN no fragments of the nucleotides were found for most of the analytes. Comparing the MALDI results between the AB 5800 and the SolariX (TOF-MS and FT-ICR) we also detected different relative intensities for the fragment ions (fig. 3-2A and 3-2B). This was especially true for CoA and ATP. These differences might be a result of the different laser sources of the two instruments, or be due to mass-dependent differences in the ion transmission of the instruments. Finally, the use of different target plates and materials could lead to different sample crystallizations and might therefore also influence the results slightly.

**Figure 3-2** The total fragmentation yield of Acetyl-CoenzymA, CoenzymA, and nicotinamidadenindinucleotide NADH, NAD+, and ATP to ADP (dark bars) and AMP (light bars). (A) depicts the yield found in the MALDI TOF instrument, and (B) the yield in the FT-ICR instrument. The analytes were freshly prepared and thus prior hydrolysis can be excluded.
Rapid estimation of the energy charge

3.4.4 Influence of the laser power

The laser power was found to have the greatest influence on the in-source fragmentation in the MALDI analysis (see fig. 3-4A). The effect is linear almost over the entire range shown in fig. 3-4A, and is in good agreement with the literature. \(^\text{71,145}\) Moreover it is known from the literature that the number of laser positions and the number of shots per position affect the scatter in the data and the S/N ratio. \(^\text{72}\)

![MALDI mass spectrum of a 2:1 mixture of ATP and \(^{13}\text{C}\)-ATP using 9-AA (95% EtOH) as a matrix. In-source decay of the metabolite leads to the formation of ADP, ADP-H\(_2\)O and AMP. (B) MALDI CID mass spectrum of ATP, (precursor mass of 505.95 m/z), 408.07 m/z can be assigned to ADP-H\(_2\)O, 177 m/z and 159.22 m/z are assigned to pyrophosphate and P\(_2\)O\(_6\)H\(_3\).](image)

**Figure 3-3** (A) MALDI mass spectrum of a 2:1 mixture of ATP and \(^{13}\text{C}\)-ATP using 9-AA (95% EtOH) as a matrix. In-source decay of the metabolite leads to the formation of ADP, ADP-H\(_2\)O and AMP (B) MALDI CID mass spectrum of ATP, (precursor mass of 505.95 m/z), 408.07 m/z can be assigned to ADP-H\(_2\)O, 177 m/z and 159.22 m/z are assigned to pyrophosphate and P\(_2\)O\(_6\)H\(_3\).
3.4.5 Influence of the extraction pulse delay

Moreover, we investigated the influence of the delayed extraction time on the in-source fragmentation. Delayed extraction is routinely used in MALDI-MS to increase the mass resolution and sensitivity of the experiment. The ADP/ATP ratio in fig. 3-4B displays the in-source fragmentation yield at different extraction delay times. Longer extraction delays led to less fragmentation of ATP. The number of energetic collisions between molecular ions and neutral matrix molecules is reduced at longer extraction delays, probably because the density of neutrals in the ablation plume, through which the ions are accelerated after the extraction field is switched on, becomes lower at longer delays. Therefore the total internal energy of the extracted ions is lower and the fragmentation ions appear less prominent.\(^{143}\) Longer extraction times also favour larger masses, i.e., the smaller fragment ions might be underrepresented.

Figure 3-4 (A) The in-source decay is laser power dependent. Higher laser power leads to increased fragmentation yield. (B) The influence of the extraction pulse delay on the in-source decay of nucleotides at 15kV acceleration voltage with a grid to source ratio of 0.77. The ADP/ATP ratio is a measure for the in-source fragmentation yield.
3.4.6 In-source decay and analyte quantification

The matrix 9-AA has attracted considerable interest due to the possibility to detect various analytes with attomolar sensitivity, even in complex biological environments. However, analyte fragmentation using 9-AA is a concern. Consequently the quantification of ADP by MALDI-MS in a mixture of metabolites using 9-AA as matrix is difficult. Without correction, it leads to an overestimation of ADP. Therefore, we performed an experiment using the isotopically labelled standard $^{13}$C-ATP, which was added to the cell extract just prior to MALDI-MS. Appearance of $^{13}$C-ADP in the spectrum can be used as an indicator for the degree of fragmentation. In a sample mixture of ATP, ADP and AMP we investigated the variation of the $^{13}$C-ATP/$^{12}$C-ADP ratio and in parallel the in-source fragmentation yield ($^{13}$C-ADP/$^{13}$C-ATP) over a concentration ranging from 0.025 µM to 10 µM. The quantitation of all three compounds with $^{13}$C-labeled ATP as internal standard was linear over the displayed concentration range (fig. 3-5A).

The $^{13}$C-ATP to $^{12}$C-ADP ratio stayed constant for different sample concentrations (ranging from 25 to 2.5 µM) and drops as expected under the limit of quantification (in this case below 2.5 µM). The fragmentation yield of the internal standard (fig. 3-5B) varied between 20 and 40%, and increased below 2.5 µM, the limit of quantification. The area of $^{12}$C-ADP can be corrected by subtracting the fragmentation yield of $^{13}$C-ATP multiplied by the measured $^{12}$C-ATP area from the measured $^{12}$C-ADP area. Accordingly, the loss via fragmentation of the $^{12}$C-ATP area can be corrected for, by adding the fragmentation yield of $^{13}$C-ATP multiplied by the measured $^{12}$C-ATP. However it must be noted that the correction can exclusively be applied in the concentration
Rapid estimation of the energy charge

range of the internal standard. Experiments with an internal standard concentration far above the or far below the analyte concentration will not yield reliable results.

3.4.7 Measuring cell batches and cell extracts with 9-AA and THAP

Both matrices 9-AA and 2,4,6-trihydroxyacetophenone, which are well known for nucleotide analysis, are suitable for the detection of the phosphorylated nucleosides ATP, ADP and AMP from cellular extracts. A cellular extract measured with 9-AA (370 ns extraction pulse delay, laser power of 3500 [arb. units]) shows ATP (505.98 m/z), ADP (426.02 m/z), AMP (346.05 m/z) and moreover UDP-N-acetylhexoseamine (606.07 m/z) as predominant peaks. The same cell extract measured by THAP (370 ns extraction pulse delay, laser power of 4400 [arb. units]) revealed a completely different picture, because the overall number of detected compounds was much lower. In both cases we used an extraction pulse delay of 370 ns and set the laser power to 10% (in arb. units) above the ionization threshold. Surprisingly, the energy charge was found to be the same for the analysis with 9-AA and 2,4,6-THAP (fig. 3-6A, 9-AA and B, THAP). Additionally, with both matrices, phosphorylated lipids and metabolites were present in the same negative ion mode spectrum.
Rapid estimation of the energy charge

**Figure 3-5** (A) Calibration curve of ATP (circles), ADP (squares) and AMP (diamonds) with $^{13}$C-ATP as internal standard over a concentration range from 1 uM to 12.5 μM (0.5 μL matrix:analyte 1:1 spotted); (B) $^{13}$C-ATP/$^{12}$C-ADP ratio measured with optimized instrument parameters circles. The in-source decay of 13C-ATP at optimized instrument parameters is displayed in triangles. (C) The energy charge calculated from $\frac{([ATP]+0.5[ADP])/([ATP]+[ADP]+[AMP])}$ over the concentration range of 12.5 μM to 1 μM
3.4.8 Estimating the energy charge in cell lysates

By evaluating the analysis parameters, we found two main influencing factors responsible for in-source decay of the selected nucleotides: laser power and the extraction pulse delay. The degree of in-source decay of ATP, which was the major source for ADP fragment ions during the analysis of cell lysates, could be determined (in each measurement) by the use of a $^{13}$C-ATP internal standard.

<table>
<thead>
<tr>
<th>THP-1</th>
<th>Uncorrected</th>
<th>Corrected</th>
</tr>
</thead>
<tbody>
<tr>
<td>energy charge</td>
<td>0.88</td>
<td>0.94</td>
</tr>
<tr>
<td>standard deviation</td>
<td>0.01</td>
<td>0.02</td>
</tr>
<tr>
<td>rel. standard deviation (%)</td>
<td>0.92</td>
<td>1.75</td>
</tr>
</tbody>
</table>

**Table 3-1** The energy charge in cultured THP-1 cells. Note: The sample was spiked with 50 µM $[^{13}$C]ATP. The relative fragmentation yield was subtracted from the measured ADP signal. The number of repeats was n = 3.

At optimized instrument conditions and the use of $^{13}$C-ATP internal standard, it was found possible to determine the energy charge of a cellular population. Without internal standard, an extract from healthy THP-1 cells showed an energy charge between 0.7 and 0.9. Since ATP is the most prominent contributor to ADP, it is often necessary to compensate the overrepresentation of ADP by the use of the correction. Subtracting the relative $^{13}$C-ATP fragmentation yield from $^{12}$C-ADP results in a higher and most probably more reliable energy charge for the cells (see table 1). The same extract was measured by a reference LC-ESI-MS method that showed values in the same range.

In order to obtain a reliable quantification, we propose to define and use reproducible instrumental reference conditions for: (1) the laser irradiance has to be set to threshold
Rapid estimation of the energy charge

irradiance, and kept constant in all measurements, (2) the extraction pulse delay has to be set to an optimized value for all measurements, and (3) further parameters like the acceleration field strength, or the residual gas pressure, which are also known to have an influence on the metastable decay, have to be monitored and ideally kept constant for the experiment. A 13C-ATP internal standard is useful for a direct estimation of the in-source decay, but was found also necessary to correct the day to day variations of the instrument performance.

5. Conclusions

Adenosine containing nucleotides and cofactors are present as highly abundant metabolites in all mammalian cells. Here we studied the in-source fragmentation behavior of AcCoA, CoA, NADH, NAD+ and ATP to ADP and AMP in negative mode MALDI-MS and discussed how this influences the estimation of the energy charge. The degree of in-source fragmentation in electrospray ionization of adenosine-phosphate molecules is
Rapid estimation of the energy charge

low; using LC-ESI-MS in-source fragmentation products are linked to their parent ions via the retention time. However in MALDI-MS, ions generated by fragmentation are not separable from those that are naturally present in the sample. Using the matrix 9-AA especially at high laser energies or too short extraction pulse delays, in-source fragmentation can exceed 60% of the analyte molecule. The extraction time delay and the laser power are critical parameters for the fragmentation yield of nucleotides. Optimizing these parameters can minimize the fragmentation yield, and enhance the quantification performance. By measuring cell extracts, ATP fragmentation was found as the major source for a false estimation of the energy charge. Using $^{13}$C-ATP as an internal standard, the in-source fragmentation could be estimated for each measurement and corrected for. Under optimized conditions, direct MALDI-MS analysis of cell extracts delivered similar results as reference LC-ESI-MS measurements. MALDI-MS can be therefore a fast alternative technique for determining the energy charge of a biological cell population.
Rapid estimation of the energy charge
High-throughput nucleoside phosphate monitoring in mammalian cell fed-batch cultivation using quantitative matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF-MS)

This chapter describes the analysis of nucleoside pools in fed-batch cultivation processes using microarray based MALDI-MS. The throughput capabilities of MALDI-MS are demonstrated by monitoring experiments.

© 2014 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim
4.1 Overview

We describe a novel high-throughput method based on matrix-assisted laser desorption/ionization (MALDI) time-of-flight mass spectrometry (TOF-MS) for monitoring intracellular metabolite levels in fed-batch processes. Current methods for monitoring multiple intracellular metabolite levels in parallel are limited in sample throughput capabilities and analyte selectivity. The MALDI-TOF-MS method presented here is based on a new microarray sample target and allows the detection of phosphorylated metabolites using stable isotope labeled internal standards. With short sample preparation steps and thus high sample throughput capabilities, the method is suitable for monitoring mammalian cell cultures in industrial environments. The method is capable of reducing the runtime of standard LC-UV methods to approximately 1 minute per sample (including 10 technical replicates). Its performance is demonstrated in an 8-day monitoring experiment of independently controlled fed-batches, containing an antibody producing mouse hybridoma cell culture. The monitoring profiles clearly confirmed differences between cultivation conditions. Hypothermia and hyperosmolarity were studied in four bioreactors, where hypothermia was found to have a positive effect on the longevity of the cell culture, whereas hyperosmolarity lead to an arrest of cell proliferation. The results are in good agreement with HPLC-UV cross validation experiments. Subsequent principal component analysis (PCA) clearly separates the different bioreactor conditions based on the measured mass spectral profiles.
4.2 Introduction

In the last decade the production of biopharmaceuticals in industrial cell cultures, including the production of monoclonal antibodies (mAbs), has undergone substantial development. To ensure reproducible cultivation conditions and product quality new process analytical tools (PATs) to supervise production steps have been introduced. 146-151 Many modern industrial processes are based on *E.coli* and mammalian cell cultures, and to a lesser extent on yeast, plant cell, or insect cultures. Such cell cultures are predominantly being run as fed-batch processes, and are constantly being optimized to achieve highest process performance, high reproducibility, good predictability. They should fulfill the high demands of the industrial environment. 146,152,153 Therefore, the development of new PATs is an active research field that grows hand-in-hand with the bioprocesses. Knowing the complexity and significance of any biopharmaceutical production process involving mammalian cell cultures, PATs should ideally offer (i) a chemically versatile and label-free method, to collect as many meaningful data as possible, (ii) a high dynamic range that is compatible with the investigated biological systems, (iii) low sample consumption, to avoid any disturbance of the cell culture process, (iv) rapid and cheap operation, and (v) the possibility to regulate the monitored culture *via* feedback. According to the 2004 FDA initiative, PATs can be grouped into multivariate tools, process analyzers, process control tools, and knowledge management tools. 154 Traditionally, monitoring strategies for mammalian cell culture physiology are based on the analysis of so-called “primary variables” such as viable cell concentration, viability, substrate consumption, waste metabolite production, and product quality. 155,156
More recently, metabolic profiles have been considered for process monitoring, because they carry useful information and provide a more extensive perspective on the cell culture state.\textsuperscript{4,155,157-160} Day-to-day and batch-to-batch variations of a running process can be followed using internal standards and a quantitation strategy.\textsuperscript{47,161} One can distinguish between metabolite \textit{profiling}, i.e., the quantitative analysis of a set of intracellular metabolites, metabolite \textit{fingerprinting} that represents an unbiased global screening approach, and metabolic \textit{footprinting} known as the analysis of extra-cellular metabolites.\textsuperscript{21}

The literature indicates that monitoring of metabolites leads to deeper insights about the cell culture process, and furthers the understanding of different process conditions.\textsuperscript{2,3,155,162} For example, it has been shown that changes in the nucleotide concentrations correlate well with cell cycle phases and proliferation, and affect enzyme activity.\textsuperscript{161,163} Moreover, nucleotide pools have been used as indicators for the growth rate of mammalian cell cultures.\textsuperscript{1} Concentration ratios of different nucleotides have been successfully correlated with the physiological state of mammalian cells.\textsuperscript{25} When followed over the time course of a bioprocess, such nucleotide concentration ratios could potentially be applied as early warning signs of process perturbations.\textsuperscript{155}

For monitoring industrial cell culture processes, one generally distinguishes between online and offline tools. The online tools include photometry, amperometry, enzymatic assays, real-time gas-chromatography (GC), near infrared spectroscopy, Raman spectroscopy, online proton transfer reaction-mass spectrometry (PTR-MS), diverse chromatographic methods, and particle size instruments coupled to flow-injection analysis systems.\textsuperscript{164-173} Among the offline tools are nuclear magnetic resonance (NMR) and
various chromatographic methods hyphenated to MS, such as liquid-chromatography (LC)-MS. 24,123,174-178 Mass spectrometry in general offers a high selectivity and sensitivity and the possibility to reliably identify metabolites according to their exact mass-to-charge ratio, or in a second dimension according to their specific fragmentation pattern. Offline metabolic profiling is currently performed with either GC-MS or high-performance liquid chromatography (HPLC-MS). Obvious disadvantages of chromatographic methods are long cycle times, demanding sample preparation and a relatively high data set complexity. MALDI–TOF-MS has the potential to overcome these difficulties and to allow an analysis with a very rapid sample preparation, without prior chromatographic separation.

Here we describe a high-throughput offline multi-analyte profiling approach for metabolic profiling, based on quantitative MALDI-TOF-MS. We aimed at simultaneous monitoring of phosphorylated nucleotides, glutathione, and nucleotide sugars using a previously introduced improved microarray MALDI sample target. The sample target consisting of a coated indium tin oxide glass slide with defined hydrophilic sample reservoirs, which allows a rapid self-aliquoting of liquid samples for technical replicates, as described by Pabst et al. 109 Accordingly, a 13C-labeled internal standard was used in the MALDI-MS method for quantification, and the results obtained were cross validated by a state-of-the-art HPLC-UV method. 22 Statistical significance was demonstrated by unsupervised principal component analysis.
4.3 Materials and Methods

4.3.1 Chemicals and solvents

EtOH (p.a.), MeOH (Chromasolv), and NH₄OAc were purchased from Sigma-Aldrich (Germany). H₂O (LiChrosolv, Merck Millipore, Germany) was used in sample preparation and dilution steps unless otherwise specified. All solvents and salts were used without further purification.

4.3.2 Metabolite standards, stable isotope internal standard.

Adenosine 5’-triphosphate (ATP) was purchased from Sigma-Aldrich (Germany) and used without further purification. To avoid hydrolysis artifacts the standard was always freshly prepared just prior to analysis. ¹³C-ATP ammonium salt (U-¹³C10, chem. pur., 98%) was purchased from Cambridge Isotope Laboratories (USA). The ¹³C-ATP salt was dissolved in H₂O (LiChrosolv) to a final concentration of 100 µmol/L and aliquoted to 250 µL volumes. The internal standard aliquots were stored for analysis at −20°C.

4.3.3 MALDI matrix

9-Aminoacridine (9-AA) hydrochloride monohydrate 99% was purchased from ABCR (Germany). 10 mg/mL 9-AA were dissolved in 90 % ethanol. To remove insoluble particles the matrix solution was centrifuged (1 min 14000 g) and the supernatant transferred to a fresh Eppendorf tube.
4.3.4 Cell culture

The hybridoma cell line CRL-1606, obtained from the American Type Tissue Collection, produces a monoclonal antibody (IgG1) against human fibronectin. The cell line was adapted and routinely expanded for 14 days in a chemically defined culture media (Turbodoma® TP6, Cell Culture Technologies, Switzerland) supplemented with 4.5 g/L D-glucose, 4 mmol/L L-glutamine and 0.1% (w/v) Pluronic-F68 at 37°C and 5% CO₂ in a humidified atmosphere.

4.3.5 Fed-batch cultivation process

A parallel bioreactor system (DASGIP Inc., Germany) was used for fed-batch cultivation of CRL-1606 cells. The culture was seeded at a viable cell concentration $N_v$ of $0.6 \times 10^6$ cells/mL in a working volume of 1 L. Temperature was controlled at 37 °C, stirring speed equal to 150 rpm, pH equal to 7.2 and dissolved oxygen set to 50% air saturation with a gas flow rate of 0.05 vvm. The feed media consists of a mixture of RPMI-1640 amino acid solution (50X conc.), RPMI-1640 vitamin solution (100X conc.), BioConcept trace element mix (1000X conc.) and L-glutamine (64 mmol/L) and was added to the reactor 30 hours after inoculation with a constant pump rate of 2.33 mL/h to aim a 1X concentration of all feed components in the bioreactor. Glucose was fed continuously from the start of the culture to the reactor in order to keep glucose concentration in the reactor at a setpoint of 20 mmol/L. The pump rate of the glucose solution (1 mol/L) was adjusted every 12 hours according to measured values of $N_v$ and the specific glucose consumption rate.

The temperature was lowered 30 hours after inoculation by simply changing the setpoint of the temperature controller to 33 °C. The time required to cool the reactor system from 37 °C
to 33 °C was negligibly short. When considering osmolarity, its value was shifted 76 hours after inoculation by addition of 6 mol/L sodium chloride solution. The required volume was determined beforehand, by measuring the osmolarity of the cell culture supernatant at the given time point using a osmometer (OsmoLab One, LLA Instruments GmbH, Germany). The experimental procedure of shift fed-batch experiments is in agreement with earlier published batch-shift experiments for osmolarity. \(^{180}\)

Cell culture samples were taken twice a day and the viable cell concentration and viability were measured using a cell counter (CedeX, Roche, Switzerland). Glucose and lactate concentrations were determined by enzymatic measurement using a compact instrument (Super GL, Hitado, Germany). The monoclonal antibody concentration was determined in the supernatant by HPLC, using affinity chromatography (PA ImmunoDetection® Sensor Cartridge, Applied Biosystems, USA). Osmolarity was measured throughout the culture with the osmometer.

### 4.3.6 Sample preparation

Immediately after sampling, \(2.5 \times 10^6\) viable cells, as determined by trypan blue, were centrifuged (5min, 1000 rpm, 100 g) and the supernatant discarded. The pale yellow cell pellet was washed with 1 mL cold (+4°C) 10 mM NH₄OAc (pH 7.6) by gently resuspending the pellet. All further steps were performed in the cold, by either keeping the sample on ice or using temperature conditioning when employing instruments. The sample was centrifuged at -4°C (3min, 1000 rpm, 100 g) and the washing supernatant fully discarded. 50 \(\mu\)L (10 nmol) of the internal standard \(^{13}\)C-ATP was added. Subsequently 250 \(\mu\)L of the extraction solution (70% MeOH) was added on
the cell pellet, as similarly described in literature. The samples were mixed and sonicated for 3 min on ice. Immediately after sonication the sample was centrifuged (5min, 14000 g) to separate the extract from degradative enzyme. The supernatant was collected in a fresh 1 mL Eppendorf tube. For MALDI analysis, 2 µL of the analyte solution were mixed with 2 µL of the matrix solution (9-AA, 10mg/mL, 90% EtOH).

4.3.7 Sample aliquoting

The sample target slides (called “microarrays for mass spectrometry”, MAMS) consist of a coated transparent indium tin oxide slide and is microstructured by creating lanes of hydrophilic spots using pulsed laser ablation. Each lane has one reservoir of 1 mm diameter and 12 sample spots of 400 µm diameter and a 35 µm depth. The 4 µL were deposited in the reservoir. Using a pneumatically assisted metallic slider, the 4 µL droplet was dragged across the sample target, which resulted in the generation of 12 very reproducible nanoliter aliquots (described further in Supplementary figure S4-1). The small aliquot reservoirs have been shown to result in a very homogenous crystallization of the matrix-analyte mixture, a prerequisite for using MALDI in a quantitative fashion. Moreover, this miniaturized sample target leads to a virtually complete sample consumption, and only a few µL of analyte solution (limit of quantification 1 µM ATP) were needed for successful analysis. After sample crystallisation (drying in air within seconds) 10 of the 12 aliquots were measured by MALDI-TOF-MS.

4.3.8 MALDI-TOF-MS

A commercial MALDI-TOF-MS instrument (AB Sciex 5800) equipped with an Nd:YAG laser was used in reflectron
negative ion mode for data acquisition. The Nd:YAG operates at 355 nm with a repetition rate of 1 kHz. A laser attenuator is installed to vary the intensity of the laser beam that reaches the sample. 110 shots using an optimized search pattern with 35 subspectra were recorded. The spectra covered the mass range from 300 m/z to 900 m/z with a focus mass of 500 m/z. The laser intensity was set to 4300 arb. units and the delay time for the extraction pulse was set to 375 ns. The signal area and the signal height found in MALDI mass spectra were used for quantification relative to the internal standard (\(^{13}\)C-labeled ATP).

4.3.9 Monitoring strategy

Viable cell density, glucose and lactate concentration of the fed-batch cultures were measured in 12-hour intervals. The metabolic profile was recorded daily. All sample preparation and MALDI instrumental parameters were kept constant during the entire experiment series. On each experiment day a new aliquot solution of internal standard was thawed from -20 °C to +4 °C prior to analysis.
4.4 Results and Discussion

The overall workflow for MALDI-MS, including sample preparation and subsequent crystallization of matrix molecules with the analyte, has been subject to extensive investigations over the past years, and enables today quantitative analysis. In combination with a MAMS sample target, metabolite profiles can be measured with high throughput. The sample target offers an optimized sample spot size and enables homogenous sample crystallization, which are both essential for monitoring experiments.

![Figure 4-1](A) The dotted lines represent the percentage of apoptotic cells of the population found by flow cytometry and the solid lines correspond to the percentage of living cells in the population found by trypan blue exclusion. (B) The dotted line shows the viable cell concentration based on trypan blue dye exclusion data, the solid line represents the calculated viable cell concentration considering flowcytometry and dye exclusion data. Triangles: control culture 37°C; squares: 33°C (after 30 hours) culture; circles: increased osmolarity (after 76 hours) culture; diamonds: 33°C (after 30 hours)/ increased osmolarity (after 76 hours) culture.

4.4.1 Viable cell concentration

Temperature effects and osmolarity changes were investigated during fed-batch cultivation of the mouse hybridoma cell line CRL 1606 in four parallel controlled
bioreactors. A shift to mild hypothermia during the late exponential growth phase is a commonly used approach in industrial fed-batch cultures due to its positive effect on culture longevity and productivity. 183-191 An increase in osmolarity require consideration during fed-batch cultivation. Due to the addition of feed solutions in concentrated form, the osmolarity of the culture can easily exceed physiological levels and inhibit cell growth. 192 Therefore we considered these two process variables as relevant test parameters for the metabolite profiling monitoring study of fed-batch processes. For a first cell viability assessment, the percentage of necrotic cells was detected by trypan blue dye exclusion (expressed as living cells in percent, fig. 4-1A filled symbols). 193 However, apoptotic cells are not detected by trypan blue dye exclusion tests, because these cells still possess an intact cell membrane that excludes trypan blue. Using flow cytometry and a propidium iodide staining for fragmented DNA it is possible according to Gorczyca et al., to detect both, apoptotic and necrotic cells (fig. 4-1A, open symbols). 194 We subtracted the number of apoptotic cells found by flow cytometry from the total number of cells counted by the CedeX cell counter. The viable cell concentration found by trypan blue (fig. 4-1B, open symbols) and the one found by flow cytometry (fig. 4-1B, filled symbols) differed strongly. The concentration of adenosine-5’-triphosphate (as a marker for cell viability) per mL cell culture is tracking the course of the cell viability concentration profile obtained from the flow cytometry measurements (fig. 4-2, open circles).
The ATP (m/z 506) concentration per cell (expressed as rel. ATP signal area in fig. 4-2, filled circles) was constant for the first two days and dropped after day 2 due to increasing fraction of apoptotic cells. Using the final amount of internal standard (2 fmol/cell) we calculated the average amount of ATP per cell for the first two days to range from 6.7 ± 0.3 to

**Figure 4-2** The relative ATP signal area per mL (open circles) is a good indicator for viable cell concentration (open triangles, calculated from trypan blue and flow cytometry method). The relative ATP signal area per cell (filled circles, expressed as rel. signal area ATP) indicates an ongoing change starting from day 3. Reactor conditions: (A) control reactor, 37 °C, (B) 33 °C (after 30 hours), (C) increased osmolarity (after 76 hours), (D) 33 °C (after 30 hours) and increased osmolarity (after 76 hours).

4.4.2 Analysis of intracellular metabolites using MALDI-MS

The ATP (m/z 506) concentration per cell (expressed as rel. ATP signal area in fig. 4-2, filled circles) was constant for the first two days and dropped after day 2 due to increasing fraction of apoptotic cells. Using the final amount of internal standard (2 fmol/cell) we calculated the average amount of ATP per cell for the first two days to range from 6.7 ± 0.3 to
High-throughput nucleoside phosphate monitoring

8.5 ± 0.3 fmol. The ATP concentration per milliliter cell batch culture (expressed as rel. ATP signal area, fig. 4-2, open circles) throughout the cell culture process perfectly tracked the course of the corrected viable cell density (fig. 4-2, open triangles), and was found to be in good agreement with literature data. As the number of apoptotic cells increased, the ATP concentration per milliliter dropped and approached zero towards the process end. The temperature reduction in two of the reactors resulted in a time lag until the maximum ATP concentration was reached, i.e. in a prolonged stationary phase (fig. 4-2 A, 37°C and B, 33°C). These findings were in good agreement with literature. The osmolarity change on day 3 resulted in a drop of the ATP concentration per milliliter (fig. 4-2 C and D). Thus we can conclude that the osmolarity change leads to a complete stop in cell proliferation and the remaining cells subsequently undergo apoptosis and necrosis (in accordance with the VCD). Interestingly, the calculated energy charge (defined as }\frac{[\text{ATP}]+0.5*[\text{ADP}]}{[\text{ATP}]+[\text{ADP}]+[\text{AMP}]}\) was constant throughout the process for both the control and the temperature reduced reactors (fig. 4-3 A and B, open circles), but dropped below 0.8 on day 5/6 in the osmolarity-affected reactors (fig. 4-3 C and D, open circles). Any methodologically induced ATP fragmentation and hydrolysis was corrected for according to the observed 13C-ATP/ADP/AMP patterns. The U-ratio (UTP/UDP-NAc-hexosamine) (fig. 4-3, filled circles) was found to be in agreement with literature values, and the trend was consistent with the calculated growth rates (fig. 4-3, filled triangles).
Nucleoside phosphate (NP) levels in the control reactor are shown in fig. 4-4. The metabolite levels appeared constant up to day 2 and subsequently declined. Monophosphates increased on day 4. Moreover, UDP-hexose, UDP-N-acetylhexosamine, and GDP-hexose showed first, a constant level throughout the process, and were found to drop at the end of the process. These trends are in good agreement with HPLC-UV measurements (HPLC-UV in Supplementary Figure S4-2).

**Figure 4-3** The energy charge (open circles) is defined as a ratio of the adenylate nucleotides [ATP + 0.5ADP]/[ATP+ADP+AMP]. The U-ratio (filled circles) is calculated as the ratio of UTP/UDPhexNAc. The errorbars are the positive and negative standard deviation of the technical replicates (n= 10). Growth rate (filled triangles) are calculated from cell viability data. (A) 37°C (control), (B) 33°C, (C) 37°C increased osm., (D) 33°C increased osm.)
High-throughput nucleoside phosphate monitoring

Concentrations calculated based on externally calibrated HPLC-UV data neglected hydrolysis during sample preparation, thus the calculated concentrations were lower compared to MALDI-MS based values.

Figure 4-4 Overview of adenosine-, uridine, guanidine-, and cytidinetri-, di-, and monophosphates as well as UDP-NAc-hexosamine, glutathione (GSH), and GDP-hexose levels throughout the cell culture process. The signal is plotted as relative intensity to the internal standard ($^{13}C$-ATP). The chemical structures (uncharged form) of the evaluated analytes are displayed.
4.4.3 Different metabolite patterns described by principal component analysis (PCA)

Finally, our data sets were subjected to statistical analysis to (i) reduce data complexity and to (ii) reveal underlying trends. The first 2 days were clearly separated from the remaining days by PCA. The profiles on day 3 and 4 are overlaying but are together entirely separated from the days 5, 6 and 7. A tight clustering of technical replicates was observed throughout the process (fig. 5A). According to the loading plots, ATP, UTP, UDP-NAc-hexosamine, ADP and UDP had the strongest influence on the first and second principal component (principal component 1 (PC1) explains 78% of the dataset and PC2 10%). Moreover, datasets of the four different culture conditions at the same time point were clearly separated (reactors on day 6, fig. 5B). ATP, UTP and UDP-NAc-hexosamine, as well as UMP had the strongest influence on the first and second component. Whereas PC1 explained 66% and PC2 explained 21% of the dataset. On day 6, the reactors 119 and 120 were already at the final state of their life cycle and thus the monophosphates appeared to be predominant. Overall, the tight clustering of the technical replicates at individual time points demonstrates the methods small standard deviation and thus its suitability to analyze nucleotides. The clear separation of the measurement time points in the PCA result indicate the usefulness of the acquired data for process modeling and controlling.
Figure 4-5 (A) Principal component analysis of the metabolic profile of one bioreactor throughout the process lifetime shows a clear separation of the individual time points. The technical replicates (n = 10) are tightly clustering. (B) PCA of four different bioreactors (on day 6). The metabolite profiles are clearly separated.
4.5 Conclusions

We demonstrated a new method suitable for mammalian cell metabolite analysis in fed-batch cultures. Clear differences in the metabolic response were detected in four individually treated bioreactors. A temperature drop resulted in a time lag to reach the maximum ATP concentration. The applied changes in osmolality strongly influenced the cell viability and consequently the metabolic profile. Time-dependent changes in the control reactor were found and discussed, highlighting the suitability of the method to monitor cell culture processes. All biological findings are in good agreement with literature, and correlate with growth rate and cell viability. Multivariate analysis revealed a tight clustering of technical replicates and a clear separation of the individual time points. This method enables high-throughput parameter screening with an analysis time for one sample (including 10 technical replicates) is only ca. 1 minute (compared to 20 minutes for a typical single LC-UV injection). Primary values as e.g. cell viability (ATP concentration) and the growth rate (U-ratio) can be accessed in this dataset. The considerable time savings achieved in this method compared to long LC runtimes is advantageous and makes the method suitable for process monitoring in industrial environments.
4.6 Supplementary Information

Supplementary figure 4-1

**Custom designed pneumatic driven slider.** A pneumatic system drives the slider at constant speed along the microarray. The slider distributes the start volume into the sample spots. The red arrow marks the working direction. The omniphobic microarray coating focuses the sample during the aliquoting procedure into the individual sample spots. In this workflow a very reproducible and automatic volume aliquoting is achieved.
**Supplementary figure 4-2**

**ATP concentrations.** The obtained absolute ATP concentrations in the cell extract during the batch process found by MALDI-MS (filled squares) and by HPLC-UV. The MALDI-MS method enables a correction of the ATP hydrolysis during sample preparation and storage, by using a $^{13}$C-ATP internal standard. This is not possible in conventional HPLC-UV methods.
Chapter 5

Microarray-based MALDI-TOF mass spectrometry enables monitoring of monoclonal antibody production in batch and perfusion cell cultures

The following chapter expanded the use of microarray-based MALDI-MS to the analysis of intact monoclonal antibodies. The detection of light and heavy chain, as well as intact antibody and antibody aggregate products is demonstrated and discussed.

©2015 Elsevier Inc.
5.1 Overview

Cell culture process monitoring in monoclonal antibody (mAb) production is essential for efficient process development and process optimization. Currently employed online, at line and offline methods for monitoring productivity as well as process reproducibility have their individual strengths and limitations. Here, we describe a matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS)-based on a microarray for mass spectrometry (MAMS) technology to rapidly monitor a broad panel of analytes, including metabolites and proteins directly from the unpurified cell supernatant or from host cell culture lysates. The antibody titer is determined from the intact antibody mass spectra signal intensity relative to an internal protein standard spiked into the supernatant. The method allows a semi-quantitative determination of light and heavy chains. Intracellular mass profiles for metabolites and proteins can be used to track cellular growth and cell productivity.

Keywords: MALDI; Antibody; IgG; Perfusion; Batch; Cell culture

Graphical abstract
5.2 Introduction

Monoclonal antibody (mAb) producing cell cultures are indispensable in biopharmaceutical production and their systematic assessment is of great interest to academic and industrial research. MAbs have undergone an impressive development from their first description by von Behring and Kitasato in 1890 to their widespread clinical applications. Today, also smaller recombinant antibody fragments such as fab and scFv, or antibody mimetics, such as designed ankyrin repeat proteins (DARPins) are studied for use in diagnostics and therapy. Three of the top ten drugs by worldwide sales are whole mAbs and consequently, their production processes are of great interest. Industrial monoclonal antibody manufacturing includes several manufacturing steps as reviewed by Wurm in 2004 and Thoemmes in 2010. Due to the required posttranslational modifications antibodies are commonly expressed in mammalian cell cultures. Typical culture modes are small-scale, batch, fed-batch and perfusion reactors. The different process designs lead to overall significant differences in antibody yield, purity and integrity. In this context, cell culture monitoring is a key element.

Besides the classical process parameters, such as cell viability, oxygen levels, and substrate sensing, more analytical techniques are implemented to support process engineering. State-of-the-art analytical methods for mAb process monitoring are based on charge separation by electrophoresis, such as capillary electrophoresis (CE), isoelectric focusing and size separation by electrophoresis as for example sodium dodecyl(SDS)-polyacrylamide gel electrophoresis. Moreover, there are a number of established high performance liquid chromatographic (HPLC) methods available for protein analysis, including ion exchange HPLC,
Microarray-based MALDI-TOF mab monitoring

reversed-phase HPLC and size exclusion HPLC with UV or mass spectrometric (MS) detection. Structural and functional analysis of mAbs is performed by HPLC-MS. Dynamic light scattering and analytical ultracentrifugation are mainly used for mAb aggregation analysis. The list of analytical tools for mAb detection is expanded by very rapid spectroscopic methods, such as FT-infrared spectroscopy, circular dichroism, and Raman spectroscopy for probing the antibodies secondary structure. Metabolite monitoring and profiling is mainly performed using mass spectrometry or nuclear magnetic resonance (NMR).

Another very promising approach for cell culture monitoring is based on matrix-assisted laser desorption/ionization (MALDI). This soft ionization method allows for the detection of small and large, biomolecules directly from complex media or extraction solutions mostly singly charged. The ions can be detected with various detector systems, including multi-channel plate (MCP), ion-conversion dynodes, Faraday cups and pixelated time-to-digital converter. Since ion-to-electron conversion is drastically decreasing with decreasing ion momentum, high mass ions are rather detected by ion-conversion dynodes or pixelated detectors. Generally, MALDI offers a high salt tolerance resulting in extremely short and simple sample preparation steps. Combined with its straightforward data analysis MALDI-MS is an ideal tool for process monitoring. The use of a microarray for mass spectrometry (MAMS) in combination with appropriate internal standards allows to reduce MALDI's poor reproducibility and to offer semi-quantitative MALDI-MS analysis. Here, we describe a bioreactor monitoring method based on MALDI mass spectrometry using a specifically optimized microarray sample target for mAb quantification and metabolite detection. The method is based on an “all-in-
one” extraction step using MeOH/water and methyl-tert-butyl ether (MTBE) as extraction solvents, which enable the analysis of intracellular proteins, metabolites and lipids. To demonstrate the performance of MALDI-MS for this application, a batch and a perfusion reactor were monitored over 6 days, respectively. Intracellular metabolite levels were measured in host cell lysates. An intracellular protein profile was recorded for the batch process and the mAb titer was determined from the intact mAb mass signal intensity as obtained directly from the unpurified supernatant. Results were cross-validated with state-of-the-art HPLC-UV methods. MALDI-MS as an at-line process analytical tool (PAT) proved to be a fast and robust technique.

5.3 Materials and Methods

5.3.1 Materials

We used Millipore water (18.2MΩ×cm). The solvents acetonitrile (Sigma-Aldrich, Switzerland) and ethanol (Fluka, Germany) were used as provided. Sinapinic acid (Sigma-Aldrich, Switzerland), 9-aminoacridine (Acros Organics, Belgium), and 2,5-dihydroxyacetophenone (DHAP) were used without prior purification. Trifluoroacetic acid (TFA) (Sigma-Aldrich, Switzerland) was likewise handled without prior dilution or purification. The used cell culture medium was chemically defined. (Merck Serono, Switzerland).

5.3.2 Reactor operation

A CHO cell line (Merck Serono) secreting an IgG1 isotype recombinant mAb was thawed from liquid N₂ and expanded in suspension for one week, and diluted with fresh media every other day. The batch experiment was inoculated at 1 x 10⁶
Microarray-based MALDI-TOF mab monitoring

cells/mL with a working volume of 40 mL in a spin tube (TubeSpin bioreactor 50, TPP, Switzerland) and cultured in humid atmosphere at 36.5 °C, 5% CO₂ and 320 rpm using a shaking incubator (Kuhner AG, Switzerland).

After one week of further expansion in a seed bioreactor, cells were inoculated to a 1.5 L working volume perfusion bioreactor (Vaudaux-Eppendorf, Switzerland) employing a tangential flow filtration (TFF) system for cell retention. The harvest rate was fixed to 1 reactor volume per day and the cell density was controlled at 20 x 10⁶ cells/mL adapting the bleed rate according to an online biomass sensor (Aber Instruments Ltd., Wales). Operating parameters were 36.5 °C, pH 7.1 and 50% dissolved oxygen tension. Daily samples were taken to monitor viable cell density, viability, mAb titer and glucose (glc) and lactate (lac) concentrations. Glc and lac concentrations were measured using an electrochemistry based biosensor system (Hitado superGL compact, Sysmex GmbH, Germany).

5.3.2.1 Perfusion cell culture

Stable culture at the viable cell density setpoint of 20x10⁶ cells/mL was achieved over the entire 6-day run. The cell viability remained high above 93.5 %. (fig. 1A) The extracellular glucose levels varied between 10.6 and 17.4 mmol/L and lactate levels were between 6 and 14.6 mmol/L (fig. 1D, filled squares and circles) After an initial transition originated from cell inoculation to the perfusion bioreactor, Glc and Lac concentrations approached constant levels. The cell diameter average was around 12 µm.
5.3.2.2 Batch cell culture

The viable cell density profile in the batch reactor followed the classic lag, exponential and stationary phases (see fig. 1B). On working day 2, exponential growth was reached and cell proliferation sharply increased. The viable cell density profile was in good agreement with literature values. \(^4\) The stationary phase was reached on day 4, characterized by little to no cell growth and consequently a constant cell density. Overall, a cell density maximum of \(8 \times 10^6\) viable cells/mL was reached. Waste product accumulations resulted in a drop in cell viability, higher death rate and consequently the end of the process. The cell viability dropped below 90\% on working day 4 and was measured at 84\% on day 5. A decline in glucose concentration (shown in Fig. 1D, open circles) from 35 mmol/L to 6.3 mmol/L throughout the process duration was observed. After an initial lactate accumulation (fig. 1D, open squares) to 14.4 mmol/L on working day 3.5, the lactate got consumed to 5.4 mmol/L at the end of the process.

5.3.3 Extraction protocol

The extraction protocol was adapted from Matyash et al. to optimize MALDI signal yield and reproducibility. \(^2\) \(^18\) \(5 \times 10^6\) cells were spun down (1000 x g, 1 min, 4 °C) and the supernatant was stored for extracellular mAb analysis at -80°C. The cell pellet was washed with 1000 µL 10 mM NH\(_4\)OAc (pH = 7.2, 4°C). All further steps were performed on ice or at 4°C to minimize sample preparation artifacts in the metabolite analysis. The washed cell pellet was vortexed to mechanically disrupt the cell membrane. 300 µL of a MeOH/H\(_2\)O (50%/50%, v/v) containing 100 µM \(^{13}\)C-ATP and 100 µM D\(_4\)-Alanine was added to the lysed cells. Subsequently 400 µL MTBE (100 µM DSPC) were added. The mixture was vortexed after each addition for 30 seconds. Afterwards, the
Microarray-based MALDI-TOF mab monitoring

solutions were incubated on ice for 3 min. The samples were centrifuged for 2.5 min at 16000 x g (4°C) to achieve phase separation. The individual phases were collected in 1.5 mL tubes and stored at -80°C for analysis.

5.3.4 Matrix preparation

9-aminoacridine (10 mg/mL) was dissolved in 100% acetone. Insoluble particles were removed by centrifugation (14000 x g, 1 min). Sinapinic acid (15 mg/mL) was dissolved in 50% aqueous acetonitrile (MeCN), containing 0.1% TFA. DHAP was mixed with MeCN, EtOH and water (25:25:50, v/v/v) containing 0.1% TFA. The aqueous phase of the extraction was mixed 1:10 (v/v) with 9-AA solution for negative ion mode analysis. The intracellular protein fraction was mixed with DHAP solution 1:10 (v/v). The extracellular protein fraction was mixed with sinapinic acid solution 1:10 (v/v). The high mass calibration was performed as described by Weidmann et al. using maltose-binding protein (MBP) as an internal standard at a concentration of 0.2 g/L. We used a self-aliquoting sample target as described elsewhere. Therefore 6 μL of the sample mixture were spotted onto the sample target plate and subsequently aliquoted using a slider either driven pneumatically or by hand. To increase the detected signal we repeated this step once. For intracellular protein profiling we used a commercially available ABSciex 384 stainless steel MALDI plate.

5.3.5 Microarray sample target production (MAMS, microarray for mass spectrometry)

A 4-inch silicon wafer (Si-Mat, Germany) was cleaned in oxygen plasma by using a plasma asher Plasma-System 200 (PVA TePla AG, Germany). The wafer was spin coated with
Microarray-based MALDI-TOF mab monitoring

polysilazane solution CAG37 (Merck KGaA, Germany), and the polysilazane was cured on a hotplate at 135°C for 4 hours. Positive photoresist (micro resist technology GmbH, Germany) was spin coated on top of the polysilazane and microstructured by photolithography using a MABAS8 mask aligner (SUESS MicroTec AG, Germany) and a transparency mask (Selba, Switzerland). Polysilazane was then removed in exposed areas using a Plasmalab 100 reactive ion etcher (Oxford Instruments Plasma Technology, UK). Residual positive resist was removed in an acetone bath using a dental brush. Individual microarray chips (75mm x 26mm) were cut from the wafer using a DAD3240 dicing saw (Disco Hi-Tec Europe GmbH, Germany).

5.3.6 MALDI-MS analysis

The metabolite analysis was performed on a commercial ABSciex MALDI 5800 TOF/TOF mass spectrometer in negative reflectron mode. The instrument is equipped with a Nd:YAG laser and operates at 355 nm with a maximum repetition rate of 1 kHz. Negative mode spectra were recorded at 4000 [arb. units] laser power with 20 shots per spectrum and a delay time for the extraction pulse of 375 ns. A total of 800 spectra were averaged. Laser shot positions were defined in a circular search pattern for the analysis on the microarray. The intracellular proteins were measured on a commercial ABSciex MALDI 4800 TOF/TOF mass spectrometer in positive linear mode, with a laser intensity of 3500 - 4500 [arb. units] and 500 to 2000 ns delay time for the extraction pulse. Overall, spectra from 1000 shots were collected (20 shots per position, 50 positions) on a commercially available stainless steel plate. The source voltage was 20 kV. The extracellular mAb was measured on the microarray with
similar MALDI settings in positive linear mode and a defined search pattern to virtually consume the entire spot. The MALDI ABSciex 4800 mass spectrometer is equipped with an Nd:YAG laser operating at 355 nm and an ion conversion dynode high-mass detector (CovalX, Zurich, Switzerland). The extracellular mAb was detected with the high-mass detector voltages HV1 and HV2 set to -3.43 kV and -20 kV, respectively. Intracellular proteins were detected using a multichannel plate detector.

5.3.7 HPLC-UV analysis

A protein A column (Poros A-20, 2.1mm x 30 mm) from Life Technologies (Carlsbad, CA, USA) was used for antibody quantification. 20 mM phosphate, 150 mM NaCl solution (pH 7.5) were used as binding and washing solution. 10 mM HCl, 150 mM NaCl (pH 2) were used as elution buffer. The column was regenerated using 20% aq. acetic acid. The antibody trace was detected using the UV-absorbance at 280 nm.

5.3.8 Size exclusion chromatography

In order to validate the aggregate content, size exclusion chromatography was performed using a TSKGel G3000 SWXL (7.8 mm x 30cm) column (Tosoh, Tokyo, Japan) on an Agilent 1200 system (Santa Clara, CA, USA). Samples were analyzed at a flow rate of 0.5 mL/min with a 20mM sodium phosphate, 100mM sodium sulfate buffer (pH 7.0) as mobile phase with detection at 280 nm.
5.4 Results

5.4.1 MALDI-MS of intracellular compounds

5.4.1.1 Targeted phosphorylated metabolite analysis in negative ion mode for batch and perfusion cultures

The aqueous extraction phase was analyzed in negative ion mode using 9-AA as a MALDI matrix. Adenosine-5’-triphosphate (ATP) and uridine-5’-triphosphate (UTP), UDP-N-acetyl-hexoseamine were found to be the most abundant metabolites present (spectra shown in Supporting information S5-1). We followed a targeted approach to monitor ATP, ADP and AMP from the extracted cells in order to confirm the cell viability data from section 3.1 in the batch and the perfusion culture. Therefore we added as described above $^{13}$C$^{15}$N-labeled ATP to the extraction solution. The intracellular ATP concentration in the batch reactor fluctuates between 4.8 to 9 fmol/cell (fig.5-1C). In the perfusion culture intracellular ATP content was constant as determined by MALDI-MS (5.6 ± 0.2 fmol/cell). Intracellular ATP levels in perfusion and batch culture were in good accordance with literature. Reason for the ATP fluctuations were found in uncertainties in the cell counting procedure (i.e. tryphan blue exclusion) at lower cell viabilities. An incorrectly determined, e.g. higher, cell per mL concentration ultimately leads to a lower metabolite level, since finally less than $5 \times 10^6$ cells were extracted. The energy charge is as postulated by Atkins et al. provided a cell number independent measure of the cell population viability. (see fig. 5-1C). Again the findings were in good accordance to current literature knowledge.
Overall, more than 100 mass features were detected in the negative ion mode, out of which 61 were identified by accurate mass (database: metlin.scripps.edu, hmdb.ca) and partially by MS/MS experiments (table1). The list comprises mainly phosphorylated nucleotides, since their ionization is favored in MALDI using 9-aminoacridine as ionization matrix.

**Figure 5-1** (A) Perfusion reactor: viable cell density profile filled circles in $10^6$ cells per mL, viability expressed as a percentage (filled squares). (B) batch reactor: viable cell density profile open circles, viability expressed as a percentage (open squares) (C) ATP concentration in fmol/cell, in batch (dashed line) and perfusion reactor (continuous line); energy charge defined as $(\text{[ATP]}+0.5\text{[ADP]})/([\text{ATP]}+\text{[ADP]}+\text{[AMP]})$ in batch reactor (open squares, dashed line) and perfusion (closed triangles, continuous line). (D) Extracellular glucose concentration in batch (open circles, dashed line) and perfusion (filled squares) cell culture; extracellular lactate concentration in batch (open squares, dashed line) and perfusion (filled circles) cell culture determined by enzymatic assays.
Figure 5-2 Intracellular protein MALDI mass spectra from 0 to 20,000 m/z for 2 different working days (WD 1 and WD5) recorded from the batch reactor. (matrix: 2,5-dihydroxy acetophenone, 10 mg/mL, MeCN:EtOH:H$_2$O, 1:1:2,v/v/v). The resolution of the MALDI mass spectrometer does not allow for a real qualitative analysis. However the profile analysis can prove process stability or irregularities.
5.4.1.1 Intracellular protein analysis

The protein phase from the extraction described in 2.4 was analyzed by MALDI-MS in positive ion mode. Figure 5-2 shows two smoothed and baseline corrected intracellular protein spectra. The protein resolubilization procedure favors water-soluble proteins and thus higher mass proteins are not well observed in this protocol. In total, proteins in the mass range of 6k - 20kDa were observed using an MCP detector. Experiments with an ion-conversion dynode detector did not result in an improvement in terms of detected species and mass range. Peak identification by MALDI-MS from the crude mixture was not possible, due to limited mass resolution. From alternative approaches in the literature we tentatively assigned the MALDI mass spectrum peaks from m/z 11k to 14k as follows: histones H4 (11kDa), H2B (13.9 kDa), and histone H2A (14kDa) (see figure 5-2). The spectra demonstrated the potential of MALDI-MS to follow intracellular proteins in bioprocesses (here exemplary for the batch process). Noteworthy, on working day 5 we observed several higher molecular weight species in the spectrum of the batch reactor, indicating a significant change in the reactor’s cell population (mainly increasing cell death). The nature of these peaks was not further investigated since this was out of scope of this work. However, intracellular protein profile variations were much lower in the perfusion reactor as compared to the batch process, similarly to the study by Feng et al. (data not shown).
<table>
<thead>
<tr>
<th>#</th>
<th>Round mass</th>
<th>Rel. Intensity</th>
<th>Metabolite</th>
<th>Adduct</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>140</td>
<td>2.16</td>
<td>O-Phosphorylethanolamine*</td>
<td>[M-H]-</td>
</tr>
<tr>
<td>2</td>
<td>146</td>
<td>3.14</td>
<td>Glutamate*</td>
<td>[M-H]-</td>
</tr>
<tr>
<td>3</td>
<td>171</td>
<td>1.05</td>
<td>Glycerol phosphate</td>
<td>[M-H]-</td>
</tr>
<tr>
<td>4</td>
<td>172</td>
<td>1.04</td>
<td>NAc-Methionine</td>
<td>[M-H,O-H]-</td>
</tr>
<tr>
<td>5</td>
<td>180</td>
<td>1.45</td>
<td>Tyrosine*</td>
<td>[M-H]-</td>
</tr>
<tr>
<td>6</td>
<td>236</td>
<td>2.03</td>
<td>N-Acetyl-D-hexosamine</td>
<td>[M-H]-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>N-Glycolyl-D-hexosamine</td>
<td>[M-H]-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Nicotinamide riboside</td>
<td>[M-H,O-H]-</td>
</tr>
<tr>
<td>7</td>
<td>259</td>
<td>3.26</td>
<td>Hexose phosphate</td>
<td>[M-H]-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>D-myo-Inositol-1-phosphate</td>
<td>[M-H]-</td>
</tr>
<tr>
<td>8</td>
<td>304</td>
<td>7.45</td>
<td>cCMP*</td>
<td>[M-H]-</td>
</tr>
<tr>
<td>9</td>
<td>306</td>
<td>91.77</td>
<td>dCMP*</td>
<td>[M-H]-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Glutathione*</td>
<td>[M-H]-</td>
</tr>
<tr>
<td>10</td>
<td>321</td>
<td>3.65</td>
<td>Glucose bisphosphate</td>
<td>[M-H,O-H]-</td>
</tr>
<tr>
<td>11</td>
<td>322</td>
<td>5.81</td>
<td>CMP*</td>
<td>[M-H]-</td>
</tr>
<tr>
<td>12</td>
<td>323</td>
<td>35.29</td>
<td>UMP*</td>
<td>[M-H]-</td>
</tr>
<tr>
<td>13</td>
<td>333</td>
<td>3.20</td>
<td>Hexosyl-glycerol phosphate</td>
<td>[M-H]-</td>
</tr>
<tr>
<td>14</td>
<td>336</td>
<td>1.50</td>
<td>(Hydroxymethyl)glutathione</td>
<td>[M-H]-</td>
</tr>
<tr>
<td>15</td>
<td>344</td>
<td>7.49</td>
<td>cGMP*</td>
<td>[M-H]-</td>
</tr>
<tr>
<td>16</td>
<td>346</td>
<td>40.25</td>
<td>dGMP</td>
<td>[M-H]-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AMP*</td>
<td>[M-H]-</td>
</tr>
<tr>
<td>17</td>
<td>347</td>
<td>4.45</td>
<td>IMP</td>
<td>[M-H]-</td>
</tr>
<tr>
<td>18</td>
<td>362</td>
<td>4.06</td>
<td>N-Acetyl-D-Hexosamine-bisphosphate</td>
<td>[M-H,O-H]-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>GMP*</td>
<td>[M-H]-</td>
</tr>
<tr>
<td>19</td>
<td>369</td>
<td>0.75</td>
<td>Glycero-manno-heptose bisphosphate</td>
<td>[M-H]-</td>
</tr>
<tr>
<td>20</td>
<td>403</td>
<td>95.29</td>
<td>UDP*</td>
<td>[M-H]-</td>
</tr>
<tr>
<td>21</td>
<td>404</td>
<td>9.20</td>
<td>UDP isotope (403 m/z)</td>
<td>[M-H]-</td>
</tr>
<tr>
<td>22</td>
<td>408</td>
<td>29.54</td>
<td>ADP*</td>
<td>[M-H,O-H]-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>PAP*</td>
<td>[M-H,O-H]-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>dGDP*</td>
<td>[M-H,O,H]-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Inositol triphosphate*</td>
<td>[M-H]-</td>
</tr>
<tr>
<td>24</td>
<td>424</td>
<td>6.73</td>
<td>GDP</td>
<td>[M-H,O-H]-</td>
</tr>
<tr>
<td>25</td>
<td>426</td>
<td>63.53</td>
<td>ADP*</td>
<td>[M-H]-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>PAP*</td>
<td>[M-H]-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>dGDP*</td>
<td>[M-H]-</td>
</tr>
<tr>
<td>26</td>
<td>429</td>
<td>20.04</td>
<td>CMP-2-aminoethylphosphonate*</td>
<td>[M-H]-</td>
</tr>
<tr>
<td>27</td>
<td>437</td>
<td>1.57</td>
<td>Riboflavin cyclic-4',5'-phosphate*</td>
<td>[M-H]-</td>
</tr>
<tr>
<td>M/z</td>
<td>Formula</td>
<td>Charge State</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>------</td>
<td>--------------------------------</td>
<td>--------------</td>
<td>-----------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>GDP*</td>
<td>[M-H]</td>
<td>5.03</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>3-Phosphoglycerol-glutathione*</td>
<td>[M-H,H2O-H]</td>
<td>4.37</td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>XDP</td>
<td>[M-H]</td>
<td>0.42</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>CDP-ethanolamine*</td>
<td>[M-H]</td>
<td>25.03</td>
<td></td>
</tr>
<tr>
<td>31</td>
<td>CTP</td>
<td>[M-H,H2O-H]</td>
<td>3.62</td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>dTTP</td>
<td>[M-H]</td>
<td>2.23</td>
<td></td>
</tr>
<tr>
<td>33</td>
<td>dCTP*</td>
<td>[M-H]</td>
<td>1.35</td>
<td></td>
</tr>
<tr>
<td>34</td>
<td>TTP</td>
<td>[M-H,H2O-H]</td>
<td>0.71</td>
<td></td>
</tr>
<tr>
<td>35</td>
<td>CTP*</td>
<td>[M-H]</td>
<td>5.97</td>
<td></td>
</tr>
<tr>
<td>36</td>
<td>UTP*</td>
<td>[M-H]</td>
<td>26.48</td>
<td></td>
</tr>
<tr>
<td>37</td>
<td>UTP isotope (484 m/z)</td>
<td>[M-H]</td>
<td>2.50</td>
<td></td>
</tr>
<tr>
<td>38</td>
<td>UTP isotope (484 m/z)</td>
<td>[M-H]</td>
<td>0.87</td>
<td></td>
</tr>
<tr>
<td>39</td>
<td>ATP*</td>
<td>[M-H,H2O-H]</td>
<td>4.75</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PAPS*</td>
<td>[M-H]</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>dGTP*</td>
<td>[M-H]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>dATP*</td>
<td>[M-H]</td>
<td>0.48</td>
<td></td>
</tr>
<tr>
<td>41</td>
<td>ATP*</td>
<td>[M-H,H2O-H]</td>
<td>44.81</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PAPS*</td>
<td>[M-H]</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>dGTP*</td>
<td>[M-H]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>42</td>
<td>Inosine triphosphate</td>
<td>[M-H]</td>
<td>7.33</td>
<td></td>
</tr>
<tr>
<td>43</td>
<td>ATP isotope (506 m/z)</td>
<td>[M-H]</td>
<td>3.23</td>
<td></td>
</tr>
<tr>
<td>44</td>
<td>GTP*</td>
<td>[M-H,H2O-H]</td>
<td>5.73</td>
<td></td>
</tr>
<tr>
<td></td>
<td>cADP-pentose</td>
<td>[M-H]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>UDP-pentose*</td>
<td>[M-H]</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>46</td>
<td>cADP-Pentose</td>
<td>[M-H]</td>
<td>8.56</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ADP-pentose*</td>
<td>[M-H,H2O-H]</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Phosphopentosyl-AMP</td>
<td>[M-H,H2O-H]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>47</td>
<td>dTDP-Hexose</td>
<td>[M-H,H2O-H]</td>
<td>0.69</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>GDP-3,6-dideoxy-D-Hexose,</td>
<td>[M-H,H2O-H]</td>
<td>6.05</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Biotinyl-5'-AMP</td>
<td>[M-H]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>49</td>
<td>dTDP-D-Hexose</td>
<td>[M-H]</td>
<td>69.38</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>UDP-Hexose*</td>
<td>[M-H]</td>
<td>69.38</td>
<td></td>
</tr>
<tr>
<td>51</td>
<td>UDP-Hexose isotope (565 m/z)</td>
<td>[M-H]</td>
<td>11.64</td>
<td></td>
</tr>
<tr>
<td>52</td>
<td>UDP-Hexuronate*</td>
<td>[M-H]</td>
<td>6.08</td>
<td></td>
</tr>
<tr>
<td>53</td>
<td>UDP-Hexuronate isotope (579 m/z)</td>
<td>[M-H]</td>
<td>2.80</td>
<td></td>
</tr>
<tr>
<td>54</td>
<td>Stearoylglycerophosphoinositol</td>
<td>[M-H]</td>
<td>0.52</td>
<td></td>
</tr>
<tr>
<td>55</td>
<td>Adenosine tetraphosphate*</td>
<td>[M-H]</td>
<td>1.11</td>
<td></td>
</tr>
<tr>
<td>#</td>
<td>Value</td>
<td>Mass (m/z)</td>
<td>Molecules</td>
<td>Formula</td>
</tr>
<tr>
<td>----</td>
<td>-------</td>
<td>------------</td>
<td>---------------------------------------------------------------------------------------------</td>
<td>-----------------------</td>
</tr>
<tr>
<td>56</td>
<td>588</td>
<td>0.79</td>
<td>ADP-Hexose</td>
<td>[M-H]-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>UDP-HexNac</td>
<td>[M-H2O-H]-</td>
</tr>
<tr>
<td>57</td>
<td>597</td>
<td>19.03</td>
<td>1-Oleoylglycerophosphoinositol</td>
<td>[M-H]-</td>
</tr>
<tr>
<td>58</td>
<td>604</td>
<td>91.54</td>
<td>GDP-Hexose</td>
<td>[M-H]-</td>
</tr>
<tr>
<td>59</td>
<td>606</td>
<td>91.54</td>
<td>UDP-HexNAc*</td>
<td>[M-H]-</td>
</tr>
<tr>
<td>60</td>
<td>607</td>
<td>17.77</td>
<td>UDP-HexNAc isotope (606 m/z)</td>
<td>[M-H]-</td>
</tr>
<tr>
<td>61</td>
<td>608</td>
<td>4.29</td>
<td>UDP-HexNAc isotope (606 m/z)</td>
<td>[M-H]-</td>
</tr>
<tr>
<td>62</td>
<td>611</td>
<td>19.86</td>
<td>GSSG</td>
<td>[M-H]-</td>
</tr>
<tr>
<td>63</td>
<td>620</td>
<td>3.86</td>
<td>ADP-ribose 1''-2'' cyclic phosphate</td>
<td>[M-H]-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>UDP-N-acetyl-2-amino-2-deoxy-D-glucuronate</td>
<td>[M-H]-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ADP-ribose 2'-phosphate</td>
<td>[M-H2O-H]-</td>
</tr>
<tr>
<td>64</td>
<td>766</td>
<td>0.72</td>
<td>CoA*</td>
<td>[M-H]-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>FAD*</td>
<td>[M-H2O-H]-</td>
</tr>
<tr>
<td>65</td>
<td>789</td>
<td>4.70</td>
<td>Glycerophospholipid (PS(18:0/18:1(9z)))</td>
<td>[M-H]-</td>
</tr>
<tr>
<td>66</td>
<td>808</td>
<td>1.57</td>
<td>Phosphatidylinositol (PI(16:0/16:1(9Z)))</td>
<td>[M-H]-</td>
</tr>
<tr>
<td>67</td>
<td>837</td>
<td>9.27</td>
<td>Glycerophospholipid (PE(MonoMe(1,3)/DiMe(11,5)) or PS(18:0/22:5(7Z,10Z,13Z,16Z,19Z)))</td>
<td>[M-H]-</td>
</tr>
<tr>
<td>68</td>
<td>850</td>
<td>0.80</td>
<td>Aceto-acetyl CoA</td>
<td>[M-H]-</td>
</tr>
<tr>
<td>69</td>
<td>862</td>
<td>55.87</td>
<td>Phosphatidylinositol (PI(20:2(11Z,14Z)/16:0))</td>
<td>[M-H]-</td>
</tr>
</tbody>
</table>

**Table 5-1** Metabolites detected in cell lysate ba MALDI-MS. Masses were extracted from high resolution FT-ICR spectra replicates. The identity of marked (*) metabolites was further verified by performing MS/MS measurements using MALDI-TOF/TOF.
5.4.2 MALDI-MS of the extracellular mAb content

Extracellular protein content in the bioreactor was directly accessible by MALDI mass spectrometry without prior purification steps. Monoclonal antibodies and their fragments have been extensively described by mass spectrometry.\textsuperscript{223} For efficient mAb detection, it was essential to create homogenous matrix-analyte spots. This was achieved using a microarray for mass spectrometry (MAMS) described in detail elsewhere.\textsuperscript{109} The homogenous spots on a MAMS target plate are in the range of nanoliter volume and can be virtually completely consumed by the laser ablation, enabling a very reproducible analyte detection. The MAMS production was optimized as described in section 2.6. This analysis is enabled by the use of a high mass ion conversion dynode detector (CovalX, Zurich, Switzerland) as specified elsewhere.\textsuperscript{224} Figure 3 gives a summary of an IgG and its characteristic building blocks. Compared to protein mass spectra recorded with electrospray ionization (ESI), the MALDI spectra are less complex and easier to interpret since the proteins are mainly detected as singly charged species. Figure 5-3A shows the mAb mass spectrum recorded from the media supernatant without prior purification. The most abundant signals detected by MALDI-MS analysis in the extracellular supernatant monitoring are - besides the entire mAb at 150 kDa - a 23 kDa and 47 kDa fragment. The 23 kDa fragments can be assigned to the light chain (Lc), the half fragment crystallizable region (Fc/2) or fd fragment (antibody heavy chain fragment consisting of V\textsubscript{H} and C\textsubscript{H}1). The 47 kDa signal can be the identified as heavy chain (Hc), Fab, or Fc. Hillenkamp et al. have assigned the spectra from a purified monoclonal antibody in a very similar way. To test whether the assignment was correct, mAbs were reduced using dithiothreitol and spectra were compared to non-reduced
Microarray-based MALDI-TOF mab monitoring

The reduction of the disulfide bonds leads to the disruption of the antibody tertiary structure resulting in isolated heavy and light chains. However, higher mass accuracy would be needed to further characterize the resulting fragments. The mAb concentration was determined in the media supernatant by MALDI-MS for every working day and was cross-validated by protein A affinity chromatography. Maltose binding protein (MBP) was used for mass calibration in the range from 40 kDa up to 260 kDa.

Figure 5-3 Antibody and antibody-fragment MALDI mass spectra. The IgG1 crystal structure (1HZH) has been imported from a protein database (http://www.rcsb.org/pdb/home/home.do) and was processed using the jmol-13 software. The reduction products using dithiothreitol (DTT) is schematically shown. (A) whole mAb mass spectra (B) reduction products after DTT reduction (2h, 10 mM DTT, 37°C) Lc, light chain; Hc, heavy chain.
Microarray-based MALDI-TOF mab monitoring

Moreover the MBP was found suitable as an internal standard for relative mAb quantification. No MBP degradation in the matrix solution was observed for several hours, which ensures the correct relative mAb quantification (data not shown).

HPLC and MALDI data revealed an average constant mAb concentration of 0.2 g/L (± 0.07 g/L) in the perfusion reactor (fig. 4A). MAb quantitation using MALDI-MS was done using the MBP protein as an internal standard and has been externally calibrated using the HPLC-UV data. The response factor of MBP to the studied mAb has not been studied in detail. In the batch reactor the mAb concentration is increasing from working day 1 to 6 from 0.02 to 0.25 g/L (fig. 4B) (example spectra in appendix figure S5-2 and S5-3). Cross validation with SDS-PAGE measurements showed an excellent agreement in (i) the number of high abundant species detected as well as (ii) the molecular size distribution of the species present (appendix figure S5-4). Interestingly, more mAb aggregates (around 4.4 % of the mAb-monomer) were detected in the MALDI-MS method compared to a cross validation study by size exclusion chromatography (SEC) (1.8% of the mAb-monomer). This might be traced back either
Microarray-based MALDI-TOF mab monitoring

to the low pH conditions during crystallization, resulting in a favorable dimerization- and multimerization environment or to protein adsorption on the SEC-resin as reviewed by Arakawa et al.. Consequently the MALDI method is potentially underestimating product quality in terms of aggregate content. The therefore investigated samples have been part of a perfusion run, which is not described in detail here.

5.5 Conclusions

MALDI mass spectrometry allows to cover a broad range of analytes (metabolites, lipids and proteins) for monitoring monoclonal antibody production. Minimal sample preparation and short analysis times, compared to traditional HPLC-UV methods, facilitate a fast feedback to a running process. The high sample throughput potentially allows for parallel monitoring of multiple bioreactor processes. The use of MAMS enabled for the first time a semi-quantitative analysis of monoclonal antibodies directly from the reactor harvest. The recorded mass spectra provide information about the antibody integrity and presence of host cell protein contaminants. Important mAb characteristics such as micro-heterogeneities require significant improvements in resolution and sensitivity of the mass spectrometer. This holds particularly true for direct measurements from a biologically complex matrix such as cell culture supernatants. The methods applicability to lower concentrated analytes/products has to be tested respectively. Although the number of detectable metabolites is limited in MALDI mass spectrometry, it is specifically sensitive towards the detection of a high number of phosphorylated species which are difficult to monitor in this comprehensiveness using HPLC-MS on the other hand.
Supplementary figure 5-1

Different examples of MALDI metabolite spectra (in negative ion mode) of CHO cells in the perfusion- (upper trace) and the batch-process (lower trace).
Supplementary figure 5-2

Typical MALDI high-mass spectra from a batch process. The presented spectra were recorded on working day 1 (WD1) and WD 4.5
Supplementary figure 5-3

MALDI high-mass spectra from the perfusion process. The presented spectra were recorded on working day 1 (WD1) and WD 6.
Supplementary figure 5-4

SDS page of a perfusion process (WD1-WD5). Stable mab expression can be seen in the upper trace >125 kDa. The numbers on the y-axis are masses in kDa.
Chapter 6

High-throughput profiling of nucleotides and nucleotide sugars in microscale bioreactor systems for method validation

This chapter introduces the monitoring experiment of 22 microscale bioreactors. The abundance of intracellular compounds such as nucleotides and nucleotide sugars is investigated in 22 parallel running batch microscale processes.

© 2016 Elsevier B.V.
**6.1 Materials and Methods**

**6.1.1 Cell culture conditions**

CHO-S cells producing an IgG monoclonal antibody were cultivated in an ambr™ micro-scale bioreactor system (TAP Biosystems, UK) with working volumes of 10-15 mL. A 300 L pilot scale bioreactor (New MBR, Switzerland) was used to obtain larger sample volumes that allowed extraction replicates. The produced antibody bears only N-linked glycosylation at the Fc region. Cells were first expanded in shake bottles and afterwards in a wave bioreactor. In both reactor setups, the cell seeding density was set to $0.3 \times 10^6$ cells/mL. A temperature shift from 36.5 °C to 33.0 °C and a pH shift from 7.1 to 6.9 were conducted on working day 5. CO₂ was used to control the pH set points and dissolved oxygen was set to 50 % air saturation. Feeds were added on day 3, 5, 7, 10 and 14 and consisted of a proprietary concentrated main feed with over 30 components, a highly alkaline amino acid solution and a glucose solution of 400 g/L. Glucose concentration set points were selected to be well above all other monosaccharide concentrations (44 mmol/L on day 3, 5, 10 and 12 and 61 mmol/L on day 7 and 14). Feed supplementation consisting of galactose (300 mmol/L), mannose (100 mmol/L), fucose (100 mmol/L), N-acetylg glucosamine (100 mmol/L), asparagine (167 mmol/L), uridine (80 mmol/L) and manganese chloride (1 and 100 µmol/L) were purchased from Merck and added with a fixed ratio after the main feeds. The experimental conditions are summarized in Table 6-1, and include one-factor-at-a-time strategies for galactose, N-acetylg glucosamine and mannose as well as several combinatory experiments. Galactose was combined with different levels of manganese because of the reported synergistic effect on galactosylation, N-acetylg glucosamine was applied together with uridine since
Monitoring experiments in microscale bioreactor systems

both are precursors of UDP-GlcNAc. \(^{227-229}\) Finally, fucose was coupled with galactose and manganese to enhance all possible enzymatic steps in the N-link glycosylation machinery including fucosylation and galactosylation.
Table 6-1 Overview of all applied conditions with the cumulative feed amount per working volume over the entire process which was applied in addition to the standard operating conditions.
Monitoring experiments in microscale bioreactor systems

6.1.2 Analytical methods for cell culture and product quality analysis

Cell counts and cell viability were measured using a Vi-Cell analyzer (Beckman Coulter, Brea, CA), metabolites with a Nova CRT (Nova Biomedical, Waltham, MA), pH, pO2 and pCO2 with a NOVA BioProfile pHox analyzer (Nova Biomedical, Waltham, MA), antibody concentration was determined on a Biacore C instrument (GE Healthcare, Waukesha, WI) and N-linked glycosylation patterns were analyzed by high-throughput capillary gel electrophoresis with laser-induced fluorescence detection (CGE-LIF, DNA genetic analyzer 3130XL, Life Technologies, Darmstadt Germany) according to previously published procedures. 230,231

6.1.3 Extraction of intracellular nucleotides and nucleotide sugars

Acetonitrile (99.9%, HPLC Grade) and ammonium acetate were purchased from Merck. 13C-ATP ammonium salt (U-13C1015N5, chem. pur., 98%) was obtained from Cambridge Isotope Laboratories (USA). The 13C15N-ATP salt was dissolved in 50 % v/v acetonitrile to a final concentration of 100 µmol/L. The internal standard aliquots were stored for analysis at -80 °C. Extractions were performed out in 96 DPW and the procedure is based on a recently optimized protocol for the extraction of nucleotides and nucleotide sugars using 50 % v/v acetonitrile (del Val et al., 2013). All centrifugation steps were performed at 4 °C and liquid handling steps were carried out with a multi-pipette system Liquidator 96 from Mettler Toledo (Switzerland) while the 96 DWP was kept on ice if not stated otherwise. Approximately 5x10^6 cells were sampled in a 96 DWP and immediately centrifuged (500 g, 5 min). After removing the supernatant, the pellet was washed with 1 mL cold ammonium acetate buffer (10 mmol/L, pH 7.4). Despite the fact that a washing step is not required for a robust
extraction (del Val et al., 2013), removing the salt ions was necessary to prevent any disturbance during the MALDI-TOF-MS measurement. After the washing step, the cells were centrifuged again (500 g, 3 min). The supernatant was discarded and the washed cell pellets were homogenized by vortexing the 96 DWP. Subsequently, 100 µL of the internal $^{13}$C$^{15}$N-ATP standard aliquots and 500 µL of ice-cold 50 % v/v acetonitrile solution were added and the suspension was incubated on ice for 10 min. After incubation, the 96 DPW was centrifuged again (4000 g, 5 min) and the clear supernatant was stored at -80 °C until MALDI-TOF-MS analysis. A schematic overview of the entire workflow is provided in Figure 2.

6.2 Results

6.2.1 Intracellular components

In order to investigate the reproducibility and robustness of the metabolite extraction and the MALDI-MS method, all major nucleosides (ATP, ADP, AMP, UTP, UDP, UMP, GTP, GDP, GMP, CTP, CDP and CMP) and six detected nucleotide sugars (UDP-HexNAc, UDP-Hex, UDP-Pent, GDP-Hex) were extracted as discussed above. The measured intracellular concentrations are generally in agreement with published values.\textsuperscript{1,23-25} In order to examine the reliability of the simultaneous extraction in the 96 DWP, the intracellular concentrations were plotted as a function of the extraction place at working day 3 Figure 6-1. The consistent value throughout the plate indicates that the impact of the waiting time during sampling and the extraction location can be neglected. A complete time profiling of the triplicate extraction from the pilot plant bioreactor is presented in Figure 6-2 demonstrating the high reproducibility of the extraction and analytical procedure. The marginal deviations
Monitoring experiments in microscale bioreactor systems

between the different extractions lie mostly within the standard deviations of the MALDI-TOF-MS measurements and thus can be disregarded. Standard deviations of the microarray based MALDI-TOF-MS method are smaller than 20 %. Therefore, we can conclude that the high-throughput extraction using 96 DWP represents a reliable protocol for measuring intracellular nucleotides and nucleotide sugars of mammalian cells.

Nevertheless, single measurement points of intracellular components should be interpreted with caution and time evolutions rather than single measurement points of different conditions are compared to reassure trends in intracellular levels. For example, the substantial change of ATP, UTP, GTP and CTP levels after peak cell density around working day 7 can be seen in all conditions as expected due to higher demand for DNA replication and other energy intensive molecular mechanism involved in cellular proliferation during the growth phase (figure 6-2 A-D). At the same time, intracellular levels of UDP-Hex and UDP-HexNAc, which are crucial for the attachment of N-acetylglucosamine and galactose during the glycosylation process, undergo significant changes along the cultivation (figure 6-2). Intracellular UDP-Hex concentrations start at around 0.5 mmol/L and drop to approximately 0.1 mmol/L, while the measured UDP-HexNAc accumulates in the cell throughout the culture from 1 mmol/L up to 10 mmol/L. An example of a well-regulated nucleotide sugar is given by GDP-Hex, which remains at constant low values throughout the process (Figure 6-2 F). Fundamental changes of nucleotide sugar levels can directly influence the glycosylation process and thus the time evolutions of intracellular profiles are used to describe the measured differences in the final N-linked glycosylation pattern.
Figure 6-1 Intracellular concentrations of nucleotides and nucleotide sugars of parallel experiments as a function of extraction location in the 96 DWP. (A) ATP, (B) ADP, (C) AMP, (D) UDP-HexNAc, (E) UDP-Hex and (F) GDP-Hex. The points represent MALDI-TOF-MS measurements of ten individual spots (n=10) where the boxes and the whiskers show the corresponding one and two standard deviation region.
Figure 6-2 Intracellular profile of measured nucleotides and nucleotide sugars of triplicate extractions of the pilot plant reactor indicates a high consistency regarding extraction as well as analytical measurement. The standard deviation stem from ten (n=10) independent MALDI-TOF-MS measurement spots.
Monitoring experiments in microscale bioreactor systems
Chapter 7

Frontiers in mass spectrometric metabolite analysis

The analysis of the metabolome – typically defined as the entire set of metabolites is still a true challenge. Unlike proteins or transcripts the metabolome is not encoded in the genome. Untargeted metabolomics has revealed that the actual number of endogenous metabolites in biological systems such as yeast is larger than originally anticipated. Recent developments in mass spectrometry, especially in LC-MS, enable a more global analysis of metabolites at systems level. In the 1980’s, soon after the discovery development of the first DNA recombination processes directed metabolic pathway modification has appeared. Pathway engineering, cellular engineering and metabolic engineering are potential approaches to convert our metabolic understanding to fruitful benefits on the level of process optimization in biotechnological engineering. Precise technologies to follow metabolic changes and alterations are of high need. Current sensitivity in mass spectrometry reaches down to detect molecules in single cells. Single-cell mass spectrometry is an emerging technology that is believed to unmask phenotypic heterogeneity on the metabolic level. Mapping the metabolome in single cells is a declared goal in analytical sciences.
Chapter 8

Conclusions

Microarrays for MALDI-MS have been developed in the lab of Prof. Zenobi at ETH in the early 2000s. Dr. Martin Pabst has introduced MAMS for quantitative MALDI experiments. The MAMS principle is inline with the high-throughput capabilities of MALDI-MS. In this work, microarray-based MALDI mass spectrometry is applied in metabolite monitoring of biotechnological production processes. Phosphorylated metabolites such as ATP, ADP and AMP are detected in negative ion mode using 9-AA as a MALDI matrix. The phosphodiester bond in phosphorylated nucleosides is prone to hydrolysis and therefore the detection of this important class of metabolites is often biased. Microarray-based MALDI MS offers a straight-forward analysis of these metabolites. The role of insource fragmentation on the ratio of detected ATP/ADP has been investigated and found to be strongly dependent on the applied laser energy. Optimization of the MALDI parameters is crucial for successful, sensitive and significant metabolite detection.

The MAMS spots confine the analyte-matrix crystals to a very small region (400 \( \mu \text{m} \) spot diameter) The applied laser focus is with 50 \( \mu \text{m} \) diameter in comparable range of the spot area and thus analyte spreading is less prone. The fast drying times of the deposited nanoliter volumes leads to homogenous MALDI crystals. Sweet spot phenomena are completely averaged out, since the entire spot is analyzed. Additionally, the sample confinement leads ultimately to higher surface concentrations.

Laser ablation and photolithography, have both been investigated to fabricate the MAMS. The newly developed
Conclusions

photolithographic approach allows precise structuring of different surface materials, including gold and silicium. These new microarray substrates allow for new applications. The sensitivity gain on the Si-based MAMS can be exploited for trace analysis in sample that are limited in quantity such as forensic hair analysis for the detection of cocaine in hair. The new MAMS generation has potential to open up the metabolic analysis of pre selected cell populations in order to unmask phenotypic differences on the metabolic level.
Overall in this thesis the MAMS platform has been further developed and applied to different monitoring tasks. New insights in the metabolic stability of perfusion and batch cell culture processes have been concluded from the obtained results. This thesis underlines the necessity of metabolic monitoring in batch and perfusion cell cultures.
Outlook

Chapter 9

Outlook

The development of a photolithographic production approach opens up a wide field of new and interesting applications for the microarray for mass spectrometry. Since the gold surface is preserved in the photolithographic approach, surface functionalization reaction based e.g. on the thiol-gold interaction will be possible. Immobilization of detection-antibodies can enable immunochemical test on the MAMS. These test coupled to MALDI MS can be interesting alternatives to traditional readouts such as fluorescence or surface plasmon resonance.

The metabolite detection from perfusion and fed batch cell culture processes in this work was limited to phosphorylated metabolites, however analyte classes such as lipids and amino acids are interesting intra- and extracellular compounds for cell culture optimization, as well. Moreover, metabolic products of 13C-labeled supplements can be traced intracellular using the presented technique. This will allow for a in depth characterization of the different process designs and their individual response times towards external changes. The detection of the intact monoclonal antibody, its fragments and aggregates by MALDI-MS has raised a couple of interesting questions regarding the method optimization. Higher resolution and increased sensitivity would convert the method into an attractive alternative to the existing characterization methods such as SEC or dynamic light scattering. The developed method, which is orders of magnitude faster than existing methods, can be optimized to absolutely quantitate mab concentrations from cell supernatants.
References


35. Bajad, S. & Shulaev, V. in *Methods in Molecular
References


55. Wei, J., Buriak, J. M. & Siuzdak, G. Desorption–


65. Hillenkamp, F. & Karas, M. Matrix-assisted laser


<table>
<thead>
<tr>
<th>References</th>
</tr>
</thead>
</table>
References


96. Dreisewerd, K., Schürenberg, K., Karas, M. & Hillenkamp, F. Influence of the laser intensity and spot size on the desorption of molecules and ions in matrix-assisted laser desorption/ionization with a


References


<table>
<thead>
<tr>
<th>References</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>126. Wei, F. <em>et al.</em> 13C NMR-Based Metabolomics for the Classification of Green Coffee Beans According to</td>
<td></td>
</tr>
</tbody>
</table>


136. Amantonico, A., Oh, J. Y., Sobek, J., Heinemann, M.


144. Burgess, K., Russell, D. H., Shitangkoon, A. & Zhang, A. J. MALDI and FAB mass spectrometry of


References


Glucose Sensor for Fermentation Monitoring. 


References


224. Wenzel, R. J., Röhling, U., Nazabal, A. &
Hillenkamp, F. A Detector Device for High Mass Ion Detection, a Method for Analyzing Ions of High Mass and a Device for Selection Between Ion Detectors.


## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>9-AA</td>
<td>9-aminoacridine</td>
</tr>
<tr>
<td>ACoA</td>
<td>Acetyl-coenzyme A</td>
</tr>
<tr>
<td>APCI</td>
<td>atmospheric pressure chemical ionization</td>
</tr>
<tr>
<td>APPI</td>
<td>atmospheric pressure photoionization</td>
</tr>
<tr>
<td>ATF</td>
<td>alternating tangential flow</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine-5' triphosphate</td>
</tr>
<tr>
<td>B0</td>
<td>magnetic field</td>
</tr>
<tr>
<td>CDK</td>
<td>cyclin dependent kinase</td>
</tr>
<tr>
<td>CDR</td>
<td>complementary-determining region</td>
</tr>
<tr>
<td>CE</td>
<td>capillary electrophoresis</td>
</tr>
<tr>
<td>CI</td>
<td>chemical ionization</td>
</tr>
<tr>
<td>CoA</td>
<td>Coenzyme A</td>
</tr>
<tr>
<td>D</td>
<td>drift distance</td>
</tr>
<tr>
<td>d</td>
<td>entrance depth in reflectron</td>
</tr>
<tr>
<td>Da</td>
<td>dalton</td>
</tr>
<tr>
<td>DART</td>
<td>direct analysis in real-time</td>
</tr>
<tr>
<td>DESI</td>
<td>desorption electrospray ionization</td>
</tr>
<tr>
<td>DHB</td>
<td>dihydroxybenzoic acid</td>
</tr>
<tr>
<td>DI</td>
<td>direct infusion</td>
</tr>
<tr>
<td>DIOS</td>
<td>desorption/ionization on silicon</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DWP</td>
<td>deep well plate</td>
</tr>
<tr>
<td>e</td>
<td>ion charge</td>
</tr>
<tr>
<td>E</td>
<td>electric field</td>
</tr>
<tr>
<td>EI</td>
<td>electron impact</td>
</tr>
<tr>
<td>ESI</td>
<td>electrospray ionization</td>
</tr>
<tr>
<td>FAD</td>
<td>flavin adenine dinucleotide</td>
</tr>
</tbody>
</table>
## Appendix

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>fc</td>
<td>fragment crystallizable region</td>
</tr>
<tr>
<td>FDA</td>
<td>food and drug administration</td>
</tr>
<tr>
<td>FIE</td>
<td>flow injection electrospray</td>
</tr>
<tr>
<td>FT-ICR</td>
<td>Fourier transformation ion cyclotron resonance</td>
</tr>
<tr>
<td>GC</td>
<td>gas chromatography</td>
</tr>
<tr>
<td>glc</td>
<td>glucose</td>
</tr>
<tr>
<td>hc</td>
<td>heavy chain</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>HV</td>
<td>high voltage</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>kd</td>
<td>specific death rate</td>
</tr>
<tr>
<td>L</td>
<td>drift length</td>
</tr>
<tr>
<td>lac</td>
<td>lactate</td>
</tr>
<tr>
<td>LAESI</td>
<td>laserablation electrospray ionization</td>
</tr>
<tr>
<td>LC</td>
<td>liquid chromatography</td>
</tr>
<tr>
<td>lc</td>
<td>light chain</td>
</tr>
<tr>
<td>LDI</td>
<td>laser desorption/ionization</td>
</tr>
<tr>
<td>m</td>
<td>mass</td>
</tr>
<tr>
<td>mAbs</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>MALDI</td>
<td>matrix-assisted laser desorption/ionization</td>
</tr>
<tr>
<td>MAMS</td>
<td>microarray for mass spectrometry</td>
</tr>
<tr>
<td>MBP</td>
<td>maltose binding protein</td>
</tr>
<tr>
<td>MCP</td>
<td>multi channel plate</td>
</tr>
<tr>
<td>MeCN</td>
<td>acetonitril</td>
</tr>
<tr>
<td>MeOH</td>
<td>methanol</td>
</tr>
<tr>
<td>MIRS</td>
<td>Mid infrared spectroscopy</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>n</td>
<td>number of replicates</td>
</tr>
<tr>
<td>NAD$^+$</td>
<td>beta nicotinamin adenine dinucleotide (oxidized form)</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinamid adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NIMS</td>
<td>nanostructure-initiator ms</td>
</tr>
<tr>
<td>NIR</td>
<td>near-infrared spectroscopy</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>PAT</td>
<td>process analytical tool</td>
</tr>
<tr>
<td>PAT</td>
<td>process analytical tools</td>
</tr>
<tr>
<td>PC</td>
<td>principal component</td>
</tr>
<tr>
<td>PCA</td>
<td>principal component analysis</td>
</tr>
<tr>
<td>PTR-MS</td>
<td>proton-transfer reaction mass spectrometry</td>
</tr>
<tr>
<td>QIT</td>
<td>quadrupole ion trap</td>
</tr>
<tr>
<td>qs</td>
<td>specific substrate consumption rate</td>
</tr>
<tr>
<td>S</td>
<td>substrate concentration</td>
</tr>
<tr>
<td>s</td>
<td>initial spatial distribution</td>
</tr>
<tr>
<td>S/N</td>
<td>signal/noise</td>
</tr>
<tr>
<td>SEC</td>
<td>size-exclusion chromatography</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of mean</td>
</tr>
<tr>
<td>SIMS</td>
<td>secondary ion ms</td>
</tr>
<tr>
<td>t</td>
<td>time</td>
</tr>
<tr>
<td>TBAS</td>
<td>tetrabutylammonium sulfate</td>
</tr>
<tr>
<td>td</td>
<td>doubling time</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>TFF</td>
<td>tangential flow filtration</td>
</tr>
<tr>
<td>THAP</td>
<td>trihydroxyacetophenone</td>
</tr>
<tr>
<td>TIS</td>
<td>timed ion selector</td>
</tr>
<tr>
<td>TOF</td>
<td>time-of-flight</td>
</tr>
<tr>
<td>UDP</td>
<td>uridine-5' diphosphate</td>
</tr>
<tr>
<td>UHPLC</td>
<td>ultra high performance liquid chromatography</td>
</tr>
<tr>
<td>UTP</td>
<td>uridine-5' triphosphate</td>
</tr>
<tr>
<td>V</td>
<td>accelerating voltage</td>
</tr>
<tr>
<td>VCD</td>
<td>viable cell density</td>
</tr>
<tr>
<td>X</td>
<td>viable cell concentration</td>
</tr>
</tbody>
</table>
Appendix

Contributions

Publication list


Appendix


Conference contributions


Steinhoff R., Metabolite and productivity monitoring of IgG producing mammalian cells in perfusion and batch processes, *PEGS Europe*, October 2015 (invited talk)


Appendix
Appendix

Curriculum Vitae

Name: Robert Friedrich Steinhoff
Date of birth: 06.05.1985
Place of birth: Lindau (Bodensee), Germany
Nationality: German
Mail: robert.steinhoff@alumni.ethz.ch

Education

Since 03/2012 PhD student in the Laboratory of Analytical Chemistry, “Mass spectrometry for biotechnological process monitoring.”
Prof. Renato Zenobi, ETH Zurich, Switzerland

10/2009 – 02/2012 Master of Science in Chemistry, with major in organic and analytical chemistry
Technical University (TUM) Munich, Germany

Master Exchange Semester (6 months), École normale supérieure (ENS) de Lyon, France

Master’s Thesis in the Laboratory of Analytical Chemistry, “The photo-physical properties of recombinant GFP in the gas-phase.“
Prof. Dr. R. Zenobi, ETH Zurich, Switzerland

10/2006 – 10/2009 Bachelor of Science in Chemistry,
Technical University (TUM) Munich, Germany

Bachelor thesis “Quantification of 1,2:3,4-Diepoxybutan in blood by LC-MS/MS.“
Prof. Dr. Filser, Helmholtz Zentrum, München

06/2005 Abitur (Bodensee-Gymnasium Lindau)
Appendix

Scholarship & Awards

  Member of an initial training network with regular scientific workshops (2 weeks per year) at:
  University of Oxford (UK),
  University of Southern Denmark,
  University Gothenborg.

- Swiss Academy of Natural Sciences (SCNAT) and the Swiss chemical society (SCS), Chemistry Travel Award, 2015

- German Society of Chemists (GDCh) travel stipend, 2013

Supervision and teaching activities

Teaching assistant, Practical Course in Analytical Chem. (3 years)
ETH Zurich, Switzerland

Teaching assistant, (during semester break, 2 months)
Technical University (TUM) Munich, Germany,
(Prof. Dr. Hermann, and Prof. Dr. Kühn)

Master student supervision, (6 months),
ETH Zurich, Switzerland

Language skills

German Native

English  Fluent in speaking and writing

French  Fluent in speaking and writing – DELF B1 (87/100)

Extracurricular activities

Rowing
Men’s eight and coxless four at international competitions,
Rowing Club Belvoir Zurich

Community activities
Voluntary fire brigade member (seven years), Lindau, Germany