Do MALDI mass spectra reflect condensed-phase chemistry? and Development of a continuous-flow interface for MALDI mass spectrometry

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Publication Date:
1999

Permanent Link:
https://doi.org/10.3929/ethz-a-003832847

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Do MALDI Mass Spectra Reflect Condensed-Phase Chemistry?

and

Development of a Continuous-Flow Interface for MALDI Mass Spectrometry

A dissertation submitted to the
EIDGENÖSSISCHE TECHNISCHE HOCHSCHULE ZÜRICH

for the degree of
DOCTOR OF NATURAL SCIENCES

presented by
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September 1999
The great thing in this world is not so much where we are, but in what direction we are moving.

Oliver Wendell Holmes
ACKNOWLEDGEMENTS

I sincerely thank Prof. Dr. Renato Zenobi for the opportunity to work on a challenging scientific topic and for his unflagging support and motivation. It was a great learning experience, both professionally and personally.

I wish to acknowledge Prof. Dr. Antonio Togni for the time he kindly invested in several discussions on various aspects of complex formation and for his immediate acceptance of being my co-examiner.

I owe special thanks to Dr. Richard Knochenmuss for his great scientific and personal support at important milestones of my thesis.

Many thanks go to Stefan Vetter for synthesizing the peptide p55F1, for collecting circular dichroism spectra, as well as for many helpful discussions and his assistance with biochemical questions.

Special thanks go to Dr. Beat Gut, Mr. Schindler, and their colleagues from the Abteilung Hochleistungskeramik at EMPA Dübendorf for their continuous commitment in the preparation of various frits for the coupling project and for numerous valuable discussions. Thanks to Dr. Beat Gut for proofreading Part B of this thesis.

I also wish to acknowledge Dr. Bernhard Linden from Linden-ChroMasSpec for the preparation of various graphite whiskers and his very kind assistance with many aspects of the coupling project.

Many thanks go to Leo Weissberg, Mr. Baumgartner, and Mr. Huber from the electronics and mechanical workshops for their highly efficient work and readiness to develop unique solutions.

Furthermore, I would like to thank Marc Suter from EAWAG Dübendorf for placing chromatographic equipment at my disposal and the time he invested in several discussions on chromatographic issues.

I wish to express my sincere thanks to my office mates Stefan Nettesheim and Martin Handschuh. We had a great time both at work and in our free time. Many thanks for this.
Special thanks also go to Elizabeth Stevenson and Thomas Roth. They have carefully undertaken the time-consuming proofreading of this work. Many thanks also for the time I spent with Elizabeth on nonscientific discussions.

Furthermore, I would like to acknowledge all my colleagues from the Zenobi group for their helpful manner, the good collaboration and the friendly atmosphere.

I also thank Daniel Meierhans for his assistance with electrophoretic mobility shift assays.

I owe my thanks to all including those not explicitly mentioned above who contributed to an open, helpful and pleasant environment at the ETH and to a great time in Zürich.

I greatly acknowledge the *Fonds der Chemischen Industrie* in Germany for a Kekulé stipend for my work performed during the period between January 1997 and December 1998.
Part of the present work has been published or has been presented as posters or talks at conferences.

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Lehmann, E.; Zenobi, R.
**DETECTION OF SPECIFIC NONCOVALENT ZINC FINGER PEPTIDE-OLIGODEOXYNUCLEOTIDE COMPLEXES BY MATRIX-ASSISTED LASER DESORPTION/IIONIZATION MASS SPECTROMETRY,**

Lehmann, E.; Zenobi, R.
**DETEKTION SPEZIFISCHER NICHTKOVALENTER ZINKFINGERPEPTID-OLIGODESOXYNUCLEOTID-KOMPLEXE DURCH MATRIXUNTERSTÜTZTE LASERDESORPTIONS/IONISATIONS-MASSENSPEKTROMETRIE,**

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September 5-10, 1998, Halkidiki, Greece.
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**SUMMARY**

The present thesis is divided into two parts. **Part A (chapters 4 to 6)** deals with the question of whether matrix-assisted laser desorption/ionization (MALDI) mass spectra reflect condensed-phase chemistry. **Part B (chapter 7)** describes the initial steps of the development of a continuous-flow (CF) interface for MALDI mass spectrometry (MS).

The process of ion formation in MALDI MS is still largely unknown. Ions may exist as preformed species in the solid state and be liberated upon laser irradiation, or ions may form by reactions initiated by the laser pulse. For the latter, many different processes are possible. The preformed ion pathway has not been studied thoroughly up to date. In **chapter 4** the contribution of preformed ions was therefore investigated from the standpoint of coordination chemistry and studied in different transition metal ion/matrix systems as well as in the copper/polystyrene system. The solution, the solid, and the gas phase were investigated by means of infrared, visible, and $^1$H nuclear magnetic resonance spectroscopy, as well as by MALDI MS. It was found that the contribution of preformed ions can be predicted on the basis of condensed-phase thermodynamics. MALDI mass spectra qualitatively reflect the amount of preformed complexes in the solid target. The experiments also gave insight into the mechanisms of charge compensation.

When studying biological systems, such as noncovalent or biomolecule-metal ion complexes with MALDI MS, it is very important to know whether the complexes are detected in the mass spectrum or whether they are destroyed during sample crystallization and laser desorption/ionization processes.

In this context, the complexation between an 18-residue zinc finger peptide of CCHC-type (CCHC = Cys-X$_2$-Cys-X$_4$-His-X$_4$-Cys, X = variable amino acid) from the gag protein p55 of human immunodeficiency virus type 1 (HIV-1) and various transition metal ions was investigated by means of circular dichroism spectroscopy (solution) and MALDI MS (gas phase) in **chapter 5.2**. This was performed as a function of pH, the nature of the metal ion used, and the molar ratio of peptide and metal ion. It was demonstrated that MALDI spectra show a specific interaction between the peptide and Zn$^{2+}$ ions. The method was found to be suitable for studying metal-binding properties of zinc finger complexes.

Additional MALDI studies on the complexation between the peptide, Zn$^{2+}$ and the oligodeoxynucleotides d(TTGTT) or d(TTTTTGTTTTT) are described in **chapter 5.3**. A specific triple complex between the three compounds could be
detected successfully. The use of a nonacidic matrix as well as physiological pH conditions were found to be crucial for this. Chemical control experiments confirmed the specificity of the triple complex. An experiment with Cu$^{2+}$ as a mimic for an antiviral HIV agent clearly demonstrated the potential of MALDI MS for rapidly screening such agents for the treatment of acquired immunodeficiency syndrome (AIDS).

Finally, in chapter 6 the question of whether noncovalent cyclodextrin inclusion complexes can be probed with MALDI MS was addressed. It was found that specific hydrophobic cyclodextrin inclusion complexes are destroyed during the MALDI process and that nonspecific electrostatic adducts are formed if an ion-dipole interaction can occur, for example, between cyclodextrin and a protonated amino group. When investigating pseudorotaxane-like complexes with similar formation constants but with electrostatic interactions between the complexing partners, it was found that MALDI MS can detect the specific complexes. The experiments demonstrated that the nature of the interaction in the noncovalent complex is decisive for whether MALDI MS can probe the specific interaction or not. The results also show that chemical controls are very powerful to complement MS in detecting the presence or absence of a specific interaction in the MALDI mass spectrum.

The coupling of liquid separation techniques to MS has many advantages, for example, it allows the fast analysis of complex mixtures. Many off-line interfaces have been reported with MALDI MS. CF interfaces, however, are less common. In chapter 7, we started to develop a CF interface for UV MALDI that relies on the two-phase MALDI methodology. A two-phase MALDI matrix consists of a solid laser absorber and a liquid matrix. First, the work focused on the search for and the modification of a broad range of different laser absorbing materials, which were tested in static and CF mode. Graphite and TiN frits, and graphite whiskers were selected and thoroughly investigated. Unfortunately, these materials had to be rejected since their wetting properties were poor, their preparation protocols were not reproducible, or the UV desorption/ionization method was not suitable. Second, important parameters governing a stable liquid flow into the mass spectrometer were identified and implemented. A CF set-up with a syringe pump and a controllable heating system were developed, and sample holders for the frits and the graphite whiskers were designed. Finally, critical parameters for a low dead volume interface were identified and are discussed in detail.
ZUSAMMENFASSUNG

Die vorliegende Arbeit besteht aus zwei Teilen. **Teil A (Kapitel 4 bis 6)** beschäftigt sich mit der Frage, ob matrixunterstützte Laserdesorptions/Ionisations (MALDI)-Massenspektren die Chemie in der kondensierten Phase widerspiegeln. **Teil B (Kapitel 7)** beschreibt die ersten Schritte zur Entwicklung eines Online-Interfaces für die MALDI-Massenspektrometrie (MS).


Für die Untersuchung von nichtkovalenten Komplexen oder Komplexen von Biomolekülen mit Metallionen ist es sehr wichtig, ob die Komplexe in den Massenspektren detektiert werden können oder ob sie während der Probenkristallisation oder dem Laserdesorptions/Ionisationsvorgang dissoziiert. In diesem Zusammenhang wurde in **Kapitel 5.2** die Komplexierung eines Zinkfingerpeptids mit 18 Aminosäuren des Typs CCHC (CCHC = Cys-X_2-Cys-X_4-His-X_4-Cys, X = beliebige Aminosäure) aus dem gag-Protein p55 des menschlichen Immunschwächevirus Typ 1 (HIV-1) und verschiedenen Übergangsmetallen mit Circulardichroismus (Lösung) und MALDI-MS (Gasphase) untersucht. Der pH-Wert, die Art des Metallions und das Molverhältnis von Peptid zu Metallion wurden variiert. Es zeigte sich, dass die MALDI-Spektren eine spezifische Wechselwirkung zwischen dem Peptid und Zn\(^{2+}\) nachweisen können. Desweiteren erwies sich die MALDI-MS als sehr geeignete Methode für die Untersuchung der Metallbindungseigenschaften von Zinkfingerkomplexen.

Weitere MALDI-Untersuchungen in **Kapitel 5.3** befassen sich mit der Komplexierung dieses Peptides mit Zn\(^{2+}\) und den Oligodesoxynucleotiden d(TTGT)
oder $d(\text{TTTTTGTTTTT})$. Ein Dreifachkomplex konnte nachgewiesen und seine Spezifität mittels Kontrollexperimenten bestätigt werden. Eine nichtsaure MALDI-Matrix sowie physiologische pH-Bedingungen waren unerlässlich für die Detektion des Komplexes. Am Beispiel von $\text{Cu}^{2+}$ als Modell für ein antivirales HIV-Agens konnte das Potential der MALDI-MS für das rasche Screening solcher Agentien für die AIDS-Behandlung aufgezeigt werden.


1 Introduction

Mass spectrometry (MS) is a technique which produces gaseous ions in the vacuum and subsequently separates them according to their mass-to-charge (m/z) ratio using a mass analyzer. The technique, which was developed in the 1920s, is nowadays an indispensable tool in the fields of chemistry and life sciences. Access to these fields was gained through the development of new desorption/ionization techniques over the last 30 to 40 years. The central problem which was addressed during this period was how to convert thermally labile, nonvolatile, high molecular weight molecules without significant fragmentation into gaseous ions. In the beginning, field desorption [1], plasma desorption [2], fast atom bombardment [3] and laser desorption led to the successful mass spectrometric detection of biomolecules with masses up to several tens of kDa. However, there were two so-called "soft", i.e. characterized by little fragmentation, desorption/ionization techniques, introduced at the end of the 1980s, that have greatly expanded the mass range and thus strengthened the role of mass spectrometry in biological research.

Matrix-assisted laser desorption/ionization (MALDI) was introduced simultaneously by Karas and Hillenkamp [4] and Tanaka et al. [5] and is usually combined with time-of-flight (TOF) mass analyzers. Electrospray ionization (ESI), which is mainly used in conjunction with quadrupole and Fourier-transform ion cyclotron resonance (FTICR) mass analyzers, was developed by Fenn and coworkers [6]. With MALDI mass spectrometry it is now possible to analyze nonvolatile, fragile compounds with masses up to several hundred kDa. Besides the large accessible mass range, the method's advantages are a sensitivity in the femtomole range, the tolerance to salt impurities, e.g. in biological samples, and the possibility to obtain structural information besides mass information. For these reasons, MALDI MS is now successfully used for the analysis of biopolymers, such as peptides and proteins [7-9], oligonucleotides [10-13], glycolipids [14], oligosaccharides [15-17], molecular aggregates with weak noncovalent interactions [18-23] and complexes of metal ions with biomolecules [24-27], synthetic polymers [28-32], as well as inorganic complexes [33].

Peptide and protein sequencing with MALDI MS has now become extremely important in proteome research. Sequencing can be performed either by mass analysis of the digested molecule or by using post-source decay and a reflectron TOF (reTOF) instead of a linear TOF instrument [8, 34-38]. The masses of the resulting peptides are subsequently compared with those from peptides registered
in data bases, and the sequence can be established.
MALDI MS is also used for DNA [36, 39-41] and RNA [42] sequencing. The
counterpart method for oligonucleotide sequencing would be too slow
[43]: the analysis of the human genome of $3.3 \cdot 10^9$ base pairs by 1000 laboratories
sequencing 30 000 bases per year would require 110 years! The potential for fast
sequencing methods is therefore enormous.
Synthetic polymers, such as polyesters, polyethers, polystyrenes or acrylics, are
studied with MALDI TOF MS with respect to their oligomer spacing, end group
distribution, molecular weight distribution, and polydispersity [28-32].

The method's success is reflected in the worldwide market for MALDI TOF mass
spectrometers. This market has grown by 30 to 40 % annually, both measured by
the number of instruments sold and the number of MALDI publications
(1992: 54; 1995: 270; 1998: 644). There are at least 1500 instruments worldwide,
with the majority being in North America (200 in California alone), Europe and
Japan. A dozen manufacturers offer commercial instruments worldwide. They
generated several hundred million US dollars in sales in 1998.

Despite the impressive range of MALDI applications, the nature of the ionization
process is not yet fully understood. Ions may exist as "preformed" species in the
solid state and be liberated upon laser irradiation, or ions may form by reactions
initiated by the laser pulse. For the latter, many different processes are possible,
e.g. multiphoton ionization, energy pooling, proton transfer or cationization. The
contribution of preformed ions or ions formed after the laser pulse may depend
on the systems investigated. e.g. biomolecule-metal ion complexes form already
in solution and MALDI signals are expected to originate from these ions [44, 45]
whereas in other systems, the contribution of preformed ions is nearly zero.
Although the presence of preformed ions is linked to the essential question of
whether MALDI spectra may reflect solution-phase chemistry, only limited work
has been done so far on this topic [24-27, 46-48].

Our aim was therefore to fill this gap with the work presented in Part A of this
thesis. The contribution of preformed ions to the MALDI ion signal was first
studied from a mechanistic point of view using model systems (chapter 4). A part
of the very initial experiments on this topic has already been described in my
diploma thesis [49]. We wanted to develop criteria based on which the relative
contribution of preformed ions could be predicted, and to demonstrate a corre-
lation between condensed-phase and gas-phase chemistries by performing both
MALDI analysis and investigations of the solution/solid phase. The knowledge
we gained during the model studies was applied to the investigation of practically relevant systems. The cationization of polystyrene, where gas-phase reactions are expected to play an important role, was investigated first (chapter 4). Subsequently, complexes between a HIV-zinc finger peptide and transition metal ions as well as oligodeoxynucleotides were studied (chapter 5). Finally, cyclodextrin inclusion complexes and pseudorotaxane-like complexes were investigated in chapter 6. Cyclodextrin inclusion complexes are based on hydrophobic interactions while the pseudorotaxane-like complexes are due to ionic interactions. Therefore this study also allowed us to probe how the nature of the interaction in the complex may influence the ability of MALDI MS to detect a specific interaction, in addition to the complexes’ formation constant. All these compounds are examples of more or less fragile complexes which may not survive crystallization and laser desorption/ionization processes.

The increasing success of mass spectrometry in biochemistry is not only due to the development of soft ionization techniques, but also due to the coupling with liquid separation techniques in continuous-flow (CF) mode, such as capillary electrophoresis (CE) or high performance liquid chromatography (HPLC). The combination allows analysis of complex sample mixtures, analyte identification with two complementary techniques and eliminates the need for time-consuming collection of the chromatographic fractions for mass analysis.

ESI MS [50-55], along with fast atom bombardment mass spectrometry (FAB MS) [56], has been most successful for the direct coupling because the eluent from the liquid separation technique can directly be introduced into the mass analyzer. Some of the drawbacks of ESI, such as difficult spectral interpretation, salt or buffer intolerance, or low speed of the quadrupole analyzer, are not of concern when using MALDI MS. Since a MALDI sample has to be co-crystallized with the matrix, mainly off-line combinations of liquid separation techniques with this method are reported [57-62]. CF interfaces are not very common and many of them are quite limited for practical use [63-70].

The aim of our work presented in Part B of this thesis was to develop a CF interface which relies on the two-phase MALDI methodology. A two-phase MALDI matrix consists of a solid laser absorber and a liquid matrix. First, an extensive search for adequate laser absorbers and liquid matrices was performed. Then, they were tested with experiments performed in static and CF mode. Finally, conditions for a stable flow of the eluent into the mass spectrometer were defined and a CF set-up was developed.
The thesis is therefore organized as follows. After this introduction, the fundamentals of MALDI TOF mass spectrometry, including possible ionization mechanisms, are described in chapter 2. The instruments used for the experiments performed in this thesis are presented in chapter 3. The remainder is then divided into Parts A and B.

In Part A (chapters 4 to 6) the experimental results on the correlation between condensed-phase and gas-phase chemistries are presented, first in model systems, and then in the polystyrene, zinc finger peptide, and cyclodextrin systems.

Part B (chapter 7) deals with the development of a CF interface for the coupling of MALDI MS with a liquid separation technique. Different approaches based on the two-phase MALDI methodology are presented. Their tests with experiments in static and CF mode are described.

1.1 References


2 Fundamentals of MALDI TOF Mass Spectrometry

2.1 Introduction

MALDI TOF mass spectrometry comprises several steps. The very first is the cocrystallization of the analyte with an excess of a solid matrix material. In contrast to the analyte molecules, matrix molecules must possess a strong extinction coefficient at the wavelength of the laser and directly absorb the laser energy. The laser pulse induces desorption and ionization processes of matrix and analyte in a very dense MALDI plume (Figure 1).

![Figure 1](image.png)

Figure 1. Schematic representation of the desorption/ionization process. Matrix molecules absorb the laser energy, volatilize explosively and liberate the analyte ions to form the MALDI plume. [MH]+ and [ANa]+ represent different matrix and analyte ions.

The desorption threshold in MALDI is about one order of magnitude lower than in laser desorption experiments without matrix. While the desorption event can now be considered as fairly well understood, the ionization mechanisms in the MALDI plume are not well understood. Both processes are neither separated in time nor space which complicates the issue. The ions formed in the MALDI plume are extracted by a strong electric field into the field-free region of the TOF analyzer, where they are separated in time according to their m/z ratio. Depending on the polarity of the electric field, positive or negative ions are
extracted. Ion extraction can be performed either promptly or delayed (time delay between laser firing and application of the electric field). Each ion induces a current in the detector, which is then monitored as a function of time yielding a mass spectrum after calibration. Several tens of mass spectra are summed up to improve the signal-to-noise ratio.

These comments show that a lot of parameters play a role in the MALDI method. In the following, the nature of the matrix, the role of the laser, the issue of fragmentation and the current knowledge on desorption and ionization processes in the MALDI plume are discussed in more detail. Subsequently, the TOF equation, the mass calibration and the performance characteristics of MALDI TOF MS are presented. This chapter should give an insight into the great diversity of possible MALDI experiments, but also into the problems that may be encountered.

2.2 Matrix

In general, a MALDI matrix has to fulfill the following requirements:

- absorption at the wavelength of the laser radiation to provide sufficient energy deposition in the sample
- low sublimation rate to guarantee vacuum stability
- for its role in the ionization mechanism, see chapter 2.6
- solid matrices, which are most commonly used, have to be soluble in the same solvent as the analyte. They have to crystallize upon sample preparation, and embed the analyte in its crystal lattice by optimally separating the analyte molecules.

The molar ratio of matrix to analyte, the sample preparation technique, and the choice of the matrix determine the success of the MALDI experiment. The optimum matrix-to-analyte ratio is generally $10^2$ to $10^3$, strongly depending on the matrix used and the size of the analyte molecules [1]. The larger the molecules, the larger the molar excess of matrix molecules needs to be. Numerous sample preparation techniques are reported in the literature. The most important are the dried droplet [2], the fast evaporation [3], as well as the layering method, which is described in chapter 4 of this work. In some cases, the use of a specific technique determines the outcome of the MALDI experiment, whereas
in others, different preparation techniques lead to the same result. For reasons of solubility, proton affinities, or other properties which are important for analyte ionization, there are specific matrix materials for each substance class. For example, benzoic acid and cinnamic acid derivatives such as 2,5-dihydroxybenzoic acid (DHB) or α-cyano-4-hydroxycinnamic (α-CHCA) acid have been recognized from early on as good MALDI matrices for proteins [4]. For oligonucleotides, 3-hydroxypicolinic acid (3-HPA) [5] or a mixture of 2,3,4-trihydroxyacetophenone (2,3,4-THAP) and 2,4,6-THAP [5-7] are used in ultraviolet (UV), and succinic acid [8] in infrared (IR) MALDI. The use of these matrix materials is combined with the application of the following methods to solve the problem of undesired alkali ion adducts to the oligonucleotides: addition of diammonium hydrogencitrate [6], drop dialysis with diammonium hydrogencitrate [9], or ion exchange using the Nafion 117 membrane [10]. 3-Aminoquinoline [11] and coumarin 120 (laser dye) [12] were found to be good matrices for polysaccharides and monosulfated oligosaccharides, respectively. Noncovalent, biomolecule-metal ion complexes [13] and double-stranded oligonucleotides [14] have been successfully analyzed with the basic matrices 6-aza-2-thiothymine (ATT) or para-nitroaniline (PNA) which, in contrast to the aforementioned acidic matrices, allow samples to be prepared at nondenaturing conditions.

A number of problems are encountered with solid matrices. The matrix and analyte have to dissolve in the same solvent and subsequently co-crystallize. Inhomogeneous co-crystallization can lead to the existence of “hot spots” on the sample probe, i.e. areas where good MALDI signals are observed while other areas yield poor or no signals. Moreover, the presence of impurities can lead to heterogeneities in the crystal lattice which may hinder ion formation. Furthermore, different matrices are known to induce varying analyte fragmentation [15, 16].

Besides solid matrices, liquid and liquid/solid two-phase matrices are also used for sample preparation. They circumvent some of the problems associated with solid matrices. The liquid matrix can dissolve the analyte and analyte diffusion can result in a homogeneous sample. The matrix can refresh its surface continuously, increasing shot-to-shot stability and the number of high quality shots at a given location. 3-nitrobenzyl alcohol (3-NBA) and 2-nitrophenyl octyl ether (2-NPOE), as well as glycerol and triethanolamine, have been successfully used in UV and IR MALDI [17-20]. Water/glycerol, ethanol/glycerol and frozen water have been used as IR matrices. If laser-absorbing particles are suspended in the liquid matrix, the advantages of the matrix are retained, and the additive can be
chosen to absorb the laser wavelength, thus extending the application of the matrices to other wavelengths. The resulting method is called two-phase MALDI or SALDI (surface-assisted laser desorption/ionization). Several combinations of particulates and liquid matrices have been used to analyze proteins, oligosaccharides, synthetic polymers and dyes [9, 21-25]. All the matrix molecules used and mentioned in the present thesis are shown below.

- 6-aza-2-thiothymine (ATT)
- para-nitroaniline (PNA)
- 2-amino-4-methyl-5-nitropyridine (AMNP)
- Glycerol
- 2-nitrophenyl octyl ether (2-NPOE)
- Triethanolamine
- 3-nitrobenzyl alcohol (3-NBA)
- Trans-3,5-dimethoxy-4-hydroxy cinnamic acid (sinapinic acid)
- Trans-3-methoxy-4-hydroxy cinnamic acid (ferulic acid)
- α-cyano-4-hydroxy cinnamic acid (α-CHCA)
- 2,4,6-trihydroxyacetophenone (2,4,6-THAP)
- 2,5-dihydroxy benzoic acid (DHB)
- 2-hydroxy-5-methoxy benzoic acid
- Nicotinic acid
- 1,8,9-trihydroxyanthracene (dithranol)
- 1,4-bis(5-phenyloxazol-2-yl)benzene (POPOP)
- 2,6-dihydroxy-acetophenone
- 3-hydroxy picolinic acid (3-HPA)
- Succinic acid
- 3-aminoquinoline
Unfortunately, there are still no clear guidelines for matrix selection, and matrices are often found after empirical screening of a large number of candidate compounds. Some guidelines for matrix selection in polystyrene analysis (chapter 4) are given. However, many uncertainties still remain, and matrix crystallization and embedding of the analyte require further study.

### 2.3 Laser

Depending on the wavelength of the laser, UV and IR MALDI experiments are distinguished. The nitrogen laser with a wavelength of 337 nm is most frequently used in UV MALDI and is the cheapest. This wavelength has also been used in this work. Other UV wavelengths are emitted from Nd:YAG lasers (frequency tripled and quadrupled at 355 nm or 266 nm, respectively) and excimer lasers (XeCl: 308 nm, KrF: 248 nm, ArF: 193 nm). More recently, radiation from IR lasers has also been used. Er:YAG lasers (2.94 μm) dominate this area now, but smaller, less expensive carbon dioxide lasers (10.6 μm) are being developed as a commercially viable alternative. In IR MALDI, owing to lower matrix absorption, pulse energy requirements and sample consumption are higher [26, 27]. Compared to UV MALDI, less metastable fragmentation and adduct formation as well as a greater tendency to form multiply charged ions is reported [28-30]. Nevertheless, IR and UV MALDI mass spectra look similar and many UV matrices work well in the IR, too. The important characteristic of the laser radiation is its fluence (in J/cm²) and not its irradiance (fluence per unit time, in W/cm²); MALDI experiments are found to be largely independent of the laser pulse width [31, 32]. Different incident angles of the laser beam with respect to the sample surface are employed in MALDI experiments. The best results are obtained for angles that are as close to 90° as possible.

### 2.4 Fragmentation

MALDI is a soft desorption/ionization method, i.e. leads to little fragmentation. Nevertheless, fragmentation may play an important role for the MALDI detection of weakly-bound noncovalent complexes or for obtaining structural information. Fragmentation results from an excess of internal energy deposited in the molecular ion during desorption/ionization, and occurs at rates that depend on the amount of excess energy, the number of degrees of freedom, and the bond strengths in the molecular ion. For small molecules with high internal energies, fragmentation may be prompt. For larger molecules, the larger number of bonds may extend the fragmentation time frame considerably [33].
Prompt fragmentation occurs in the ion source very shortly (ns) after ionization. In MALDI it takes place on, or very near to, the sample surface. Fragment ions that are formed promptly appear at their correct daughter masses with a mass resolution comparable to that of the parent molecular ion.

Post-source fragmentation results from metastable unimolecular decomposition or collisions of the ions with the background gas in the drift region. The resulting fragments have the same velocities as their parent molecular ions and the same flight times. In a linear TOF analyzer, i.e. with one acceleration region, this results in loss of fragment-ion information. A reTOF analyzer comprises two acceleration and drift regions, enclosing an angle of < 10° leading to the reflection of the ions onto the detector. Using this analyzer, ions produced by post-source fragmentation are dispersed according to their masses. This phenomenon can be effectively exploited to obtain structural information, e.g. amino acid sequences for peptides. Post-source decay (PSD) [34, 35] in a reTOF instrument is therefore an alternative method to chemical sequencing by enzymatic digestion with subsequent analysis in a linear TOF instrument.

2.5 Desorption Mechanisms and the MALDI Plume

Hillenkamp and coworkers [32, 36] introduced a quasi-thermal desorption model for MALDI that involves a pressure-driven disintegration of the matrix material into microscopic particles. Thermal desorption from these particles leads to molecular matrix and analyte gas-phase species. Garrison and coworkers [37, 38] as well as Handschuh et al. [39] demonstrated that the desorbed material consists of single molecules as well as clusters or aggregates of matrix. The ratio of particulates to single molecules ejected decreases with laser fluence. In accordance with this result, particulate ejection plays a much more important role in IR than in UV MALDI since the laser fluence is much lower. The MALDI plume formed by the desorbed material can be described by a very rapid, even explosive phase transition [40]. In the first few nanoseconds it has a very high density comparable to that of a solid, which is characterized by a high collision rate. After tens of nanoseconds the plume completely expands into the collision-less region of the vacuum. Plume molecular velocities are about 1000 m/s [41]. After the initial temperature jump upon formation of the dense plume, jet expansion induces cooling. Nevertheless, temperatures are still relatively high (about 500 K) after expansion owing to the inefficient cooling of large polyatomic molecules [42].
2.6 Mechanisms for Ion Formation in UV MALDI

UV MALDI is characterized by a high energy density in the sample and a low penetration depth (nm range) of the laser due to high absorption of the matrix. In contrast, the resonant vibrational excitation of the matrix molecules in IR MALDI leads to less absorption, therefore a greater penetration depth (μm range) of the IR laser irradiation and a low energy density in the sample. This is often not sufficient for complete sublimation of the ablated sample volume. Therefore, a desorption model based on spallation of the matrix material due to mechanical stress is commonly accepted [43].

2.6 Mechanisms for Ion Formation in UV MALDI

The ionization processes in MALDI are less well understood. A number of mechanisms are discussed in the literature, and as a result of the better knowledge about ion formation, a number of possibilities for better control of the MALDI experiment are suggested. This section summarizes the most important findings. A more detailed description of this topic can be found in a review paper of Zenobi and Knochenmuss [44] and in the references therein.

In positive ion mode, which is most often used in MALDI experiments, the commonly observed ions are protonated molecular ions, cationized molecular ions in the form of metal ion adducts, and radical cations (less common for analyte). Cationization may either occur with ions which are ubiquitous in any sample as impurities, such as sodium or potassium, or by deliberate addition of metal ions. Protons for protonation are assumed to stem from matrix molecules. Depending on the preference of the analyte for protons or cations, metal ions are either removed (oligonucleotides) or added (apolar polymers).

Small analyte molecules up to 1000 Da are only observed as singly charged ions. Larger ions can carry multiple charges, but no more than triply charged ions are typically observed in MALDI spectra. The number of charges on a molecule is controlled by Coulomb energy. Since the Coulomb energy of multiply charged small size species is greater than the proton binding energies, charge compensation to yield singly charged ions is commonly done by deprotonation or complexation with deprotonated matrix molecules.

The current view of the MALDI ionization process is that primary ions (initial ions from neutral molecules, often matrix-derived species) are created in a medium hot, dense bath of neutral matrix molecules and clusters. Many will undergo collisions leading to secondary ions (observed ions, in particular analyte
ions. The ions are then accelerated and extracted into the field free region of the mass spectrometer. Since the typical ion-to-neutral ratio in the plume is \(10^{-4}\), the detected ions are minority species. Primary ionization processes involve multiphoton ionization, energy pooling, excited state proton transfer, disproportionation reactions, preformation, and thermal ionization. The reactions producing secondary ions include proton transfer, cationization and electron transfer [44].

The charge separation in a matrix molecule and in a NaCl crystal to produce protons and metal ions for protonation and cationization in the vacuum requires 14 eV and 8.4 eV, respectively. It is suggested that ionization processes in the MALDI plume initiate charge formation and separation processes, and that collisions with neutrals in the expanding plume may finish charge separation.

### 2.6.1 Primary Ion Formation

Multiphoton ionization to form matrix radical cations has long been considered as a possible mechanism for UV MALDI primary ionization [45]. Today, this process is assumed to be rather unlikely since values of ionization potentials for some matrices are known. It would require simultaneous deposition of three nitrogen laser photons (3.7 eV each) to ionize the common UV-absorbing matrix DHB (ionization potential: 8.05 eV [46]).

It is known that excited states of matrix molecules play an important role in UV MALDI [47-50]. One suggested ionization mechanism is energy pooling in which two or more separately excited matrix molecules pool their energy to yield one matrix radical cation. This multicenter model takes into account that MALDI is a collective phenomenon which is demonstrated by the existence of clusters as well as strong interactions between closely packed chromophores. The multicenter ionization is strongly supported by the matrix suppression effect reported by Knochenmuss et al. [1]. This effect describes the observation that no matrix signals are observed in the MALDI mass spectra below a certain matrix-to-analyte ratio. Matrix suppression occurs if all excited matrix molecules are in close proximity to analyte molecules. Only analyte ions are then formed. The molar ratio of analyte to matrix under matrix suppression conditions depends on the size and concentration of the analyte. Experimental findings were consistent with the theoretical predictions.

Excited-state proton transfer (ESPT) is a frequently proposed MALDI ionization mechanism [51, 52]. A single excited matrix molecule is suggested to become much more acidic than in its ground state, and analyte or matrix molecules in its
proximity then accept the labile proton. This model is favored since it requires only one nitrogen laser photon for ion formation. However, none of the commonly used matrices are known to exhibit a particular ESPT activity and none of the traditional ESPT compounds generally functioned well as MALDI matrix.

A MALDI matrix often performs well in positive and negative ion mode. This suggests the possibility that disproportionation of an excited matrix pair forms deprotonated and protonated or oxidized and reduced matrix ions. Only two photons are required for these processes. For example, the energetics for the proton disproportionation reaction can be calculated from recent data on gas-phase basicities of matrix neutrals and anions determined by Taft and Topsom [53] and Breuker et al. [54]. Nevertheless, little experimental evidence for the disproportionation mechanism is currently available.

Another attractive possibility for primary ionization in MALDI is the existence of preformed ions. In this mechanism, ions observed in the MALDI spectrum are already present in the solid sample prior to desorption and are liberated by the laser pulse. The charge is located on a large macromolecular molecule in the solid phase. As mentioned above, charge separation for such a case is thermodynamically rather favorable and laser energy requirements for desorption are relatively low. This possibility has not been investigated extensively up to now and is the topic of Part A of this thesis. It is discussed in great detail in chapter 4.

A thermal ionization mechanism is very probable in two-phase MALDI. The liquid matrix containing the analyte is generally transparent to the laser radiation which is absorbed by the particulates. The extent of ionization, $\alpha$, assuming thermal equilibrium between all degrees of freedom, is given by the Saha-Langmuir equation [55]:

$$\alpha = C \cdot \exp((\phi - \text{IP})/kT)$$

where $C$ is a constant near unity, $\phi$ is the work function of the particle surface, $T$ is the temperature of the particle surface, and IP is the ionization potential of the liquid matrix. According to this equation, the extent of ionization increases with the temperature and the work function of the particle surface. Peak temperatures of 700 to 900 K are reached in a two-phase MALDI experiment using a 2 $\mu$m graphite/glycerol mixture. Smaller particulates lead to higher peak temperatures and thus a higher ion yield. Schürenberg [23, 24] estimated the peak temperatures of 35 nm particles to be above 10 000 K. Nitrides especially have a very
high work function and TiN is a particularly good two-phase particulate material. Even relatively large TiN particulates give a high ion yield. In contrast to two-phase MALDI, temperatures of ~500 K are reached in a MALDI plume desorbed from a solid matrix preparation [42]. A thermal ionization mechanism at this temperature is clearly not significant. However, temperatures of up to 3000 K are measured in laser desorption experiments without matrix. Due to the thermal ionization mechanism involved in two-phase MALDI as well as in laser desorption without matrix, higher laser fluences are required for these experiments than for MALDI experiments with solid matrices.

2.6.2 Secondary Ion Formation

Proton transfer in the gas phase may occur between two matrix molecules or between a matrix and an analyte molecule. For example, experimental evidence for proton transfer between matrix and analyte was found by Bökelmann et al. [56]. The authors investigated ion angular and velocity distributions in the MALDI plume and found depletion of matrix ions in high density areas of the protonated analyte species.

Gas-phase cationization has been suggested by many authors as an important secondary ionization mechanism. The cationization of polystyrene which is extensively discussed in Part A, chapter 4 of this thesis follows this mechanism.

Electron transfer reactions are proposed as a secondary mechanism for MALDI analysis of nonpolar compounds with ionization potentials lower than that of the matrix [57]. In this mechanism, a matrix radical cation transfers an electron to the analyte to form an analyte radical cation. This mechanism is not expected to occur very often since ionization potentials for typical UV MALDI matrices are relatively low.

Ionization in MALDI is most probably not the result of one single mechanism, but several mechanisms may play a role. Depending on the physical properties of the matrix and the analyte, some mechanisms may be favored over others. Knowledge on MALDI ionization mechanism leads to a better control of the experiment, especially in terms of fragmentation, ion yield, and matrix selection. For example, oligonucleotide fragmentation in MALDI has been suggested to result from a protonated base. The use of matrices with high proton affinities should therefore reduce fragmentation. Another example is the chemical derivatization of analytes with a charge tag to yield preformed ions. These preformed ions are detected with a higher ion yield than the nonderivatized counterparts [44].
2.7 TOF Equation and Mass Calibration

In a TOF mass spectrometer, ions of different m/z ratios are accelerated by a constant electric field to the same kinetic energy, and are then dispersed in time in a field-free region. The relationship between time of flight and m/z can be derived as follows.

The total voltage U accelerates an ion with m/z to a kinetic energy E of

\[ E = \frac{1}{2} m v^2 = z e U \]

where \( m \) is the mass of the ion, \( z \) the number of charges, \( e \) the elemental charge, and \( v \) the ion velocity in the flight tube.

The velocity is defined as

\[ v = \frac{l}{t} \]

where \( l \) is the length of the flight tube and \( t \) the time needed to cross this length.

Using these equations, the following relationship between the m/z ratio of an ion and its TOF is obtained:

\[ m/z = 2 U e t^2/l^2 \]

In reality, the relationship is more complicated and has the form [58]:

\[ m/z = a t^2 + b t + c \]

This is due to the fact that the acceleration path is not infinitely small but has a defined length. Thus, immediately after their formation, the ions do not have the velocity \( v \) yet. In practise, calibration allows determination of the constants \( a \), \( b \), and \( c \) and transformation of the TOF spectrum into a mass spectrum.

The problem with mass calibration is that it is often difficult to obtain calibration samples of exactly known mass and high purity. Adduct formation of the calibrant with matrix molecules and cations may falsify the calibration and limit mass accuracy. The use of internal instead of external calibrants generally improves mass accuracy.
2.8 Performance Characteristics of MALDI TOF Mass Spectrometry

2.8.1 Mass Resolution
Mass resolution is defined as $m/\Delta m$, with $m$ the ion mass and $\Delta m$ the full width at half maximum (FWHM) of the ion signal. It depends on instrument characteristics as well as on the ion formation process. The former is described in the following, section 4.1.2 deals with the latter.

The ions in a TOF analyzer may have a temporal, spatial, and/or initial kinetic energy distribution. These can be corrected with instrument set-ups using time-lag-focusing [59] (time delay between laser firing/ion formation and ion extraction), dual-stage acceleration, or ion reflection.

2.8.2 Mass Range
The highest masses detected by MALDI so far are 700 kDa for nucleic acids [60] and 1 million Da for polystyrene samples [61]. The upper mass limit is not known, but it is probably not so much determined by the desorption/ionization processes, but rather by ion detection. Large ions are much slower than light ions. They have a lower impact velocity on the detector and therefore a low ion-to-electron conversion [62]. Multichannel plate (MCP) detectors in a TOF instrument exhibit a decrease of signal intensity with $m/z$. Work on mass independent or less mass dependent detectors is currently in progress [63].

2.8.3 Sensitivity
Usually no more than 1 pmol of analyte is used for a MALDI sample preparation since the analyte is very much diluted in the matrix. One of the highest sensitivities so far was demonstrated by Kerstin Strupat from the Hillenkamp group [64]. She showed in her diploma thesis that 1 fmol of cytochrome c yielded 100 mass spectra. The sample consumed for one spectrum was 10 attomol. This amount could not be determined more accurately, but it is suggested that the actual amount consumed is 1 to 2 orders of magnitude lower. The high shot-to-shot stability in MALDI suggests that the laser radiation does not cause any radiation damage. At least in principle, the remaining sample could be retrieved for further use.
2.9 References


3 Experimental Set-up of the MALDI TOF Mass Spectrometer

3.1 Introduction

The experiments described in this thesis were done on two linear home-built MALDI TOF mass spectrometers without delayed ion extraction. One, in room C61, was used for the experiments described in Part A and for part of the experiments in static mode in Part B. The investigations using CF mode in Part B were done on the instrument in room B55, because the sample introduction system on this instrument is more adequate for the coupling with liquid separation techniques. The C61 mass spectrometer is described first. Due to similarities of the instruments, the subsequent section only deals briefly with the differences of the B55 instrument. A schematic view of both instruments is shown in Figure 2.

Figure 2. Schematic set-up of both MALDI TOF mass spectrometers used.
3.2 Room C61 MALDI TOF Mass Spectrometer

This mass spectrometer can be divided into four parts: sample introduction system and ion optics, TOF tube with pumps, nitrogen laser with optics to focus the laser beam, and ion detection with subsequent data acquisition.

The sample holder is made of insulating plexiglass. The probe tip, made of stainless steel, is screwed into it (Figure 3). This holder is introduced via a two-stage pumping system into the ion optics of the mass spectrometer. The ion optics for two-stage acceleration consists of three cylindrical electrodes made of stainless steel: repeller, extractor and ground plate with high voltages of +26, +21 kV (30 kV power supplies, model 2341, Bertan High Voltage, NY, USA) and ground potential, respectively. Additional plates are installed for the deflection of undesired ions. The probe tip fits exactly into the repeller plate in a manner such that the tip desorption surface is flush with the repeller surface on the extractor side. The set-up of the sample holder permits the probe tip to be put at the same potential as the repeller while insulating it from the rod for sample introduction. Extractor and ground plates have holes in the middle for ion extraction from the ion source. The electrodes are insulated from each other through distance pieces made of ceramics and are connected to the flange of the TOF tube via four insulating rods made of polyoxymethylene homopolymer (Delrin). This set-up allows easy removal of the ion optics for cleaning purposes or troubleshooting.
The TOF tube (length of 2 m), can be divided into two chambers via a valve. A vacuum of $5 \times 10^{-7}$ mbar is maintained by means of two turbomolecular and two mechanical pumps. The vacuum is necessary to eliminate background gas which would lead to loss of ions through collisions. The valve between the chambers is closed during sample introduction, allowing the vacuum to be maintained in the second chamber while only the first chamber has to be pumped subsequently. The turbomolecular pump connected to the first ("ionization" chamber) has recently been changed to a diffusion pump which is more tolerant to particle contamination resulting from the use of two-phase MALDI matrices. A liquid nitrogen trap is used to condense remaining water molecules and to improve the vacuum.

The nitrogen laser beam (pulse width 3 ns, energy per pulse max. 250 µJ, wavelength 337 nm, model VSL-337ND-T, Laser Science Inc., MA, USA) is focused onto the sample in the ion optics via three mirrors and a lens (focal length 47 cm). The incident angle is ca. 60° to the surface normal. The laser power is attenuated by glass plates and an adjustable iris. The energy after attenuation was measured with a pyroelectric detector (model ED 100, Gentec, Quebec, Canada). Laser pulse energies were in the range of 20 µJ to 40 µJ for solid matrices and 30 to 60 µJ for two-phase matrices. A photodiode using the fraction of the laser beam which is transmitted by the first mirror serves as a trigger for the oscilloscope.

The detector consists of two multichannel plates (MCPs, models S40-10-D and E40-10-D, Scientific Instruments, Gilching, Germany). A MCP plate contains $10^4$ to $10^7$ parallel channels made of specially treated lead glass with semiconducting properties. Metal coatings on the upper and lower sides of the plates assure electrical contacts [1]. MCPs transform the ion current into an electron current: an ion which hits the detector frees an electron, which is accelerated by the electrical field applied to the detector (3.5 kV, max 5 kV). Successive collisions with the channel walls create an electron avalanche, resulting in an electron current which is amplified by a factor of $10^8$. The current is again amplified by a preamplifier (bipolar low noise amplifier, model 322-6, Laser 2000, Wessling, Germany) and is then monitored by the digital oscilloscope (model 9420, LeCroy, MA, USA; 350 MHz) as a function of time. The MCPs are fixed on the TOF tube’s back flange, which permits easy detector replacement. MCPs are not supposed to be used at pressures above $10^{-6}$ mbar since collisions with the background gas may perturb the detection process. MCP detectors are very sensitive owing to the high ion-to-electron conversion rate, which is,
however, mass dependent: large ions hit the detector with a smaller kinetic energy and create a smaller current and therefore a smaller ion signal is generated than for small ions. The time resolution of MCPs is in the nanosecond range. After receiving the "start" signal from the trigger, the digital oscilloscope monitors the electron current (converted to a voltage in the oscilloscope) as a function of time. For each laser shot, a complete spectrum is acquired and transferred via a general purpose interface bus (GPIB) using a GPIB card (NI-488.2, model AT-GPIB/TNT, National Instruments Corp. TX, USA) to a 486 PC. The MALDI TOF software from Hewlett-Packard sums up single spectra to a total spectrum, which can then be exported to other programs for further modification. Using the previously determined calibration constants a, b and c, the TOF spectrum can be transformed into a mass spectrum (voltage as a function of m/z). Addition of several single shot spectra increases the signal-to-noise ratio since the noise only increases with the square root of the number of spectra taken. If not mentioned otherwise, spectra in this thesis are taken from 100 shots in positive ion mode.

### 3.3 Room B55 MALDI TOF Mass Spectrometer

A heating set-up is fixed on the repeller plate to prevent freezing of the solvent which expands into the vacuum during CF experiments on the B55 instrument. The set-up is described in detail in Part B. Since the ion optics are heated, the rods on which the ion optics are fixed are made of polyvinyliden fluoride (PVDF). Besides its excellent properties for minimizing leakage currents it is also stable for temperatures up to 150 °C. Delrin used in the C61 instrument is only stable up to 100 °C. The distance pieces between the electrodes are made of ceramics or polyether-etherketone (PEEK, maximum temperature 200 °C). The electrodes are made of aluminum. A picture of the ion optics of the B55 instrument is shown in Figure 4.

The TOF tube of the B55 instrument has a length of 2.2 m. In the static mode, a pressure of 4·10^{-6} mbar is maintained in the vacuum chamber. This pressure in the B55 chamber is higher than that in the C61 instrument because the valve through which the sample is introduced is not as well-sealed. During CF experiments, the pressure is about one order of magnitude higher, depending on the flow rate into the mass spectrometer.

The incident angle of the laser beam is only 40° to the surface normal, compared to 60° in the C61 instrument. In order to realize this steeper angle, one of the
optic's mirrors is installed inside the vacuum chamber and holes are drilled into the extractor and ground plates. The laser power is adjusted with an attenuator and an iris.

The voltage applied to the detector is 2.7 kV (max 4.5 kV). The digital oscilloscope (model 9450, LeCroy, MA, USA; 500 MHz) is utilized at sampling rates of 250 to 500 MHz. The GPIB card is a CEC-488 (model PC488, 8-bit ISA, Capital Equipment Corp., MA, USA) and the TOFWARE program (Ilyys software, Pittsburgh, PA, USA) is used for data acquisition.

The resolution of both instruments is 300 to 400 for m/z ratios smaller than 1500 Da and ca. 50 to 100 for m/z ratios larger than 10 kDa.
3.4 High Resolution Mass Spectrometry

For an experiment testing the mechanism of charge compensation in the singly charged copper-polystyrene adducts (see section 4.5.4), a mass resolution exceeding the one of a TOF instrument was needed. For this purpose, the spectrum was recorded on a FTICR mass spectrometer, consisting of a 4.7 Tesla superconducting magnet (Bruker, Fällanden, Switzerland), a cylindrical cell, and a workstation-based data acquisition system (Odyssey, Finnigan/Extrel FTMS, Madison, WI, USA). The sample was irradiated with 355 nm from a frequency-tripled Nd:YAG laser (Continuum, Model Surelite II, 5 ns pulse width). The working pressure was below $1 \times 10^{-8}$ mbar.

3.5 References

1. Wiza, J. L. Product Description, Galileo Electro-Optics Corporation, Sturbridge, Massachusetts, USA.
PART A

DO MALDI MASS SPECTRA REFLECT CONDENSED-PHASE CHEMISTRY?
4 Ionization Mechanisms in MALDI: Preformed Ions vs. Gas-Phase Cations

In this chapter, the term "gas-phase reaction" as opposed to "preformation of ions" generally refers to post-desorption processes, without specifying the exact place and time after the desorption event, and the exact ion formation mechanism. "Preformed ions" refer to ions already present in the solid sample and released into the gas phase upon desorption.

4.1 Introduction

4.1.1 Ion Formation Before and After Laser Desorption

As was demonstrated in chapter 2 when presenting possible mechanisms for ion formation, MALDI studies have mainly focused on post-desorption reactions to date [3-7]. For example, Wang et al. [3] suggested gas-phase formation of the cationized and protonated gramicidin S molecules based on their observations. They studied the influence of delayed ion extraction on the formation of protonated and cationized species. The authors found that the amount of these species increases with delay time due to the increasing probability of ion-molecule reactions. The metal ions were provided by irradiating neat NaI to produce a plume of sodium ions before the desorption laser was fired.

Only a few experimental studies on preformed ions have been performed. Liao et al. [8] found that under the conditions of their experiment, peptides which had been derivatized with a charged triphenylphosphonium group yielded a signal. This was not the case for underivatized peptides. The authors attributed this to the fact that derivatized peptides are already ionized in the solid and only have to be desorbed, which decreases the laser energy requirement.

Hutchens et al. [9, 10] evaluated the metal binding properties of a histidine-rich peptide. These authors report the capability of MALDI MS to determine the peptide-metal binding capacity which agreed with the results from direct titration in solution. The absence of Mn²⁺- and Co²⁺- but presence of Cu²⁺-peptide complex signals in MALDI spectra also demonstrated that specific metal ion-peptide interactions can be detected with MALDI MS. Further evidence for the ability of MALDI MS to probe solution-phase binding and the presence of preformed ions was given by Nelson and Hutchens [11] who worked with the
same histidine-rich peptide. After washing a sample containing matrix, peptide, and metal salt with water, copper-peptide adducts were still observed in the MALDI spectra, whereas sodium and potassium adducts disappeared. This observation was attributed to the fact that the copper adducts already exist in solution, but not the sodium and potassium adducts.

Hornshaw and Hutchens [12] presented further experiments demonstrating the liberation of preformed ions. Matrix containing a metal-peptide complex with an excess of free metal was deposited next to matrix containing another peptide without metal. Irradiation of a region containing both types of crystals did not lead to the pick-up of the metal by the second peptide. Nevertheless, some doubt remained about efficient mixing in the plume which may depend on the size of the crystals.

Dubois et al. [13] studied the effect of salt addition on the MALDI spectra of proteins from cow milk. At the mass of the proteins, metal ion adducts could not be resolved. When investigating smaller matrix molecules, they obtained experimental evidence that adducts between matrix and calcium may already be preformed in the crystal. The authors found that the extent to which these adducts are formed strongly depends on the solution pH and on the solvent. High pH values and polar solvents increase adduct formation. To rationalize the result, the following explanation was given. The observed clusters contain deprotonated DHB. Under basic pH conditions, preferential formation of this species is observed, leading to the presence of the calcium-(DHB-H) adducts in the solid crystal, which are simply desorbed by the laser shot. Apolar solvents and acidic pH values are considered as optimum conditions for observing few adduct formation of large biomolecules in the presence of salt.

Ehring et al. [14] compared front-side and back-side laser illumination of samples deposited on a 200 nm gold film. With back-side illumination, photochemical processes are not possible, yet small cationized peptides such as gramicidin were observed. This was interpreted as thermal desorption of preformed ions.

### 4.1.2 Undesired Adduct Formation and Desired Cationization Using Metal Ions

Shaler et al. [15] investigated the effect of impurities on the MALDI spectra of oligodeoxynucleotides. This class of molecules is easily detectable as protonated species in the MALDI spectra. Addition of alkali or alkali earth ions led to a
decrease of mass resolution and accuracy, which resulted from multiple adduct formation between the oligodeoxynucleotide and metal ions. Addition of metal-chelating agents, such as ethylenediamine tetraacetic acid (EDTA), did not completely restore the initial spectra before salt addition, even if an excess of EDTA was used. After salt addition, Dubois et al. [13] (see chapter 4.1.1) also observed poor mass resolution and accuracy for their proteins from cow milk, because metal ion adducts could not be resolved.

Apolar polymers, such as polystyrene, are preferentially observed as cationized and not as protonated species in the MALDI mass spectra. Scivener et al. [16] described MALDI cationization experiments of polystyrene with various transition metal ions. The authors quoted the transition metals’ d-orbitals interacting with the π-system in polystyrene as well as their ability to form singly charged ions as a reason why Ag⁺ and Cu⁺/2⁺ form polystyrene adducts. However, they did not check whether singly charged polymer adducts form as a result of singly charged cations or whether another mechanism such as proton loss in polystyrene takes place.

Llenes and O’Malley [17] performed LD experiments with polystyrene and cationizing agents including transition metal salts and alkali metal salts. They, as well as Thomson et al. [18], evoked the principle of hard and soft acids and bases (HSAB) of Pearson to account for Ag⁺ (soft acid) forming adducts and for alkali metal ions (hard acids) not forming adducts with the benzene ring (soft base) in polystyrene. As they state correctly, there is no quantification possible using this principle. Therefore, only differences between the spectra of transition metal and alkali metal ions were discussed; small differences between the transition metal ions could not be discussed on the basis of HSAB. The authors also compared experiments using transition metal ions in the oxidation state I with those using ions in a higher oxidation state. In the latter case, higher laser powers were needed and intensities were lower than in the former case. This observation was attributed to the necessity of reducing ions to oxidation state I with electrons desorbed by the laser beam. However, their conclusion is not necessarily true. The higher laser power could also be caused by the abstraction of polystyrene protons to compensate the higher charges. Furthermore, two different laser desorption techniques were compared. In one experiment, the sample holder was made of the desired metal, which desorbed as ions at high laser powers. In this case, the cationized analyte molecules result from gas-phase reactions. In another experiment, the metal ion in oxidation state I was added as a salt before laser desorption. This technique required a lower laser power resulting in a better resolution of the analyte signal.
case, the cationized analyte signals may also be preformed in the solid phase. When LD and MALDI experiments with cations in oxidation state II and III were compared, a remarkable phenomenon was observed. Whereas the LD studies done with Cu(II), Cr(III), Fe(II), Fe(III) [17] and Cr(II) [19] all resulted in a singly charged polystyrene distribution, only Cu(II) was successfully applied among MALDI experiments using Pb(II), Cr(II), Fe(II), Fe(III), Mn(II), Zn(II) and Cu(II) as cationizing agents [16].

These statements clearly show that addition of metal salts has an enormous influence on MALDI mass spectra. In some cases, it leads to multiple adduct formation with a loss of resolution and mass accuracy. In other cases, the ion yield can be improved significantly by addition of metal salts. The statements also indicate that there is no uniform picture on MALDI cationization of polystyrene. It is widely assumed that cation attachment takes place in the gas phase, but little experimental evidence exists to support this statement.

The purpose of this study was to investigate the contribution of preformed ions to the ion signal observed in MALDI. We were especially interested in predicting this contribution based on possible correlations between the chemistry in the condensed phase and in the MALDI mass spectrum. An approach with coordination chemistry as the basis for cationization in MALDI has never been taken before, although it is known that MALDI matrices form metal ion-mediated adducts with certain analytes, often resulting in degraded mass resolution and accuracy [13]. As the complexing ability of matrices has not been studied systematically, we investigated the question of preformed ions by studying matrix complexation using model systems in the solution, in the solid, and in the gas phase. Following the study on preformed ions, the cationization mechanism of polystyrene is further investigated at the end of this chapter.

4.1.3 Systems Investigated

The following chemical systems were investigated: Ni\(^{2+}\) and Co\(^{2+}\) were chosen as coordination centers because transition metal ions are potential MALDI cationizing agents. Ortho-phenanthroline (ophen) and 2,2'-bipyridine (bpy) are models for heteroaromatic (bio-)molecules and served as bidentate nitrogen ligands for the transition metal ions, forming the octahedral complexes [Co(bpy)\(_3\)]Cl\(_2\) and [Ni(ophen)\(_3\)]Cl\(_2\). Ni\(^{2+}\) is more strongly bound to ophen than Co\(^{2+}\) to bpy [20]. 2,5-dihydroxybenzoic acid (DHB) was chosen as a MALDI matrix. Its complexation behavior in the condensed phase is not cited in the literature. However, it is assumed to be similar to that of 2-hydroxybenzoic acid,
reported by Perrin [21]. Deprotonated DHB, [DHB-H]–, is therefore expected to complex with transition metal ions as a bidentate oxygen ligand, which can compete with the nitrogen ligands for the metal ions. We investigated ligand exchange reactions between DHB and ophen or bpy in the transition metal complexes [Co(bpy)3]Cl2 and [Ni(ophen)3]Cl2.

In addition to these model systems, the question of preformed ions in MALDI was also investigated in a system including an analyte, namely polystyrene (PS), using dithranol (1,8,9-anthracenetriol) as a matrix and Cu(TFA)2H2O as cationizing agent. Besides 1,4-bis(5-phenyloxazol-2-yl) benzene (POPOP) [22], dithranol was found to be the most effective matrix for polystyrene [18, 23, 24]. Other matrices were, however, also investigated in the present work and gave some insight into the ionization mechanism.

4.1.4 Methods Used
Solutions were examined by visible spectrophotometry and, in the case of [Ni(ophen)3]Cl2, with proton nuclear magnetic resonance (1H NMR) spectroscopy. Characterization of the solid state was done by infrared spectroscopy, whilst gas-phase chemistry was monitored by MALDI MS.

Visible Spectrophotometry
Visible spectrophotometry is suited to investigate ligand exchange because nitrogen ligands create a different ligand field than the oxygen ligand [DHB-H]–, resulting in the appearance of new bands or a band shift in the electronic absorption spectra of the transition metal ions. The fundamentals of this widely used method are described in various publications [25-27].
$^1$H NMR Spectroscopy of Paramagnetic Compounds

Ni$^{2+}$ is a paramagnetic metal ion (3d$^8$ configuration). There is a strong interaction between the nuclear spin of the ligand protons and the spin of the metal ion’s unpaired electrons leading to a strong shift and broadening of the signals in the $^1$H NMR spectrum of the ligand, indicating a complexation of the ligand to Ni$^{2+}$. If there is an excess of ligand, then unshifted, fine signals of the uncomplexed ligand are observed additionally. The fundamentals of NMR spectroscopy of paramagnetic compounds are extensively described in the book of La Mar et al. [28]. A summary of the most important features of this method can be found in my diploma thesis [1].

Infrared Spectrophotometry

Metal-ion-bound [DHB-H]$^-$ is characterized by other IR bands than free DHB, allowing a distinction of both species in the IR experiment of solid samples. The fundamentals of the widely used IR spectrophotometry are described in the books of Ebsworth [29] and Wedler [30].
4.2 Experimental

4.2.1 Materials
Polystyrene 5000 standard, tetrahydrofuran (THF), diethylether, ethanol, DHB, bpy, ophen monohydrate (ophen·H₂O), silver(I) trifluoroacetate (Ag(TFA)), benzene, trifluoroacetic acid (TFA), and pH indicator strips (pH 1-11) were purchased from Fluka Chemie AG (Buchs, Switzerland). Polystyrene 2500 standard, dithranol, copper(II) trifluoroacetate monohydrate (Cu(TFA)₂·H₂O), and copper(I) chloride (CuCl) were obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). Acetonitrile, sodium hydrogen carbonate (NaHCO₃), and sodium (Na) were purchased from Merck (Darmstadt, Germany). Nickel(II) acetate tetrahydrate (Ni(ac)₂·4H₂O), nickel(II) chloride hexahydrate (NiCl₂·6H₂O), and copper(II) chloride dihydrate (CuCl₂·2H₂O) were obtained from Siegfried Handel (Zofingen, Switzerland). Substance P and melittin were from Sigma Chemie (Buchs, Switzerland). Pyrene was purchased from Chem Service Inc. (West Chester, PA, USA). KBr powder was obtained from single crystals grown in our laboratory. Deuterated water (D₂O) and deuterated methanol (CD₃OD) were acquired from Glaser AG (Basel, Switzerland). All materials obtained from commercial sources were used without further treatment or purification. If not otherwise mentioned they were of highest purity available, and solvents were of spectrophotometric grade. Aqueous solutions were prepared using bidistilled water.

4.2.2 Spectroscopic Measurements

Visible Spectrophotometry
The spectra were taken on a commercial double beam spectrophotometer (model Uvikon 940, Kontron Instruments, Switzerland). The preparation of the transition metal ion solutions is described below. Polystyrene 2500, dithranol and Cu(TFA)₂·H₂O were dissolved in THF. Concentrations and molar ratios of the solutions are indicated in the figure captions.

1H NMR Spectroscopy
This method was used for the investigation of [Ni(ophen)₃]Cl₂ in solution. 1H NMR samples were measured on a 300 MHz spectrometer (model Gemini 300, Varian, MA, USA) equipped with a 7 Tesla magnet. CD₃OD was used as a solvent.
40 4 Ionization Mechanisms in MALDI

**Infrared Spectrophotometry**

The measurements were performed on a single beam Fourier-transform IR spectrophotometer (model 1600, Perkin-Elmer, CT, USA). 0.5 to 1 mg of the ground solid sample was thoroughly mixed in a mortar with KBr and pressed into a pellet under vacuum.

### 4.2.3 Syntheses and MALDI Sample Preparation

**Transition Metal Complex Solutions**

Solutions of \([\text{Co(bpy)}_3]\)Cl₂ and \([\text{Ni(ophen)}_3]\)Cl₂ were prepared according to Kauffman and Takahashi [31], yielding a red \([\text{Ni(ophen)}_3]\)Cl₂ and a yellow-brown \([\text{Co(bpy)}_3]\)Cl₂ solution. For the MALDI, IR, and visible analyses, water was used as a solvent for \([\text{Co(bpy)}_3]\)Cl₂, and a mixture of acetonitrile and water (1:2, v:v) was used to prepare \([\text{Ni(ophen)}_3]\)Cl₂. The pH of the solutions was adjusted with NaHCO₃ and TFA. \([\text{Ni(DHB-H)}_2\cdot2\text{H₂O}]\) was prepared according to the following equation:

\[
\text{Ni(ac)₂.4H₂O} + 2\text{DHB} \iff \text{Ni(DHB-H)₂.2H₂O} + 2\text{Hac} + 2\text{H₂O}
\]

DHB (5 mmol) was dissolved in 50 mL of water, and a 0.5 molar equivalent of \([\text{Ni(ac)}_2\cdot4\text{H₂O}]\) dissolved in 30 mL of water was added. The azeotrope water/acetic acid was distilled off with 75 % of the solution, and the rest of the mixture was left to cooler. The excess of DHB was eliminated by two extractions with 5 mL of diethylether. After partial removal of the solvent on the rotary evaporator, the product precipitated overnight. It was recrystallized from water, filtered and dried under vacuum. The yield was ~31 %. Synthesis and purification of \([\text{Cu(dithranol-H)}_2]\) were performed according to Van Duuren et al. [32].

For the \(^1\text{H} \)NMR study, the crystal water of the reactants \([\text{NiCl}_2\cdot6\text{H₂O}]\) and \([\text{ophen-H₂O}]\) would have disturbed the measurement. Therefore it was exchanged using D₂O, and the product was dried for several hours under vacuum. The dried product is very hygroscopic and was immediately dissolved under nitrogen atmosphere in an appropriate volume of CD₃OD to yield a concentration of 1 M. The same procedure was applied for the preparation of a 0.1 M solution of dried ophen, with the only difference that CD₃OD with a few drops of benzene was used to eliminate the crystal water. \([\text{Ni(ophen)}_3]\)Cl₂ was then synthesized from these solutions. DHB was used as a 0.1 M solution. A 2 M solution of sodium deuterio-methylate Na(OCD₃) was used as a base to deprotonate DHB. For the
preparation of Na(OCD$_3$)$_2$, elemental sodium was cautiously added in 1:1 molar ratio to CD$_3$OD under a nitrogen atmosphere.

**Solids**

The solids were prepared by evaporating the corresponding solutions on the rotary evaporator. Solid [Co(bpy)$_3$]Cl$_2$ and [Ni(ophen)$_3$]Cl$_2$ samples used for the MALDI and IR analyses were prepared as solid mixtures of the complex and DHB, as well as cocrystallized from solutions containing both complex and DHB.

Similarly, for the study of the copper/dithranol system, solid mixtures of Cu(TFA)$_2$H$_2$O and dithranol, as well as the solid cocrystallized from a solution containing both components were investigated. In the copper/polystyrene 2500 system, dithranol and polystyrene were always cocrystallized. Again, solid mixtures of the polystyrene/dithranol cocrystal and Cu(TFA)$_2$H$_2$O, as well as a solid cocrystallized from a solution containing all three components, were prepared. The molar ratios used for the different components are indicated in the figure captions.

**MALDI Sample Preparation**

For the analysis of the [Co(bpy)$_3$]Cl$_2$ and [Ni(ophen)$_3$]Cl$_2$ transition metal complexes, DHB was used as a matrix. Aqueous complex (10 mM) and aqueous DHB solutions (100 mM) were mixed to yield the molar ratios indicated in the figure captions. 1-2 µL of the resulting solution were deposited on the probe tip, partially dried in a warm air stream, and then completely dried in the vacuum. This fast evaporation resulted in a microcrystalline, optically homogeneous sample layer over the whole probe tip. NaHCO$_3$ and TFA were used as base and acid in order to vary the pH value of the sample solutions.

Solutions of polystyrene 2500 (3.7 mM) and dithranol (53 mM) were prepared in THF. Solutions of the different cationizing agents (2.4 mM) were prepared with the following solvents. Ag(TFA) and Cu(TFA)$_2$H$_2$O were dissolved in water and THF. Acetonitrile was used as a solvent for CuCl. CuCl$_2$·2H$_2$O was dissolved in water. Fresh solutions of matrix, polystyrene and metal salts were prepared daily. 200 µL of the dithranol solution were mixed with 25 µL of the polystyrene solution. Typically, 1-2 µL of this mixture were deposited on the probe tip and allowed to air dry. An equal amount of the cationizing agent solution was deposited on top and also dried. This order of preparation steps was sometimes reversed. The procedure resulted in a molar ratio of polystyrene 2500:dithranol:
cationizing agent of 1:114:5.7. For the analysis of polystyrene 5000, doubly concentrated polystyrene and matrix solutions in THF were used, resulting in a molar ratio of polystyrene 5000:dithranol:cationizing agent of 1:114:2.9. This layering method allows the use of different solvents for the cationizing agent and the matrix/analyte mixture [16]. Solutions of cationizing agents in volatile solvents were air dried, aqueous solutions were partially dried in a warm air stream, and then completely dried in the vacuum. Inspection under an optical microscope showed that this fast evaporation resulted in an evenly distributed metal salt layer covering the whole probe tip rather than selective crystallization at edges, scratches, etc. Additionally, for cationizing agents soluble in THF, the following preparation technique was applied. Polystyrene, dithranol and metal salt solutions were mixed prior to deposition onto the sample holder in the same molar ratio as indicated above.

For the analysis of solid samples, double-sided adhesive tape was used on the probe tip. The tip was dipped into the sample, the sample was further pressed with a spatula and then the nonsticking material was shaken off. Since the use of this preparation technique can lead to contamination of the MALDI ion source and can damage the turbomolecular pumps, we used a diffusion pump instead in the desorption chamber. Low laser powers were used to minimize the amount of particles ejected from the sample.
4.3 Ligand Exchange in \([\text{Co(bpy)}_3]\text{Cl}_2\)

Figure 5 shows MALDI mass spectra of \([\text{Co(bpy)}_3]\text{Cl}_2\) with DHB as a matrix.

**Figure 5.** MALDI mass spectra of \([\text{Co(bpy)}_3]\text{Cl}_2\) with DHB as a matrix in 1:1000 molar ratio at different pH. Different cluster signals observed are marked with stars, crosses and circles. Each signal is a multiplet and corresponds to mixed complexes of cobalt with varying ratios of bpy and DHB (the mass difference between DHB and bpy is only 2 Da). These complexes are summarized with the notation \(',\text{bpy/DHB}\)' . The repeat unit within a given cluster series corresponds to \([\text{Co(DHB-2H)}]^{+}\) with a mass of 211 Da. The data were normalized to the lowest mass starred cluster signal. For a given cluster signal observed in spectra A. and B., one additionally observes different sodium adducts (from NaHCO₃) of this cluster in C., i.e. the intensity is distributed over several signals. The relative cluster intensities were not influenced by the laser power.

In the middle panel (Figure 5B, unmodified DHB matrix), many peaks have been assigned. No signal corresponding to the 1:3 complex between \(\text{Co}^{2+}\) and bpy was observed. Instead, three cluster series were identified. The cluster signals are
multiplets representing mixed complexes of cobalt with varying ratios of bpy and DHB. Free [bpy+H]+ stemming from the exchange between bpy and DHB is also observed. The repeat unit in a given cluster series always corresponds to neutral [Co(DHB-2H)]. Cluster ions are all singly charged. The excess positive charge of Co2+ in the cluster series marked with stars and circles are compensated by deprotonation of a corresponding number of DHB molecules. These observations agree with the results of Dubois et al. [13]. They found an analogous repeat unit, as well as deprotonation of DHB in clusters formed with Ca2+. The observed mechanism of charge compensation by deprotonation of DHB is also consistent with the complexation behavior of DHB [21]. In the cluster series marked with crosses, the excess charge is compensated by the addition of chloride ions.

This MALDI experiment with [Co(bpy)3]Cl2 revealed an exchange of DHB and bpy. Clearly, it is important to know if this exchange takes place in the condensed phase or in the MALDI plume.

The first ligand exchange reaction between DHB and bpy in solution is:

\[
[\text{Co(bpy)}_3]^{2+} + \text{DHB} \rightleftharpoons [\text{Co(DHB-H)(bpy)}_2]^+ + \text{bpy} + \text{H}^+ \quad (1)
\]

The first equilibrium constant for the formation of [Co(bpy)]2+ from Co2+ and bpy is log K1 = 6.1, and is log K1 = 6.8 for [Co(DHB-H)]+ [20, 21]. The Co-bpy bond is weaker than the Co-[DHB-H]- bond and therefore an increase of the amount of mixed complexes of cobalt with bpy and [DHB-H]- is expected upon DHB addition. Addition of base shifts the equilibrium to the right. This was confirmed by visible spectrophotometry (Figure 6). Figure 6A shows the spectrum of aqueous [Co(bpy)3]Cl2. Upon addition of DHB (Figure 6B), a new band appears at ca. 600 nm, which increases substantially in intensity when base is added (Figure 6C). As [DHB-H]- induces a smaller ligand field than bpy, the d-d transition of the [DHB-H]--containing cobalt complex appears at longer wavelengths than that of the [Co(bpy)3]Cl2 complex. These findings indicate that at least part of the transition metal ion-matrix clusters observed in MALDI spectra do form in solution and that their quantity can be controlled by manipulation of the relevant equilibria.

If MALDI spectra reflect solution behavior, then changes in the quantity of preformed ions caused by variation of solution conditions should have an impact on cluster formation in the MALDI spectra. Figure 5 also shows MALDI spectra of [Co(bpy)3]Cl2 when the pH of the sample solutions was varied.
An increase in the relative intensity (peak integrals) of larger to smaller clusters in all series was observed with increasing pH of the sample solutions. Larger clusters are assumed to be fragments of a preformed solid of mixed complexes of cobalt with bpy and [DHB-H]−, crystallized from the corresponding solution. If the pH of the solution increases, DHB is deprotonated, and more of the sample is found as a preformed solid of mixed complexes of cobalt with bpy and [DHB-H]−, causing an increase in the quantity of larger compared to smaller clusters.
4.4 Ligand Exchange in $[\text{Ni}(\text{ophen})_3]\text{Cl}_2$

In contrast to the $[\text{Co}(\text{bpy})_3]\text{Cl}_2$ system, the Ni-ophen bond in the complex $[\text{Ni}(\text{ophen})_3]\text{Cl}_2$ is stronger than the Ni-[DHB-H]$^+$ bond ($\log K_1 = 8.8$ for $[\text{Ni}(\text{ophen})]^2+$ and 7.0 for $[\text{Ni}(\text{DHB-H})]^+$ [20, 21]). By means of $^1\text{H}$ NMR (Figure 7) and visible spectroscopy it was shown that mixed complexes of nickel with ophen and [DHB-H]$^+$ are not preformed in solution. $^1\text{H}$ NMR spectra of a solution of $[\text{Ni}(\text{ophen})_3]\text{Cl}_2$, DHB, and base (Figure 7B) in 1:10:10 molar ratio showed only signals of complexed ophen, as in the spectrum of $[\text{Ni}(\text{ophen})_3]\text{Cl}_2$ alone (Figure 7A). No signals of complexed DHB (Figure 7C) were detected. In solutions of the complex and DHB in 1:1 molar ratio, no visible absorption corresponding to the cobalt ion in the ligand field of DHB was observed.

![Figure 7. $^1\text{H}$ NMR spectra of solutions of A. $[\text{Ni}(\text{ophen})_3]\text{Cl}_2$ (100 mM), B. $[\text{Ni}(\text{ophen})_3]\text{Cl}_2$, DHB, and base in 1:10:10 molar ratio, C. NiCl$_2$, DHB, and base in 1:10:10 molar ratio.](image)

In order to find out whether preformed ions were present in the solid, IR spectra of the solid cocrystallized from a solution of $[\text{Ni}(\text{ophen})_3]\text{Cl}_2$ and DHB, where preformed ions were in principle possible, were compared to spectra of a solid
mixture of $[\text{Ni}(\text{ophen})_3]\text{Cl}_2$ and DHB, where preformed ions were not possible (Figure 8).

Figure 8. IR spectra of A. a solid mixture of $[\text{Ni}(\text{ophen})_3]\text{Cl}_2$ and DHB in 1:1 molar ratio and B. a solid cocrystallized from a solution of $[\text{Ni}(\text{ophen})_3]\text{Cl}_2$ and DHB in 1:1 molar ratio.
In addition to the bands appearing in the latter experiments due to ophen and free DHB, a characteristic band of complexed deprotonated DHB (symmetrical streching vibration of COO⁻ at 1483 cm⁻¹) was detected, indicating some preformed [DHB-H]⁻-containing nickel complex in the solid. Successive addition of complexed [DHB-H]⁻ (as Ni(DHB-H)₂₂H₂O) to a mixture of solid DHB and [Ni(o phen)₃]Cl₂ showed that 33 % of the total amount of DHB was complexed to Ni²⁺ in the solid crystallized from a solution with a molar ratio of [Ni(o phen)₃]Cl₂ to DHB of 1:5. IR spectra of all components of the described samples with the assignments of all the bands are depicted in my diploma thesis [1]. ¹³C NMR and visible absorption spectrophotometry were also applied to investigate the solid phase. They were not suitable due to their dependence on the solid phase structure (amorphous, crystalline) or the diffraction and reflection of the solid, respectively.

The absence of preformed ions in solution and their presence in the solid phase can be understood by considering the equilibrium relevant for the crystallization of [Ni(DHB-H)(o phen)₂]Cl from a solution containing [Ni(o phen)₃]Cl₂ and DHB:

\[
[Ni(o phen)₃]Cl₂ + DHB \rightleftharpoons [Ni(DHB-H)(o phen)₂]Cl + ophen + HCl
\]  

Upon solvent evaporation HCl is removed from the right hand side, shifting the equilibrium to this side. Preparation of the solid therefore has the same effect on the equilibrium as adding base.

In analogy to equation 2, the amount of preformed ions in the solid state in the [Co(bpy)₃]Cl₂/DHB system is also higher than in solution. This amount is higher than in the [Ni(o phen)₃]Cl₂/DHB system because ions are already preformed in solution. Relative cluster intensities are thus expected to be higher in the MALDI spectra of [Co(bpy)₃]Cl₂ than of [Ni(o phen)₃]Cl₂. Moreover, the MALDI spectrum of [Ni(o phen)₃]Cl₂ is expected to be less pH dependent than that of [Co(bpy)₃]Cl₂. Figure 9 depicts the MALDI spectra of [Ni(o phen)₃]Cl₂ with DHB as a matrix at different pH. A comparison of the corresponding spectra in Figure 5 and Figure 9 reveals that the relative intensities of the nickel clusters are smaller than those of the corresponding cobalt clusters. If the pH of the solutions is increased (Figure 9A through Figure 9C) relative cluster intensities do not change as much as they did in Figure 5, confirming the expectations outlined above.
4.4 Ligand Exchange in [Ni(o phen)_3]Cl_2

Figure 9. MALDI mass spectra of [Ni(o phen)_3]Cl_2 with DHB as a matrix in 1:1000 molar ratio at different pH. Stars and circles mark the two cluster series observed. The repeat unit within a given cluster series corresponds to [Ni(DHB-2H)] with a mass of 211 Da. The signals were normalized to the lowest mass cluster of the circle series. The signal corresponding to [Ni(o phen)(DHB)-H]^+ is not included in a cluster series. The relative cluster intensities were not influenced by the laser power.

An additional experiment demonstrating a direct correlation between the behavior of a species in the condensed phase and in MALDI MS supported these results (Figure 10). The MALDI spectrum of a solid mixture of [Co(bpy)_3]Cl_2 and Na(DHB-H) in which no preformed ions were possible (Figure 10A) was compared to the spectrum of the corresponding cocrystallized solid where it is known from the previous experiments that part of the ions are preformed (Figure 10B). It can be seen that strong clustering occurs at higher masses when preformed ions contribute to the signal (Figure 10B) which is not the case in Figure 10A.
Figure 10. MALDI mass spectra of A. a mixture of the solids [Co(bpy)$_2$]Cl$_2$ and Na(DHB-H) in 1:20 molar ratio, B. a solid cocrystallized from an aqueous solution of [Co(bpy)$_2$]Cl$_2$ and Na(DHB-H) in 1:20 molar ratio. Cluster signals are denoted in the same way as in Figure 5. The spectra were taken at the same laser power.
4.5 Cationization of Polystyrene

We next considered a more complicated system containing an analyte in addition to the transition metal salt and the matrix. Polystyrene was chosen as an analyte since it is typically observed in MALDI mass spectra cationized with transition metal ions. Dithranol was used as the matrix.

Figure 11. MALDI mass spectra of polystyrene 2500 with dithranol as a matrix and Cu(TFA)$_2$·H$_2$O as cationizing agent in 1:114:5.7 molar ratio. A. Copper-dithranol clusters are observed in the low mass range that can be divided in different cluster series, marked with circles, stars, crosses, and diamonds. The repeat unit within a given cluster series corresponds to [Cu(dith-2H)]$^+$ with a mass of 288 Da. Signals of the cross and diamond series could not be identified.
Figure 11 depicts the MALDI mass spectra of polystyrene 2500 using Cu(TFA)$_2$H$_2$O as cationizing agent. In the higher mass range (Figure 11B) 1:1 adducts of polystyrene with copper are observed, while in the lower mass range (Figure 11A) clusters between copper and dithranol are observed. The latter can interfere with polymer signals at low molecular weight. All signals represent singly charged ions. For both subsystems we examined whether copper adducts were preformed in the condensed phase or resulted from reactions in the MALDI plume.

Beforehand, experiments on the correct identification of the polystyrene adduct signals in the MALDI mass spectrum were done. They are described in the following section. This was very important since polystyrene samples do not consist of molecules with only a single mass, but of a distribution of polymer molecules.

4.5.1 Calibration and Identification of the Polystyrene Spectra

The mass corresponding to a cationized polystyrene signal is given by:

\[ \text{m(endgroups)} + n \times \text{m(C}_8\text{H}_8) + \text{m(cation)} \]

with \(n\): number of monomer units of the polymer molecule, \(m\): molecular weight.

![Figure 12. Isotopic distribution for the 20mer of polystyrene 2500 cationized with copper ions. Compound: 20mer + Cu [C164 H170 Cu], nominal mass: 2201.0 Da, most intense peak: 2204.3 Da.](image)
4.5 Cationization of Polystyrene

The end groups are tert-butyl $\text{C(CH}_3\text{)}_3$ (molecular weight = 57 Da) on one end and H (molecular weight = 1 Da) on the other. When calculating the mass of the cationized polymer ions, one cannot simply take the mass of the most abundant isotope of an element into account, e.g. 12 for C and 1 for H (nominal mass), but has to consider the isotopic distribution of each element. Otherwise, expected masses do not coincide with the experimental values (they correspond to the most intense peak of the isotopic distribution, if the MALDI signal represents the envelope of the distribution). The isotopic distribution for the 20mer polymer ion cationized with copper is depicted in Figure 12 (calculated with SpecTools). A mass difference of 3.3 Da between nominal mass and mass of the most intense peak is already observed for the 20mer.

At first glance, a calibration of the MALDI spectra based on the polymer distribution seems very promising, since the spectrum consists of several equidistant peaks observed in a relatively broad mass range. However, the mass difference between two successive polymer signals is not exactly 104 Da (which corresponds to the nominal mass of the monomer unit $\text{C}_8\text{H}_8$) but a little higher and is not constant over the polymer distribution. This is because the mass difference between the most intense peak in the isotopic distribution and the nominal mass increases with increasing number of monomer units. Example: 20mer+Cu: $\Delta m = 3.3$ Da, 30mer+Cu: $\Delta m = 4.9$ Da. For this reason a calibration of the MALDI spectra based on the polymer distribution is rather difficult to do. A calibration procedure was tried to be developed based on the constant mass difference of 104 Da but this failed since knowledge of the exact mass difference between two successive peaks is crucial for getting an accurate calibration. Therefore, an external calibration with substance P (molecular weight = 1347.7 Da) and melittin (molecular weight = 2846.5 Da) was performed before taking the polymer spectra. The masses of these two peptides are at either edge of the polymer mass distribution.

Taking into consideration a mass deviation of +/- 1 Da for the TOF instrument after a thorough calibration, comparison of the experimental mass with the mass of the calculated most intense peak in the isotopic distribution of the corresponding polymer ion unambiguously revealed that the cation from the metal salt complexes to the polymer.

4.5.2 Copper-Dithranol Clusters

Cluster formation between the matrix dithranol and copper was investigated in more detail. Van Duuren et al. [32] report a formation constant of $10^{9.6}$ for
Cu(dithranol-H)₂. Similar to the DHB matrix, deprotonated dithranol acts as a bidentate ligand, and the excess positive charge in the dithranol clusters is assumed to be compensated by deprotonation of dithranol. Because of the large formation constant in solution, we expected clusters between copper and dithranol to be partly preformed. Furthermore, we expected the relative cluster intensity to be influenced by shifting the thermodynamic equilibrium for the formation of Cu²⁺-dithranol complexes.

![Figure 13](image)

**Figure 13.** Visible spectra of dithranol (2 mM) **A.** without addition of Cu(TFA)₂·H₂O, **B.** with Cu(TFA)₂·H₂O in 1:0.2 molar ratio, **C.** with Cu(TFA)₂·H₂O in 1:1 molar ratio.

In this case, the equilibrium was shifted to the right by increasing the concentration of Cu(TFA)₂·H₂O. Our hypothesis was checked by visible spectra (Figure 13) and MALDI spectra (Figure 14) of solutions with different molar ratios of dithranol and Cu²⁺. The amount of dithranol was always kept constant. When adding Cu(TFA)₂·H₂O to a solution of dithranol (Figure 13A) a band due to Cu²⁺ complexed to dithranol appears at ~440 nm (Figure 13B) in agreement with van Duuren et al. [32]. This band increases with increasing molar ratio of Cu²⁺ to dithranol (Figure 13C). In the MALDI spectra one notes an enormous increase of the relative intensity of larger to smaller clusters when increasing the
molar ratio of Cu$^{2+}$ to dithranol. From IR spectrophotometry of the solid phase, 33% of the total quantity of dithranol was found to be complexed to Cu$^{2+}$ in the solid crystallized from a solution with a molar ratio of Cu(TFA)$_2$·H$_2$O to dithranol of 1:0.2. As noted above for cobalt and nickel complexes, it is expected from equilibrium considerations that the amount of preformed Cu$^{2+}$-dithranol clusters in the solid state is higher than in the diluted solution.

It should be emphasized that it is the correlation between the behavior in MALDI MS and in the condensed phase, not the mere presence of clusters, which indicates a contribution of preformed ions.

The MALDI spectrum of PS 2500 using Ag(TFA) as cationizing agent revealed clustering between Ag$^+$ and dithranol to be much less pronounced than in the Cu$^{2+}$-dithranol system. Ag$^+$ is a much softer Lewis acid than Cu$^{2+}$ leading to lower formation constants with dithranol (a hard Lewis base). Therefore the
amount of preformed complexes is lower, as is the relative cluster intensity in the MALDI spectra. This confirms again that MALDI spectra reflect at least qualitatively solution thermodynamics.

### 4.5.3 Copper-Polystyrene Adducts

Transition metal ions bind to polystyrene by interaction of the metal d-orbitals with π-orbitals of the phenyl side groups. Thermodynamic data have only been reported for Ag⁺-benzene. Fisher et al. [33] found that the reaction between Ag⁺ and benzene in solution only takes place in the presence of aluminum trichloride, a strong Lewis acid. A formation constant of $10^{0.2}$ for the reaction of Ag⁺ with benzene (a soft Lewis base) in solution to form the 1:1 complex supports this [34]. Assuming an even lower formation constant for Cu²⁺-benzene complexes in solution, Cu²⁺-polystyrene adducts are not expected to be preformed in solution. Various experiments were performed to check this assumption.

The MALDI analysis of a solution of polystyrene 5000, dithranol, and Cu(TFA)$_2$H$_2$O in THF (Figure 15B) was compared to another MALDI experiment (Figure 15A) in which dithranol and polystyrene 5000 were cocrystallized from THF, and Cu(TFA)$_2$H$_2$O dissolved in water was crystallized on top. Water does not redissolve polystyrene, and therefore preformed Cu²⁺-polystyrene adducts cannot form in the second experiment. Both MALDI spectra are very similar which implies that preformed transition metal ion-polystyrene adducts do not contribute to the MALDI signal. This must, however, be regarded as tentative since the conclusion is based on signal strengths, which are not always very reproducible in MALDI.

The experiment also demonstrates that the layering method is as effective and reproducible as the conventional dried droplet method for the production of polystyrene adducts, which was confirmed by further studies with Ag(TFA), CuCl$_2$·2H$_2$O and CuCl as cationizing agents. The advantage of the layer method is that it can be applied when both the analyte and the cationizing agent are not soluble in the same solvent as well as when they are and that it does not require any premixing. It is also applicable to systems consisting of matrix and analyte [16].

The result is in contrast to findings of Deery et al. [24], who crystallized Rb and Cs salts from water, and applied a solution of polystyrene and dithranol in THF on top. They failed to produce spectra of polystyrene cationized with Rb⁺ and Cs⁺ claiming inhomogeneity of the sample due to the preparation technique. The
4.5 Cationization of Polystyrene

reasons possibly include too small sample loads, a metal ion inappropriate for cationization, or an unsuitable drying procedure for the aqueous salt. If water is not evaporated very quickly in the vacuum but rather slowly in a stream of air, the salt concentrates on a small area on the probe tip. This area is probably quite difficult to hit with the laser beam. Other groups have also described the importance of fast evaporation to form homogeneous sample layers [19, 35].

Spectra like the ones shown in Figure 15 were also recorded with other matrices. Polar matrices such as DHB did not yield any polystyrene signals, whereas apolar matrices, for example pyrene, produced spectra of reasonable quality. We believe that incorporation of the polystyrene and the cationizing agent in the matrix crystals is crucial for obtaining good MALDI spectra. This is shown schematically in Figure 16. We define preformed ions as the case where the metal ion-polystyrene adduct crystallizes separately from the counter ion (Figure 16A), or if it crystallizes as an ion pair with the counter ion (Figure 16B). In contrast, when polystyrene cocrystallizes with the matrix, and the metal salt

![Figure 15. MALDI mass spectra of polystyrene 5000 with dithranol as a matrix and Cu(TFA)$_2$H$_2$O as cationizing agent in 1:114:2.9 molar ratio. A. Dithranol and polystyrene 5000 were crystallized from THF, and Cu(TFA)$_2$H$_2$O dissolved in water was crystallized on top. B. Dithranol, polystyrene and Cu(TFA)$_2$H$_2$O cocrystallized from THF. The laser power was the same for both spectra.](image-url)
crystallizes separately (Figure 16C), we expect that only reactions after desorption can lead to metal ion-polystyrene adducts. As polystyrene is an apolar molecule, apolar matrices are expected to favor the case of Figure 16C, consistent with the experimental findings.

The same conclusion could be drawn from the visible spectrophotometric analyses. No band due to copper in the ligand field of polystyrene was observed when adding Cu(TFA)$_2$·H$_2$O to a solution of polystyrene. Furthermore, the band corresponding to copper complexed to dithranol in Figure 13 did not decrease when polystyrene was added to the solution.

The results obtained for the transition metal ion-dithranol clusters and the transition metal ion-polystyrene adducts can be explained on the basis of thermodynamics in solution. The formation of Cu$^{2+}$-dithranol complexes in solution is favored by several orders of magnitude over the formation of Cu$^{2+}$-polystyrene adducts. Therefore when mixing polystyrene, dithranol and Cu(TFA)$_2$·H$_2$O in solution, Cu$^{2+}$-dithranol complexes form whereas polystyrene
adducts do not form. The competition in solution between polystyrene and dithranol for the cationizing agent seems to be completely won by the matrix, as shown by visible spectrophotometry.

The MALDI experiments with polystyrene demonstrated that gas-phase cationization with copper is the dominant ion formation pathway. In the following, this mechanism is investigated further.

4.5.4 Mechanism of Charge Compensation

Singly charged copper-polystyrene adducts are observed in MALDI spectra, although using Cu(II) for cationization. Two mechanisms of charge compensation are conceivable:

\(a)\) Reduction of Cu(II)

\[
\text{Cu}^{2+} + e^- \leftrightarrow \text{Cu}^+ \quad \text{Cu}^+ + \text{PS} \leftrightarrow [\text{Cu(PS)}]^+
\]

\(b)\) Proton Abstraction in Polystyrene

\[
\text{PS} \leftrightarrow (\text{PS-H})^- + \text{H}^+
\]

\[
(\text{PS-H})^- + \text{Cu}^{2+} \leftrightarrow [\text{Cu(PS-H)}]^+
\]

\[\text{Figure 17.} \ \text{FTICR mass spectrum of the polystyrene pentamer cationized with copper.} \ \text{Besides the base peak with reduced copper(I) (C}_\text{60h}\text{H}_{30}\text{Cu})^+ \text{(calculated m/z 641.32), peaks corresponding to (C}_\text{61h}\text{H}_{30}\text{Cu})^+ \text{(calculated m/z 642.32), (C}_\text{60h}\text{H}_{30}\text{Cu})^+ \text{(calculated m/z 643.32), and (C}_\text{61h}\text{H}_{30}\text{Cu})^+ \text{(calculated m/z 644.32). The difference between calculated and experimentally determined masses was 0.04 Da.}\]
The molecular weight of the product of mechanism b) is 1 Da less than that of the product of mechanism a). The issue was resolved by investigating the polystyrene pentamer at a resolution of 10 000 in a FTICR mass spectrometer (Figure 17).

The deprotonated species \((C_{44}H_{80}Cu-H)^+\) is not observed. Instead, the pentamer cationized with Cu(I) \((C_{44}H_{80}Cu)^+\) forms the base peak. A similar result was obtained by Wong and Chan [36] for polar polymers with copper(II) used for cationization. However, it is not known whether the metal ion is reduced before or after complex formation with polystyrene.

### 4.5.5 Competition between Matrix and Polystyrene

In contrast to the solution, where the competition between dithranol and polystyrene for the cationizing agent is won by the matrix (log \(K\) for copper-dithranol is 9.6 and for copper-polystyrene is < 0.2,), a real competition does occur in the gas phase. Figure 18 depicts the MALDI spectra of polystyrene 2500 at different polystyrene-to-matrix ratios, always keeping the molar ratio of polystyrene to cationizing reagent constant at 1:5.7. With increasing molar ratio of polystyrene to dithranol, Cu\(^{2+}\)-matrix clusters decrease with respect to the Cu\(^{2+}\)-polystyrene adducts.

The observation points to the existence of reactions and possibly an equilibrium in the MALDI plume between polystyrene, matrix and the metal ion, involving a competition between polystyrene and matrix for the transition metal ion.

\[
\text{Cu}^{2+} + 2\text{dithranol} \rightleftharpoons \text{Cu}(\text{dithranol-H})_2 + 2\text{H}^+ \quad (3)
\]

\[
\text{Cu}^{2+} + \text{PS} \rightleftharpoons [\text{Cu(PS)}]^{2+} \quad (4)
\]

Equilibrium (3) is already shifted to the right side in solution as shown above. In contrast, reaction (4) mainly takes place in the gas phase because the solvent is absent. This allows the intrinsic properties of the bare reactants to come into play. An increase of the polystyrene concentration compared to the matrix concentration forces equilibrium (4) towards the right, leading to a shift of equilibrium (3) to the left. The influence of the increased copper concentration is the same on both equilibria. Provided that there is a competition between polystyrene and dithranol for the transition metal ion in the gas phase, Cu\(^{2+}\)-polystyrene adducts should not be observed if dithranol binds all of the cationizing agent in a complex. This was found to be the case if Cu(dithranol-H)\(_2\) is used as matrix and cationizing agent in one.
Figure 18. MALDI mass spectra of polystyrene 2500 with dithranol as a matrix and Cu(TFA)$_2$H$_2$O as cationizing agent in different molar ratios. The laser power was kept constant for experiments A. to C., and had to be slightly increased for experiment D.. Intensity units are the same from A. to D..

MALDI experiments performed with a solid mixture of Cu(dithranol-H)$_2$ and polystyrene as well as with a sample cocrystallized from a solution of
Cu(dithranol-H)₂ and polystyrene did not yield any Cu²⁺-polystyrene adducts; only Cu²⁺-dithranol clusters were detected. If however Cu(TFA)₂·H₂O and dithranol are mixed in solution, some uncomplexed Cu(II) salt exists in the solid phase because the complexation is not complete. This can be desorbed, forming Cu²⁺-adducts with polystyrene in the gas phase as was observed in Figure 11.

4.5.6 Further Experiments

Cationizing experiments of polystyrene with different transition metal ions (silver, nickel, copper) and transition metal ions having different counterions (chloride, trifluoroacetate, acetate) were also performed to gain insight into the cationization mechanism. Antibonding orbitals in transition metal ion/benzene complexes become more occupied and the binding energies decrease when increasing the number of electrons of the transition metal ion in the same period of the periodic table [37]. The unfavorable occupation of antibonding orbitals can be avoided by reduction of hapticity which rationalizes why Ag⁺ in Ag⁺-benzene is localized over one of the C-C bonds. This is valid for the solid and gas-phase complexes [33, 38]. Cu⁺ contributes the same number of electrons and therefore it also binds only to one C-C bond of benzene. No references on the hapticity in Ni²⁺ and Cu²⁺-benzene complexes were found; these transition metals are expected to bind to at least two benzene carbon atoms. Steric and electronic reasons result in transition metal ion binding to a maximum of two benzene rings. This was confirmed by a high energy MS/MS study [39]. Therefore, polystyrene molecules are not as much wrapped around the transition metal ions as polyethylene glycols are around alkali metal ions. In the latter case, seven oxygen atoms from along the polymer chain solvate one sodium ion [40]. At first glance it seems possible that several transition metal ions stick onto one polystyrene molecule because it contains as many benzene rings as monomer units. These benzene rings are, however, very close to one another and it is most probable that one transition metal is common to several benzene rings. The binding energies for different transition metal ion-benzene complexes in the gas phase are indicated in Table 1.

Values for doubly charged metal ion complexes or information on the stoichiometry of the different complexes is unknown. Based on the available data, significant differences in the cationization efficiencies of the different transition metal ion salts were expected. However, there was no experimental evidence for a preference of polystyrene for a certain transition metal ion.
Table 1. Binding energies of different transition metal ion-benzene complexes in the gas phase. Experimental values were determined from ion beam experiments. Theoretical values are given in parenthesis [41].

Similarly, the effect of the counterion could not be clearly separated from other perturbing effects, e.g. different solubilities and crystallization behaviors of different salts, and therefore no statements concerning the influence of the counterion could be made.
4.6 Summary and Perspectives

The contribution of preformed ions to the ion signal observed in MALDI was investigated using different analytical methods, such as IR, visible and \(^1\)H NMR spectroscopy as well as MALDI mass spectrometry. Complexes between transition metal ions and matrix, as well as polystyrene have been studied.

It was found that MALDI mass spectra qualitatively reflect the amount of preformed complexes between transition metal ions and MALDI matrices or analytes in the solid sample. The contribution of preformed ions can be predicted on the basis of condensed phase thermodynamics: preformed ions play an important role in the case of transition metal ion-matrix complexes because solution-phase thermodynamics are favorable for these reactions. However, owing to low formation constants in solution, gas-phase processes dominate MALDI ion formation of metal ion-polystyrene adducts.

If thermodynamic solution data on the complexation behavior are not available, which is often the case, no prediction on the contribution of ions preformed in solution can be made. If thermodynamic solution data are in favor of these ions, they are formed and will be detected in the MALDI mass spectra. If the solution data are not favorable, formation can take place in the solid phase and/or in the gas phase.

MALDI mass spectra of samples containing transition metal ions and matrix show extensive clustering. Interference with signals of analytes of low molecular weight is possible. The quantity of preformed ions influences the relative cluster intensity. The amount of preformed ions in the solid state is higher than in diluted solution provided that volatile byproducts form during complexation which are then eliminated upon crystallization.

The gas-phase cationization of polystyrene using dithranol as a matrix and \(\text{Cu(TFA)}_2\cdot\text{H}_2\text{O}\) as cationizing agent was further investigated. It was found that singly charged Cu(I)-polystyrene adducts were detected, although Cu(II) was used as cationizing agent.

It was also shown that matrix and polystyrene compete for the cationizing agent in the gas phase, whereas in solution, the competition is completely won by the matrix. Naively, the combination of matrix and cationizing agent in one compound could be considered as ideal, because no perturbing counterions
would be present. However, using Cu(dithranol-H)$_2$ instead of dithranol and Cu(TFA)$_2$·H$_2$O, no copper-polystyrene adducts could be detected, most probably because no uncomplexed copper ions are available for polystyrene.

Based on the competition model, the cationization of polystyrene is assumed to be most effective if the matrix has a lower affinity for the metal ion than polystyrene. Matrices without functional groups would be potential candidates. However, besides the desired complexation behavior, the matrix also has to fulfill the requirements of a UV MALDI matrix. The necessity of the matrix to absorb at 337 nm requires an aromatic ring system in the molecule, which may complex through its $\pi$-orbitals to the metal ion, as is the case for polystyrene. One of the best UV matrices conceivable would be pyrene which has no functional groups. However, polystyrene spectra using dithranol yielded data of much higher quality. This example shows that, besides solution-phase thermodynamic data, incorporation of the analyte in the matrix plays a crucial role. Cations can be selected for their likely binding strength to polystyrene. It was found that transition metal ions are better cationizing agents than alkali ions, as is expected from the HSAB model. However, there was no experimental evidence that transition metal ions with different binding strengths have an influence on the cationization efficiency.
4.7 References


5 Detection of Specific Zinc Finger Peptide Complexes

5.1 Introduction

5.1.1 Noncovalent and Other Interactions

Proteins are biopolymers made from amino acid residues that are joined by covalent amide bonds. The primary amino acid sequence folds into the tertiary structure necessary for biological activity. Forces involved in protein folding and in interactions of proteins with molecules from other substance classes are of noncovalent nature: ionic, hydrophobic, and van-der-Waals interactions, as well as stacking and hydrogen bonds.

As the environment of a folded protein is altered by changing pH, temperature or pressure, its structure and biological activity exhibits only little change until a denaturation point is reached. At this point, noncovalent bonds are disrupted and the tertiary structure as well as the biological function are suddenly lost [1]. Therefore, noncovalently bound protein complexes and complexes involving tertiary structures are mainly stable under physiological conditions in solution.

In this chapter, the complex between the zinc finger peptide p55F1 and Zn\(^{2+}\), called Zn-p55F1, and the complex between Zn-p55F1 and oligodeoxynucleotides is investigated. The interactions in the latter complex are noncovalent since hydrogen bonds, stacking and electrostatic interactions are involved. The bond in Zn-p55F1 is coordinate-covalent with polar character. Zn-p55F1 can be called a polar coordinate-covalent compound or a polar covalent complex. In the MALDI MS literature, complexes are often considered to be noncovalent, although they are not. Therefore, the terms coordinate, covalent, and polar are defined below.

A bond is called coordinate or the compound containing the bond is called a complex, if free electron pairs or orbitals of the binding partners are involved in the binding. In the case of Zn-p55F1, 3d, 4s and 4p orbitals of Zn\(^{2+}\) as well as free electron pairs of the thiolate sulfur in cysteine and of the nitrogen in the histidine’s imidazole ring are involved.

Covalent, in contrast to ionic, can be explained with the valence bond model, based on electronegativity values. The higher the electronegativity differences between the binding partners, the more ionic and the less covalent the bond is. The binding strength of Zn-p55F1 is mainly based on the interaction between
Zn$^{2+}$ and S$^-$. This interaction is known from ZnS, which crystallizes in the “wurtzit” lattice, similar to that of diamond. The lattice of diamond can certainly be considered as covalent, and therefore one also considers Zn-p55Fl as a covalent complex.

The intermediate between a covalent and an ionic bond is called a polar covalent bond. Due to the positive and negative charges that are involved in the binding, the interaction between Zn-p55Fl is also partly ionic and therefore the complex is completely described by polar covalent. It should not be considered as noncovalent, although this is often done in the MALDI literature on similar metal ion-peptide complexes.

The critical points in the MALDI detection of this complex are that it is only stable under defined solution conditions and that it can form nonspecific adducts in the MALDI plume. These are also the critical points in the MALDI analysis of noncovalent complexes. Therefore, the discussion on noncovalent complexes in the following introduction and in the summary is valid for both sorts of complexes.

### 5.1.2 Techniques for Investigating Noncovalent Complexes

There are several established instrumental techniques that have been applied for the study of tertiary structures and noncovalent complexes [2]. Chromatographic and electrophoretic-based assays, such as size exclusion chromatography and gel electrophoresis, have traditionally been used for determining stoichiometry and molecular weights. Spectroscopic approaches to this field are circular dichroism, light scattering, fluorescence, NMR and X-ray crystallography, as well as absorption spectroscopy and electron paramagnetic resonance (ESR) spectroscopy (for metal ion-protein complexes). NMR and X-ray crystallography yield high resolution and detailed three-dimensional information of biomolecules and complexes in solution and the solid phase. In general, the structures from NMR and X-ray crystallography agree. Both of these techniques require relatively large, usually mg quantities of material and are time-consuming. Often, the protein precipitates at the high concentrations necessary for NMR. NMR is generally limited to the analysis of molecules of up to 40 kDa although higher magnetic fields allow access to larger molecular weights. Due to the high exchange rates of protons with the solvent water, no information on the number of protons in a structure is obtained. This may, however, be crucial for the structural determination of complexes whose formation is pH dependent.

A recent analytical tool to overcome some of the problems of these techniques and/or complement them is mass spectrometry, especially when it is coupled
5.1 Introduction

with soft ionization techniques like ESI and MALDI. While the characterization of tertiary structures and noncovalent complexes has already been demonstrated with ESI MS (see section 5.1.4), it is just starting to be explored with MALDI MS (see section 5.1.5).

5.1.3 Detection of Specific Interactions vs. Nonspecific Aggregation

Noncovalent complex formation in solution is often directed by specific molecular recognition. The notion “specific” is also not used very consistently in the literature, and therefore we would like to give a correct definition in the following. If a saturation is observed when plotting the concentration of the complex as a function of the concentration of one of its components, then the interaction between the components of the complex is specific. When using the logarithm of the molar ratio of the components instead of the concentration of one component, the stoichiometry of the complex can be read at the projection of the curve’s inflection point onto the x-axis.

The mass spectrometric technique used must distinguish between specific complex formation and nonspecific aggregation which may be introduced by the mass spectrometric technique itself. To avoid misinterpretation of mass spectra, adequately planned control experiments have to be performed. In his book on mass spectrometry in biochemistry, Lehmann [3] proposes competition experiments, in which one binding partner is fixed and the other varied, and vice versa and which allow conclusions to be drawn based on relative ion intensities. Further possibilities to identify specific complex formation are to follow the dependence of the complex on the pH value or concentration or to follow the influence of specific chemical modifications on complex formation. Small changes in solution conditions or small structural changes of one of the binding partners should lead to dissociation of the specific complex. In general, experiments demonstrating a correlation between solution-phase and gas-phase chemistries suggest that the interactions in the detected complex are specific. In the following, an overview of the detection of noncovalent complexes with ESI and with MALDI MS is given.

5.1.4 ESI MS of Noncovalent Complexes

ESI MS produces gas-phase macromolecular ions directly from solution by spraying the solution into the mass spectrometer. This is in contrast to MALDI MS, where the solution has to be crystallized before analysis. This is the reason why macromolecular complexes have predominantly been investigated by ESI
MS. In 1991, work by Katta and Chait [4] on the globin-heme interaction of myoglobin and work by Ganem et al. [5] on a receptor-ligand complex demonstrated for the first time that specific noncovalent interactions could be detected with ESI MS. Since these initial reports, several other types of noncovalent complexes have been studied, including protein-protein [6, 7], protein-ligand [8, 9], antibody-antigen [10], protein-oligonucleotide [11, 12], protein-double-stranded DNA [13], and protein subunit complexes [14, 15]. The application of ESI MS to the detection of noncovalent protein complexes has been the subject of extensive reviews [2, 16, 17]. Their authors emphasize the necessity of performing control experiments as a crucial point to avoid misinterpretation of ESI spectra.

ESI MS has also proved useful for studying zinc finger proteins. Nucleocapsid protein NCp7 contains two zinc fingers (one of which is p55F1 used in the present MALDI study) that are involved in the encapsulation of genomic RNA during HIV viral assembly. Surovoy et al. [18] and others [19, 20] have demonstrated that ESI MS can determine the zinc stoichiometry for NCp7. The ejection of zinc from NCp7 caused by the covalent binding of various inhibitors to the protein can also be monitored by ESI MS [20, 21].

Solution-phase methods and ESI MS have already been compared [22], and it was found that solution-phase binding can be detected in certain cases [23]. An example for this is the binding of various peptide inhibitors to Src SH2 domain protein. When working under competitive binding conditions, the relative abundances of the Src SH2 protein-phosphopeptide complexes observed in the ESI mass spectrum were consistent with the measured solution-phase binding constants [24]. As a further example, the association of albumin protein with oligonucleotides was measured to have dissociation constants in the micromolar range by a Scatchard analysis of ESI MS data. This was independently verified by capillary electrophoresis experiments [25].

The nature of the noncovalent interaction may have an effect on the result of the mass spectrometry experiment. Going from solution to a solvent-free environment, i.e. gas phase, may change the forces involved. For example, electrostatic interactions may be strengthened in vacuum. Feng [26] studied the binding of highly basic spermine to an acidic spermine-binding peptide. Despite a weak binding constant in solution ($10^7$ M$^{-1}$), the complex could be readily detected indicating an unusual stability in the gas phase. Similarly, the gas-phase stabilities of noncovalent complexes between bovine carbonic anhydrase and para-sub-
stituted benzenesulfonamide inhibitors were found to have no direct correlation with the hydrophobicity of the inhibitors [27]. Obviously in systems where hydrophobic interactions play a major role in solution, for example, in leucine zipper peptides and receptor-ligand complexes, the fraction of complex species in the gas phase is low (10-20 %) since such interactions are in large part due to the role of the solvent [8]. Whether ESI mass spectra reflect solution-phase behavior depends on the system investigated, especially the forces involved in the binding. The general applicability of ESI for biochemical problems has not yet been fully demonstrated and further work on the comparison of gas-phase and solution behavior has to be done.

Another problem is that typical ESI MS operational conditions, such as organic additives or high temperature, are often not compatible with the stability of non-covalent complexes [2, 28]. For these reasons, there is still plenty of room for improvements in the field of the detection of noncovalent complexes. On the instrumental side, the development of microspray and nanospray interfaces which work with pure aqueous solutions and the development of interfaces which operate at low temperatures are extensively studied topics [8, 29-32]. On the mechanistic side, the comparison of gas-phase and solution structure are heavily investigated topics.

5.1.5 MALDI MS of Noncovalent Complexes

MALDI MS has some advantages over ESI MS. Its tolerance to salt impurities and buffers allows direct analysis of biological samples without further purification. A TOF analyzer is characterized by a higher sensitivity and a shorter analysis time compared to quadrupole analyzers commonly used for ESI. However, ESI is now also starting to become available with TOF analyzers. In MALDI, triply charge molecular ions are at maximum observed, which facilitates spectral interpretation. This is in contrast to ESI, where ions with very high charge states are detected, necessitating deconvolution of the spectra. MALDI spectra are taken from crystalline samples. Compared to ESI MS, additional crystallization of the sample from the solution may introduce further modification of the analyte structure. This renders MALDI experiments of non-covalent complexes and tertiary structures more complicated and the method’s performance in this area lags behind.

Since the discovery of MALDI, analysis of quarternary structures of proteins was constantly pursued. Intermolecular noncovalent interactions are responsible for the aggregation of folded polypeptide chains into multimers determining a
protein system's quaternary structure. Initially, quaternary structures could only be observed after crosslinking of the subunits. Nelson et al. [33] observed human immunoglobulin IgM pentamers (980 kDa) which contain covalent disulfide bonds linking subunits into stable aggregates. Dimeric glucose-6-phosphate dehydrogenase (104 kDa) and tetrameric avidin (68 kDa) were detected by Farmer and Caprioli [34] only after crosslinking with glutaraldehyde. Aerolysin heptamers (300 kDa), observed by Moniate et al. [35], are exceptionally stable oligomers that retain noncovalent bonds even if exposed to extreme conditions such as low pH values, heat or buffer. Quaternary structures without crosslinked subunits have also been detected, predominantly by researchers from the Hillenkamp group in Münster. Examples are complexes of streptavidin [36] and glucoseisomerase (172 kDa) [37], which have been observed using the matrix nicotinic acid. Unfortunately, nicotinic acid suffers from the disadvantages of a strong tendency for matrix-adduct formation, intolerance to salt contamination, and does not absorb the 337 nm wavelength emitted by commonly used nitrogen lasers. A follow-up study with matrices suitable for the 337 nm desorption wavelength identified ferulic acid in THF to yield spectra of the trimeric membrane protein porin and the tetrameric streptavidin [38]. Good results were also obtained with beef liver catalase of mass 233 kDa using 2,6-dihydroxyacetophenone in THF or acetonitrile/TFA [39].

The most striking observation the researchers from the Hillenkamp group made during these experiments was that dominant signals of the quaternary protein complex could only be detected for the first laser exposure of a given sample location. All subsequent exposures of a given location yielded spectra with monomeric units as the base peak and an exponential decrease of the signal intensities with increasing aggregate order. The authors considered a specific noncovalent complex (i.e. its formation is directed by specific molecular recognition in solution) if the MALDI spectrum depicted the target complex as dominant species [40, 41]. On the other hand, an exponential decrease of the intensities of the complex's components was considered to be indicative of nonspecific aggregation. It was assumed to take place in the MALDI plume after desorption where the collision probabilities decrease with the number of colliding partners. The basis for this assumption was the observation that properties such as ion yield and analyte aggregation depend strongly on the parameters of the ion extraction and the electric field strength. The authors' criteria for specificity are somewhat different from those of Lehmann [3] (see section 5.1.3). However, the authors also stress the need for control experiments...
5.1 Introduction

to distinguish MALDI-inherent nonspecific aggregation from specific complexation in solution.

Ion generation and extraction into the mass spectrometer is a sequence of complicated steps, which all can interfere with the detection of noncovalent complexes. The successful analysis of noncovalently-bound compounds by MALDI MS requires that the intact complex is transferred from solution to the solid phase, and is then incorporated into the matrix crystal. Subsequently, the complex has to survive the laser shot and the transition from the solid state to the gas phase, and has to be stable in the gas phase on a time scale of some 100 µs for detection. The noncovalent complex must be assumed to be substantially influenced by the dramatically changing environment during both crystallization and desorption processes. The details of these processes during most of the steps are not yet fully understood. This is manifested in the fact that even for the first step of sample preparation, completely different protocols are suggested in the literature.

Since noncovalent interactions are destroyed under harsh acidic conditions, Woods et al. [42] as well as Glocker et al. [43, 44] point out the need for using a nonacidic matrix for the analysis of enzyme-substrate, zinc finger peptide-metal ion complexes, leucine zipper dimers, and RNase S complexes. ATT as well as sinapinic acid and α-CHCA in different ammonium buffer solutions were used as matrices. A number of other UV MALDI matrices have recently been developed by Fitzgerald et al. [45] which can be crystallized from aqueous solution in the pH range of 2 to 8. To our knowledge, their feasibility for the analysis of noncovalent complexes has not yet been evaluated.

On the other hand, as Hillenkamp pointed out [40], the best results for the detection of intact quarternary proteins were obtained for matrix/solvent compositions, such as ferulic acid/THF which are expected to lead to dissociation of the complexes. There are only a few minutes between mixing of the complex in water with the matrix solution and the complete evaporation of the solvent. Kinetics of denaturation might be slow on this time scale. Since organic solvents have a higher vapor pressure than water, a concentration gradient of the mixture of organic solvent, originating from the matrix solution and the water, originating from the sample solution is expected to develop during solvent evaporation. Immediately before complete solvent evaporation, a predominantly aqueous solution remains and the quarternary proteins may precipitate onto the matrix crystals or get incorporated into the top layer of the crystals, thus leading to the
observation of the intact complex for the first shot only. The author points out, however, that strong differences between chemically quite similar matrices such as ferulic acid (intact quarternary complex) and other cinnamic acid derivatives like sinapinic acid (only subunits were observed) are difficult to understand with this model. It was also found that the exclusive detection of protein subunits for all but the first exposure was not the result of UV photodissociation [38].

The processes in the MALDI plume are the least understood in the whole sequence [40], as has already been noted in chapter 2. They take place on a nanometer (thickness of the desorbed layer in UV MALDI) to micrometer (plume expansion) length scale and on a nanosecond time scale, rendering investigation very difficult. The stability of the noncovalent complex is certainly influenced at this stage: first, by strengthening some of the noncovalent forces and weakening others upon transfer into the vacuum, and second, by collisions in the MALDI plume.

Besides quarternary protein complexes, other noncovalent complexes or tertiary structures have been detected with MALDI. A review from Hillenkamp on the topic of MALDI MS of noncovalent complexes has recently appeared [40]. Winston et al. [16] also describes the subject in his review on MS techniques for investigating protein structure and function.

Examples for the detection of nonspecific ionic interactions are complexes of anionic compounds such as single- and double-stranded oligodeoxynucleotides [46, 47], sulfated oligosaccharides [48, 49] or dyes [50] with strongly basic peptides. The mass of these complexes was a few kDa. Aqueous ATT matrix solutions with a pH value of 7 were successfully used. Acidic pH values led to the disruption of the ionic interactions. An important observation of Lin et al. [47] was that no complexes were observed using oligonucleotide sequences lacking thymidine. ATT was also shown to allow detection of double-stranded DNA [47, 51, 52].

As for quarternary complexes, some authors also observed the “first shot” phenomenon in their experiments, i.e. the dominance of the specific noncovalent complex in the spectrum only for the first shot. For subsequent shots, the complex’s constituents dominate the spectrum. In contrast to the quarternary protein structure, where the first shot phenomenon was observed using denaturing matrix solutions, the use of nonacidic sample preparation conditions solely lead to a successful MALDI experiment for these chemical systems.
As an example, work of Gruic-Sovulj et al. [41] can be cited. These authors investigated specific complexes formed by yeast seryl-tRNA synthease and tyrosyl-tRNA synthease with tRNA\textsubscript{Ser} and tRNA\textsubscript{Ytr}, with complex masses above 100 kDa. The matrix used was ATT in 12.5 mM ammonium acetate (pH 7).

Strupat et al. [53] detected specific noncovalent heterotetramer complexes of the calcium-binding myeloid-related proteins MRP8 and MRP14 with a mass of ca. 48 kDa. The number of calcium ions attached to the tetramer was also determined. The first-shot phenomenon was observed for the tetramer complex. Sub-unit-calcium ion complexes were observed for consecutive laser shots. 2,6-dihydroxyacetophenone and ATT in water/acetonitrile (3:1, v:v) were successfully used, although ATT led to less reproducible results. No tetramers were observed using super-DHB (2-hydroxy-5-methoxybenzoic acid:DHB molar ratio is 1:10).

Other authors also detected specific noncovalent complexes, but did not observe the first-shot phenomenon. Signals of the specific complex could be observed from consecutive laser shots. Spectra were dominated by signals of the individual components of the complex, and the complex signal was rather small in intensity (except Jespersen et al. [54]). Specificity was checked by performing control experiments. The authors of these papers (except Jespersen et al. [54] and Borchers et al. [55]) also assume that a physiological pH of the matrix/sample solution is a prerequisite for the detection of the intact complex.

In this sense, Glocker et al. [43, 44] detected the RNase S complex of S-protein and S-peptide, as well as a leucine zipper dimer complex. Two different control experiments, one with the unrelated peptide angiotension and the other at acidic pH conditions proved the specificity of the complexes. The matrix solutions were composed of ATT in 10-50 mM ammonium bicarbonate, citrate, or acetate buffers (pH 5.5).

Woods et al. [42] reported the intact desorption of complexes of the three enzymes aminopetidase I, trypsin and arylamidase with the substrates bovine growth hormone releasing factor (GH), rat parathyroid hormone and again GH, respectively in the mass range 25-35 kDa. The matrix was a saturated solution of sinapinic acid in 1M ammonium citrate:ethanol (1:1). Control experiments to ensure the detection of specific complexes involved the use of three proteins lacking affinity and the variation of pH conditions.

To our knowledge, the only MALDI experiment on zinc finger peptide-metal ion complexes have also been described by Woods et al. [42]. Two experiments with a zinc finger peptide of Cys\textsubscript{2}-His\textsubscript{2} type have been performed. In one of them, the authors varied the peptide:Zn\textsuperscript{2+} molar ratio. Molar ratios smaller than or equal to 1:1 yielded similar spectra, whereas an excess of peptide resulted in a decrease of
the Zn$^{2+}$-peptide complex signal. In another experiment, the authors used Co$^{2+}$ instead of Zn$^{2+}$. In contrast to the Zn$^{2+}$-complex, the Co$^{2+}$-peptide complex was barely visible in the MALDI spectra. This was explained by a difference of four orders of magnitude between the dissociation constants of both complexes. However, it is known that conclusions drawn from absolute intensities are problematic in MALDI mass spectrometry. It was clearly visible in the MALDI spectra that the relative signal intensity of the Co$^{2+}$-peptide complex was not lower than that of the Zn$^{2+}$-complex. The matrix was a saturated solution of $\alpha$-CHCA in 1M ammonium bicarbonate:ethanol (1:1). Control experiments at acidic pH conditions had not been performed. All metal ion-peptide complex signals observed by the authors correspond to singly charged ions in which the excess charge of the metal ion is compensated by deprotonation of the peptide.

Borchers et al. [55] detected the complex of the viral envelope glycoprotein HIV-gp120 and its cellular receptor CD4 with a mass of ca. 140 kDa. Specificity was checked for with bovine serum albumin lacking affinity. In contrast to the previously mentioned experiments, ATT matrix did not yield molecular complex ions, but $\alpha$-CHCA in organic and acidic solutions did.

Thiede et al. [56] identified specific noncovalent interactions between different peptides and ribonucleic acids in the mass range 5-9 kDa. A solution of 2,4,6-THAP in methanol was used as the matrix (peptides and ribonucleotides were dissolved in water). It was claimed that the affinities of different molecules for a target molecule could be derived from the relative intensities of the corresponding complexes. A comparison with the complexes’ solution-phase behavior had not been done.

Jesperersen et al. [54] investigated noncovalent complex formation of the tetrameric recombinant streptavidin and the dimeric glutathione-S-transferase. Signals of both multimers could only be observed as base peaks using the acidic matrix 3-HPA and not using other matrices, such as the acidic matrix DHB and the nonacidic matrices PNA, ATT, and 2-amino-4-methyl-5-nitropyridine (AMNP). No first-shot phenomenon was observed. $\alpha$-Chymotrypsinogen A which is known to exist as a monomer only, was used as a control. No signals due to nonspecific aggregation of the monomer were observed in the MALDI spectra. From this, the authors concluded that the multimers observed with streptavidin and glutathione-S-transferase were specific.

The experiments presented up to now were done using UV MALDI. The mild desorption conditions of IR MALDI using frozen water from an aqueous sample solution as the matrix also appear very promising [57, 58]. Expensive IR laser instrumentation has prevented its routine use up to now. With the development of
carbon dioxide lasers, IR MALDI will surely become an alternative to UV MALDI. One example shall be cited here. Proteins carrying a noncovalently bound prosthetic group such as myoglobin are observed as apoproteins in UV MALDI spectra. IR MALDI experiments of myoglobin using a physiological solution as a matrix showed, however, clear signals of the heme protein [59].

The aforementioned examples clearly show that the potential of MALDI for detecting noncovalent complexes and tertiary structure has not been fully realized yet. Many of the reported observations are inconsistent or contradictory, for example, sample preparation protocols or specificity criteria, to mention the most important. Moreover, in contrast to ESI, there is no extensive MALDI study which compares solution- and gas-phase chemistries of noncovalent complexes. Using different model systems described in chapter 4 we have already shown a correlation between solution-phase and gas-phase behavior in MALDI. In the study described in this chapter, we sought to demonstrate this correlation for more practically relevant peptide-metal ion and noncovalent complexes, which are mainly stable under physiological conditions in solution. Our studies concentrated on the MALDI detection of zinc finger peptide complexes with metal ions (chapter 5.3) as well as with oligodeoxynucleotides (chapter 5.4). Special emphasis was put on control experiments to distinguish between specific and nonspecific aggregation.

5.1.6 Chemical Systems

All retroviruses, including human immunodeficiency virus type 1 (HIV-1) encode a gag precursor polyprotein which contains zinc binding domains of CCHC type (CCHC = Cys-X2-Cys-X4-His-X4-Cys, X = variable amino acid) [60]. These zinc-coordinated motifs are known to play an important role in the recognition of viral ribonucleic acid and replication of the virus. An 18-residue peptide of CCHC type with a sequence corresponding to the first zinc finger domain from the gag protein p55 of HIV-1, called p55F1, was chosen for this study. This peptide has never been studied with MALDI before. The apopeptide has no defined secondary structure in the absence of metal ions. Metal ion binding induces peptide folding which is required for the interaction with nucleic acids [61, 62]. CCHC-type peptides bind Zn$^{2+}$ very strongly, whereas the complexes with other transition metal ions are weaker [63, 64]. The three-dimensional structure of the Zn$^{2+}$-complex of p55F1 had first been solved by Summers et al. [65] (Figure 19). Zn$^{2+}$ is tetrahedrally coordinated by three cysteine and one histidine residue. It is still controversially discussed for peptides and proteins containing CCHC arrays whether all of the thiol groups are
deprotonated when the molecule is complexed to the metal ion. Based on the fact that neither NMR spectroscopy nor X-ray crystallography has detected the presence of thiol protons, it was assumed that all cysteines involved were deprotonated [66]. Using electrospray mass spectrometry and a strategy based on the different susceptibilities of thiols and thiolates to alkylating agents, Fabris et al. [66] suggest that two protons are released upon zinc complexation. As individual zinc finger domains are small (a few kDa) and behave as almost complete independent units, they are synthetically accessible and are ideal model systems for entire nucleocapsid proteins [67].

The metal ion affinity of p55F1 was evaluated using Zn$^{2+}$, Cd$^{2+}$, Co$^{2+}$, and Ni$^{2+}$. A molar excess of the metal ion compared to p55F1 had to be used to detect a strong MALDI signal of the metal ion-p55F1 complex, whereas an equimolar amount of metal ion and peptide was sufficient to completely form the complex in solution.

![Figure 19. Schematic view of the Zn-p55F1 complex. The histidine and the three cysteine residues (all black) of the CCHC motif bind to the zinc(II) ion. The binding heteroatoms are depicted in grey.](image)

The complexation of the folded Zn-p55F1 to single-stranded nucleic acids is essential for the replication of HIV-1. Therefore, it has been extensively studied by various methods in solution [61, 68], mainly in view of developing antiviral agents for the treatment of acquired immunodeficiency syndrome (AIDS) [69]. Antiviral agents function by preventing peptide complexation to the nucleic acid. This aim can be achieved in two ways. The first is removal of Zn$^{2+}$ by addition of a chelating agent which has a higher affinity for Zn$^{2+}$ than the peptide [70, 71]. The second possibility is a chemical modification of the peptide’s metal-coordinating cysteine residues, mainly their oxidation to disulfide bonds which
5.1 Introduction

prevents complexation to Zn\textsuperscript{2+}. Efficient peptide modification capability has been demonstrated for different compounds: nitroso-containing compounds such as 3-nitrosobenzamide (NOBA) \cite{72}, cupric ions \cite{70}, cysteamine, thiamine disulfide and disulfiram \cite{73}, derivatives of disulfide benzamides (DIBA) \cite{74}, and azodicarbonamide \cite{75}. The latter two compounds are already used in clinical trials in the United States and Europe. Considering that the effort on finding new antiviral HIV agents was based on relatively time- and material-consuming methods, our aim was also to test the capability of MALDI for rapidly screening such antiviral agents.

South et al. \cite{68} investigated different triple complexes consisting of nucleic acid, Zn\textsuperscript{2+} and p55F1 with NMR in solution. In the noncovalent triple complex, the nucleic acid binds within a hydrophobic cleft onto the peptide surface. The interaction involves hydrogen bonds with guanosine, stacking of aromatic rings of both peptide and oligonucleotide, and an electrostatic contribution from the interaction of the positively charged side chain of arginine with the phosphodiester backbone of the nucleic acid. The authors showed that single-stranded oligodeoxynucleotides could be used as structural probes for ribonucleic acid-binding CCHC zinc fingers. The MALDI results presented here were carried out using oligodeoxynucleotides, mainly d(TTGTT), which had also been used in the NMR study of South et al. \cite{68}.

5.1.7 Methods Used

Solutions were studied by means of circular dichroism (CD) spectroscopy, while gas-phase behavior was studied using MALDI MS. Circular dichroism spectroscopy is suited to investigate the structure of the peptide and therefore of the peptide-metal ion complex, because the secondary structure elements (ß-sheet, ß-turn, or random coil) show characteristic CD spectra \cite{76}.

ATT was used as a MALDI matrix for the analysis of p55F1 and the metal ion-p55F1 complexes. Different authors showed that this matrix was suited for the detection of noncovalent compounds \cite{42-44, 51, 52}. It works at a physiological pH of the sample solution, which is necessary for complex formation. Such pH conditions are in contrast to normal MALDI conditions, where acidic matrices are used.

AMNP was used as a MALDI matrix for the analysis of the Zn-p55F1-oligodeoxynucleotide complexes. This nonacidic matrix is optimized for MALDI MS of oligodeoxynucleotides \cite{45}, but also allows the simultaneous detection of both
Specific Zinc Finger Peptide Complexes

p55Fl and the oligodeoxynucleotide in the positive ion mode. Finally, it permits work at physiological conditions of the sample solution, using ammonium bicarbonate to adjust the pH.

5.2 Experimental

5.2.1 Materials
The zinc finger peptide was synthesized and characterized in solution by Dr. Stefan Vetter, Department of Biochemistry, ETH Zürich (see below). The oligodeoxynucleotides d(TTGT), d(TTTTTGTTTTT), d(TTATT), and d(ACGCC) were purchased in the desalted form from Microsynth, Balgach, Switzerland. Zinc(II) chloride (ZnCl₂), cobalt(II) chloride hexahydrate (CoCl₂·6H₂O), nickel(II) chloride hexahydrate (NiCl₂·6H₂O), EDTA, DHB, TFA, and pH indicator strips (pH 1-11) were purchased from Fluka Chemie AG (Buchs, Switzerland). Diammonium hydrogen citrate, ammonium bicarbonate (NH₄HCO₃), and ATT were obtained from Aldrich Chemie (Buchs, Switzerland). Cadmium(II) chloride dihydrate (CdCl₂·2H₂O) and copper(II) chloride dihydrate (CuCl₂·2H₂O) were purchased from Siegfried Handel (Zofingen, Switzerland). Tris(hydroxymethyl)aminoethane (Tris), dithiothreitol (DTT), gramicidin S, luteinizing hormone releasing hormone (LHRH), AMNP, 2-amino-5-nitropyridine (ANP), 2,3,4-THAP, 2,4,6-THAP, and 3-HPA were acquired from Sigma Chemie (Buchs, Switzerland). [γ-32P]-ATP was obtained from Amersham (England) and T4-polynucleotide kinase from New England Biolabs (Beverly, MA, USA). The VS-dialysis membrane (0.025μm) was obtained from Millipore (Volketswil, Switzerland). The TentaGel S resin was purchased from Rapp Polymere (Tübingen, Germany). Fmoc amino acids were obtained from Novabiochem (Läufelfingen, Switzerland). All materials obtained from commercial sources were of highest purity available and were used without further treatment or purification. Aqueous solutions were prepared using bidistilled water.

5.2.2 Peptide Synthesis and Purification
The zinc finger peptide p55Fl has the sequence acVKCFNCGKEGHIA RNCRA-OH. A second peptide was also used for the experiment involving the variation of the molar ratio of peptide and zinc(II). It differs from p55Fl only by substitution of isoleucin by valine at position 12 (p55Flm). Judging from CD spectroscopy, the modification had no effect on the metal binding behavior. The peptides were acetylated at the N-terminal end and therefore have molecular
weights of 2049 and 2034 g/mol, respectively. They were synthesized manually on TentaGel S resin using Fmoc-protected amino acid derivatives. The peptides were purified on a reversed-phase HPLC C18 column.

5.2.3 Oligodeoxynucleotide Purification

The oligodeoxynucleotides were purchased in the desalted form. However, MALDI spectra of oligodeoxynucleotide-containing samples still showed various undesired alkali ion adducts to the oligonucleotide. Therefore, further desalination of the oligodeoxynucleotides using the Millipore “drop dialysis” method was required [77, 78]. With this method, alkali ions are exchanged against ammonium ions and upon crystallization of the exchanged sample with the matrix, ammonia evaporates. Overall, alkali ions are exchanged with protons. The MALDI mass spectra of the exchanged samples show only the protonated molecular ion peak of the oligodeoxynucleotide and hardly any alkali ion adducts. Typically, 10 µL of the aqueous oligodeoxynucleotide solution were placed on a VS-membrane, which was then allowed to float on a 100 mM solution of diammonium hydrogen citrate. After 0.5 to 1 minute, the sample was removed from the membrane.

5.2.4 Oxidation Behavior of the Peptide

The thiol groups of the CCHC motif’s three cysteine residues are oxidation sensitive. The HPLC-purified peptide was stored after lyophilization under an argon or nitrogen atmosphere at room temperature. No significant oxidation was observed during storage. When dissolved in degassed water at pH 7.5 and stored in a closed Eppendorf tube, less than 15 % of the peptide was oxidized within 4 hours (followed by using Ellman’s reagent and visible absorption spectroscopy at 412 nm [79]). Since we always worked with solutions that were freshly prepared or not older than 2 hours for MALDI and CD sample preparation, it is justified to not protect them from air. For the investigation of the metal ion specificity of p55F1, it was also important to know whether metal ion addition induces more extensive peptide oxidation, as transition metal ion-thiolate complexes are potentially oxidation sensitive. Within 1.5 hours, all metal ions studied (1.5 molar equivalents added to peptide) did not lead to any significant oxidation. As a control, we used Cu^{2+}, which we found in similar experiments to be much more effective in oxidizing the peptide. Using again a 50 % molar excess, Cu^{2+} led to 70 % oxidation of the peptide, most probably accompanied by reduction of Cu(II) to Cu(I).
5.2.5 MALDI Sample Preparation

Zn-p55F1 Complex
An aqueous ATT (35 mM, pH 4-5) matrix solution was used for MALDI sample preparation of the Zn-p55F1 complex. In order to vary the solution pH, ATT was dissolved in aqueous Tris (10 mM), NH₄HCO₃ (20 mM) or 0.1 % TFA in water to yield pH values of 7.5, 7 and 1.5, respectively. For one experiment, an aqueous DHB (100 mM, pH 2) matrix solution was used. Equal volumes of aqueous zinc finger peptide (0.1 mM), matrix, and aqueous metal chloride solutions (0.3 to 10 mM) were mixed to yield the molar ratios indicated in the figure captions. 2-4 µL of the resulting solution were deposited on the probe tip, partially dried in a cool air stream, and then completely dried in the vacuum. If not otherwise mentioned in the figure captions, the final sample solution had a pH value of 5. For the zinc complexation experiment with EDTA (Figure 23), the aqueous ligand solution (2.2 mM) was first desalinated for 15 minutes using the Millipore drop dialysis method, which was also used for desalination of the oligodeoxynucleotide solutions. An aliquot of the EDTA solution was added to a solution containing the peptide, matrix, and ZnCl₂ in order to yield the molar ratios indicated in the corresponding figure caption.

Zn-p55F1-Oligodeoxynucleotide Complex
10 µL of the aqueous p55F1 solution (0.5 mM) were mixed with 2.5 µL of an aqueous solution of ZnCl₂ (10 mM) and 2.5 µL of an aqueous oligodeoxynucleotide solution (6.1·10⁻⁵ M). Desalination of the oligodeoxynucleotide solutions was performed according to the procedure described above. The concentration of ZnCl₂ is unusually high for MALDI experiments but was found to work well. The pH value was adjusted to 6.5-7 by addition of NH₄HCO₃. 3.5 µL of this mixture were mixed with 10 µL of a saturated, aqueous AMNP solution. 2-4 µL of the resulting solution were deposited on the probe tip and dried in a cold stream of air.

5.2.6 Circular Dichroism Spectroscopy
Measurements were performed on a Jasco J-600 spectropolarimeter. The temperature (22-23°C) was controlled using a water-jacketed cuvette holder and an external water bath. Spectra were recorded between 190 and 280 nm in a 0.1 mm pathway cuvette. The spectra were averaged over three scans. The peptide was dissolved in 50 mM Tris buffer pH 7.5 (1 mM). Concentrations of metal salts in 50 mM Tris buffer pH 7.5 were chosen in order to obtain the final solution by a 1:1 (v:v) mixing with the peptide solution.
5.2 Experimental

5.2.7 Electrophoretic Mobility Shift Assays

Radioactive oligodeoxynucleotide labeling was performed by incubation with $[\gamma^{32}P]$-ATP in the presence of T4-polynucleotide kinase. For the affinity measurements, labeled oligodeoxynucleotides in water ($10^{-7}$ M) were incubated with increasing amounts of Zn-p55F1 (molar ratios from 1:0.25 to 1:2500) in EMSA (electrophoretic mobility shift assays) buffer (50 mM Tris-HCL, pH 7.9, 6 mM MgCl$_2$, 40 mM ammonium sulfate, 0.2 mM EDTA, 1 mM DTT). The Zn-p55F1 concentration range was chosen in order that the dissociation constant of the triple complex be in between. The final volume for all mixtures was 20 µl. The mixtures were applied on a 10 % polyacrylamide gel (14.8x18x0.1 cm, gel preparation see [80]). Electrophoresis was performed in 0.9xTris-acetate-EDTA (TAE) buffer pH 7.9 [80] using 200 to 300 V during 2 to 4 hours at 4 °C. Gels were dried on a BioRad Model 583 Gel Drier and exposed with Kodak X-OMAT-S films at −70°C.
5.3 Zinc Finger Peptide Complexes with Metal Ions

![Diagram of MALDI mass spectra](image)

**Figure 20.** MALDI mass spectra of A. peptide p55F1 alone, B. peptide and Zn$^{2+}$ in 1:10 molar ratio, and C. peptide and Zn$^{2+}$ in 1:10 molar ratio, with gramicidin S.

Figure 20 depicts the MALDI mass spectra of p55F1 with ATT as a matrix. The spectrum in Figure 20A of the apopeptide is dominated by the protonated molecular ion peak, [P+H]$^+$ (m/z 2050). Minor signals correspond to the sodiated molecular ion, and impurities at masses P+48 and P-43, the latter possibly representing the nonacetylated peptide. Upon addition of 10 molar equivalents of Zn$^{2+}$ (Figure 20B), a signal corresponding to the peptide adduct with Zn$^{2+}$, [P-H+Zn]$^+$ (m/z 2114), appears. This composition has been confirmed by FTICR mass spectrometry: the difference between the monoisotopic masses of the protonated peptide and the zinc-peptide adduct equals 62.0, corresponding to the monoisotopic mass of (Zn-2H).

The observation that singly charged metal ion-peptide complexes are detected, even though doubly charged metal ions are used, is in agreement with results
from Woods et al. [42] (see section 5.1.5) and Wong et al. [81]. Wong et al. report deprotonation of the analyte instead of reduction of the metal ion to obtain singly charged ions if labile protons, e. g. present in polar molecules, are available in the analyte molecules. Furthermore, due to its redox behavior, no reduction is expected if Zn$^{2+}$ is used as the metal ion.

The spectra in Figure 20 and in the other figures in this section were normalized to the [P+H]$^+$ signal. In the following, the ratio of the intensities (peak integrals) of the [P-H+Zn]$^+$ signal to the [P+H]$^+$ signal is called relative intensity of [P-H+Zn]$^+$.

The [P-H+Zn]$^+$ signal can either represent a specific 1:1 complex or a non-specific adduct. To check this, the peptide gramicidin S, which does not form specific adducts with Zn$^{2+}$, has been added to the peptide- and Zn$^{2+}$-containing solution (Figure 20C). The relative intensity of [P-2H+Zn+H]$^+$ is the same as without addition of gramicidin S. The Zn$^{2+}$-adduct of gramicidin S, [G-H+Zn]$^+$, is negligible. This experiment indicates a specific 1:1 complex of Zn$^{2+}$ with p55F1. With this knowledge, [P-H+Zn]$^+$ can be written as [P-2H+Zn+H]$^+$ in order to account for the fact that p55F1 loses two thiol protons upon Zn$^{2+}$ complexation, and acquires an additional proton to form a singly charged species [66]. Clearly it is important to know whether this specific complex is formed in the condensed phase or in the gas phase after desorption.

The association reaction of Zn-p55F1 in solution can be written as:

$$p55F1 + Zn^{2+} \rightleftharpoons [(p55F1)+Zn]^{2+} \quad (5)$$

Equilibria similar to (5) exist for metal centers other than Zn$^{2+}$, but unfortunately, complex formation constants with p55F1 are not available in the literature. They are assumed to be similar to those reported for a CCHC sequence in murine leukemia virus, i.e. $10^{10}$ M$^{-1}$ for the Zn$^{2+}$, and between $10^5$ and $5\cdot10^7$ M$^{-1}$ for the Co$^{2+}$ complex [63, 64]. Cd$^{2+}$ has the same valence electronic configuration as Zn$^{2+}$ but a larger ionic radius. Krizek et al. [82] indicate a similar formation constant for the Cd$^{2+}$- and the Zn$^{2+}$-complex with a zinc finger peptide containing the sequence Cys-X$_2$-Cys-X$_2$-His-X$_3$-Cys. It is not known whether these data are also valid for the p55F1 peptide, because the effect of a change in the chain length between the cysteine and histidine residues and of a different amino acid sequence cannot easily be estimated. For these reasons, the affinity of p55F1 for different metal ions was investigated using CD spectroscopy and MALDI MS.
Figure 21 shows the CD spectra of p55F1 alone and in the presence of various metal ions. The spectrum of apo-p55F1 (Figure 21D) is typical for an unstructured peptide (random coil). It is characterized by a negative minimum at 198 nm. Addition of various metal ions induces significant changes in the spectrum. A nuclear magnetic resonance study of the Zn$^{2+}$-complexed peptide revealed a β-turn as the dominant secondary structural element and a short β-sheet [65]. β-Turns are reported to show a positive CD band between 205 and 220 nm [83]. Indeed a positive band with a maximum at 212 nm is found in the Zn$^{2+}$-p55F1 complex besides a 5 nm red shift of the negative minimum (Figure 21A). However, CD active charge transfer bands appear between 200 and 230 nm in spectra of Zn$^{2+}$-containing metallothioneins [84]. It is difficult to attribute the band at 212 nm either to the β-turn or to the charge transfer effect and therefore no unambiguous conformational analysis based on these CD spectra was possible. The CD region between 190 and 200 nm was chosen to investigate the metal-peptide interaction. This region is not affected by CD bands related to metal peptide charge-transfer effects. Since complexation induces folding, the CD spectra give a qualitative estimate of the formation constants of the different metal ion-p55F1 complexes. In terms of binding hierarchy, the formation constants with p55F1 decrease in the series from Zn$^{2+}$ over Cd$^{2+}$/Co$^{2+}$ to Ni$^{2+}$. The CD spectrum of Co$^{2+}$-p55F1 was about the same as the one of the Cd$^{2+}$-complex (data not shown).

If MALDI spectra reflect solution behavior, then these differences should be reflected in the relative intensities of the metal ion-peptide complex signals.
Figure 22 shows MALDI spectra of the corresponding samples. The relative intensities of the metal ion-peptide signals are smaller for Cd\(^{2+}\) (m/z 2161) and Ni\(^{2+}\) (m/z 2107) than for Zn\(^{2+}\). Relative signal intensities for the Ni\(^{2+}\) and Cd\(^{2+}\)-complexes as well as for the Co\(^{2+}\)-complex (not depicted) are roughly the same. MALDI spectra therefore clearly suggest a high affinity for Zn\(^{2+}\) and a lower affinity for Cd\(^{2+}\), Ni\(^{2+}\), and Co\(^{2+}\). This was observed using both the basic matrix ATT and the acidic matrix DHB.

![Figure 22. MALDI mass spectra of p55F1 alone and with different metal ions in 1:10 molar ratio.](image)

The [P-2H+Ni+H\(^+\)] signal in C is broader than the other metal ion-peptide complex signals because the Ni\(^{2+}\)-complex signal overlaps with the impurity signal at [P+48]\(^+\).

To sum up, these experiments indicate a correlation between the behavior of the metal ion-peptide complexes in solution and in the gas phase. Moreover, they show that in contrast to the above-mentioned zinc finger sequence Cys-X\(_2\)-Cys-X\(_{12}\)-His-X\(_3\)-Cys from Krizek et al. [82], the sequence Cys-X\(_2\)-Cys-X\(_4\)-His-X\(_4\)-Cys in p55F1 has a lower affinity for Cd\(^{2+}\) than for Zn\(^{2+}\). As a corollary, our
results also support the discrimination between Zn$^{2+}$ and Co$^{2+}$ by the same CCHC zinc finger motif in the murine leukemia virus [63, 64].

It should be emphasized that, in the present study, a correlation between the behavior of the metal ion-peptide complex in solution and in the gas phase is said to exist if changes in solution conditions lead to corresponding changes in the MALDI spectra. Absolute complex abundances in solution and complex peak intensities in the MALDI spectra cannot be directly compared.

![ MALDI mass spectra of A. p55F1 and Zn$^{2+}$ in 1:10 molar ratio, and B. p55F1, Zn$^{2+}$, and EDTA in 1:10:12 molar ratio.](image)

**Figure 23.** MALDI mass spectra of A. p55F1 and Zn$^{2+}$ in 1:10 molar ratio, and B. p55F1, Zn$^{2+}$, and EDTA in 1:10:12 molar ratio.

The formation constant for the Zn$^{2+}$-EDTA complex in solution is $10^{16}$ M$^{-1}$ [85]. It is several orders of magnitude larger than the formation constant of the Zn-p55F1 complex. Addition of EDTA to a solution containing this Zn-p55F1 complex is expected to shift equation (5) to the left side since Zn$^{2+}$ is eliminated from the equilibrium through complexation with EDTA. This was confirmed by CD spectroscopy. The spectrum of a solution of p55F1 and Zn$^{2+}$ (Figure 21A) which showed an ordered structure of the peptide changed its appearance to that of the unfolded peptide as shown in Figure 21D when a slight molar excess of EDTA compared to Zn$^{2+}$ was added. The corresponding MALDI spectra are depicted in Figure 23. It can clearly be seen that the [P-2H+Zn+H]$^+$ signal in Figure 23A disappears when EDTA is added (Figure 23B). The obtained spectrum looks like the one of the peptide alone (Figure 20A). These results re-
5.3 Zinc Finger Peptide Complexes with Metal Ions

reveal a reversible complex formation between Zn$^{2+}$ and p55Fl which can be monitored both by CD spectroscopy and MALDI MS.

![Figure 24.](image)

**Figure 24.** Titration of p55Fl with Zn$^{2+}$. The signal intensity in presence of Zn$^{2+}$ relative to the signal intensity of the unfolded peptide in the CD spectra at 212 nm is depicted as a function of the molar ratio of Zn$^{2+}$ to peptide. Solvent: 50 mM aqueous Tris, pH = 7.5.

We next investigated the effect of an increase of the molar ratio of Zn$^{2+}$ to p55Fl in solution on the MALDI spectra. According to equation (5), an increase of the amount of complexes formed in solution is expected because the equilibrium is shifted to the right. CD spectroscopy confirmed this observation. Spectra were taken at different molar ratios of Zn$^{2+}$ to peptide. Figure 24 shows the relative signal intensity in the CD spectra at 212 nm as a function of the molar ratio. The intensity rises up to a molar ratio of about 1:1 and then reaches saturation. The same is observed when taking the relative signal intensity at 198 nm. This agrees with observations from Frankel *et al.* [86]. The behavior reflects an increase of the peptide’s ordered structure with the molar ratio of Zn$^{2+}$ to peptide, i.e. an increasing amount of complexed peptide, and a specific interaction between Zn$^{2+}$ and p55Fl (see section 5.1.3 for the definition of specificity). Figure 25 shows the MALDI spectra of p55Flm at different molar ratios with Zn$^{2+}$ using DHB as a matrix. An increase of the relative intensity of the [P-2H+Zn+H]$^+$ signal with the molar ratio of Zn$^{2+}$ to peptide is clearly visible. This result correlates with the result from CD spectroscopy.
We have seen that in solution a molar ratio of Zn$^{2+}$ to peptide of 1:1 is sufficient to completely form the Zn$^{2+}$-peptide complex, whereas a large excess of Zn$^{2+}$ is needed to see a significant [P-2H+Zn+H$^+$]$^+$ signal besides the [P+H]$^+$ signal in MALDI spectra. This observation can possibly be explained as follows. First, the desorption, ionization, and detection efficiencies of [P+H]$^+$ and [P-2H+Zn+H$^+$]$^+$ are different. The same amount of both the peptide and the Zn$^{2+}$-peptide complex in solution can probably lead to a peptide MALDI signal that is much more intense than the complex signal. Second, the pH of the solution for the MALDI experiments was lower than the one for the CD experiments, resulting in a decrease in the relative intensity of the [P-2H+Zn+H$^+$]$^+$ signal. (see pH study below). Third, it is possible that a certain amount of the complex ions present in

![MALDI mass spectra](image-url)

**Figure 25.** MALDI mass spectra of p55Flm and Zn$^{2+}$ at different molar ratios. The final solution has a pH value of 2.5. The signal at 2200 Da (marked with a question mark in spectrum D) could not be identified.
the solid phase dissociates during the MALDI process. Fourth, partial oxidation of the peptide during MALDI sample preparation may adversely affect the complexation. As CD spectroscopy and MALDI mass spectrometry rely on completely different principles, the results can therefore not be compared quantitatively, but only qualitatively. This is sufficient to establish a correlation between the behavior of a species in solution, probed by CD spectroscopy, and in the gas phase, probed by MALDI MS.

A further MALDI experiment revealed that besides the ions formed in the condensed phase prior to desorption, some are also formed by gas-phase reactions after desorption. A solid mixture of peptide in DHB was thoroughly mixed in different molar ratios with a solid mixture of ZnCl₂ in DHB. All components were dried before use and manipulated in a glove box in order to prevent dissolution on the particles' surface and thus complex formation prior to the MALDI experiment. The MALDI spectra showed a [P-2H+Zn+H]+ signal, whose relative intensity increased with the molar ratio of Zn²⁺ to peptide. Due to the reactions in the MALDI plume, no saturation of the intensity of the metal ion-peptide signal is observed. It would have been expected if preformed ions alone contributed to the ion signal.

In order to account for the pH dependence of the complexation of p55F1 and Zn²⁺ in solution, which is not included in equation (5), a modified complexation equilibrium must be considered. At neutral pH, the basic side chains of the two arginine and the two lysine residues of p55F1 are protonated, and glutamic acid, as well as the terminal carboxylic acid group are deprotonated. This results in a doubly protonated peptide. Upon formation of the tetrahedral metal ion-zinc finger peptide complex, at least two of the three cysteine -SH groups are deprotonated [66]:

\[
[(\text{p55F1})+2\text{H}]^{2+} + \text{Zn}^{2+} \rightleftharpoons [(\text{p55F1})+\text{Zn}]^{2+} + 2\text{H}^+ \quad (6)
\]

According to equation (6), addition of base leads to an increase in the amount of metal ion-peptide complexes. Above pH 6, CD spectra of solutions of peptide and Zn²⁺ in molar ratio 1:1 in the presence of ATT matrix reveal an ordered peptide structure similar to the one seen in the CD spectrum in Figure 21A, which disappears at lower pH values. This means that the complex between p55F1 and Zn²⁺ is not stable at a pH < 6. Figure 26 shows MALDI spectra of p55F1 in the presence of Zn²⁺ ions at various pH values of the sample solutions.
ATT could successfully be used as a matrix at different pH values which was not possible for an acidic matrix such as DHB. The relative intensity of the [P-2H+Zn+H]+ signal increases with pH. In the MALDI spectrum of the solution buffered with Tris to pH 7.5, peptide adducts with two Zn²⁺ ions, with ATT, as well as with Zn²⁺ and ATT are also detected besides sodium and potassium adducts. Binding of the second Zn²⁺ ion is assumed to be nonspecific because the zinc finger motif exclusively complexes to one Zn²⁺ ion. A further experiment with NH₄HCO₃ to adjust the pH yielded the same MALDI spectrum as with Tris buffer. Obviously, Tris, which is not lyophilizable as is NH₄HCO₃, does not deteriorate the MALDI spectra. This confirms the salt tolerance of this method [87]. The study on the pH dependence of the complexation between peptide and Zn²⁺ demonstrated that MALDI results reflect solution behavior. For the size of peptide used in the study, singly charged species are commonly observed in
MALDI mass spectra. In the MALDI plume, the charge state of the species in solution is balanced by protonation or deprotonation.

Note that the MALDI spectra at a pH below 6 exhibit Zn$^{2+}$-peptide complex signals, whereas CD spectra of the solutions at this pH do not. This is most probably due to the fact that MALDI spectra are taken from crystalline samples. We have shown in chapter 4 that the amount of complexes present in the solid phase is higher than in solution if volatile byproducts, such as HCl, can form upon complexation. This is the case here, because ZnCl$_2$ was used as metal salt. The reason for this is that HCl is eliminated during crystallization, resulting in a shift of equilibrium (6) to the right.
5.4 Zinc Finger Peptide-Oligodeoxynucleotide Complexes

5.4.1 MALDI Sample Preparation

An adequate sample preparation procedure for the detection of Zn-p55F1-oligodeoxynucleotide had to be found initially. It had to permit simultaneous detection of the oligodeoxynucleotide and the peptide as well as permit work at neutral pH. Generally, the matrix selection for oligonucleotides is more limited than that for peptides. Therefore, mainly matrix compounds used in the literature for the MALDI detection of oligodeoxynucleotides were tested: AMNP and ANP [45], ATT [43, 44, 51, 52], a mixture of 2,3,4-THAP and 2,4,6-THAP [88-90], 3-HPA [88], and DHB. AMNP and ANP worked equally well for all oligodeoxynucleotides tested. In positive ion mode, the peptide and the oligodeoxynucleotide signals were of about the same intensity; the peptide could not be seen in negative ion mode. Using the same analyte molar ratios, ATT led to the detection of the peptide and the oligodeoxynucleotide in the negative ion mode, whereas only the peptide could be observed in the positive ion mode. We chose to work with AMNP in positive ion mode with a molar ratio of peptide to oligodeoxynucleotide of 33:1 which resulted in about the same intensity of both species. The molar ratio of AMNP to the analytes was chosen as to result in good quality MALDI spectra. About the same molar ratio of Zn\(^{2+}\) to peptide as used in the experiments on Zn-p55F1 complexation was used. AMNP also permitted work at neutral pH conditions using ammonium bicarbonate to adjust the pH. During crystallization of a sample containing this salt, carbon dioxide, water and ammonia evaporate. No perturbing ions are present in the sample, which would be the case if, for example, sodium bicarbonate was used.

MALDI spectra of oligodeoxynucleotides exhibit undesired alkali ion adducts, which have to be eliminated prior to analysis. This task can be performed using three different methods: addition of diammonium hydrogen citrate [89], drop dialysis with diammonium hydrogen citrate [91], or ion exchange using the Nafion 117 membrane [92]. These methods are described in detail in the indicated literature. Here, only a brief overview is given. By adding diammonium hydrogen citrate to the sample, alkali ions are replaced by ammonium ions. Upon sample crystallization, ammonia evaporates and the overall result is the exchange of alkali ions with protons. The same result, but without addition of diammonium hydrogen citrate to the sample can be achieved using the drop dialysis method. Here, the sample solution is placed on the VS-dialysis membrane which floats on
5.4 Zinc Finger Peptide-Oligodeoxynucleotide Complexes

a solution of diammonium hydrogen citrate. Ammonium ions are exchanged against alkali ions over the membrane. In the method using Nafion 117 membrane, a 3N HCl solution is used instead of an diammonium hydrogen citrate solution. This method is more efficient than drop dialysis. We found that 0.5 to 1 minute of drop dialysis led to MALDI spectra of oligodeoxynucleotides without alkali ion adducts. Longer exchange times and the use of the Nafion 117 membrane led to glossy amorphous samples after cocrystallization with the matrix, which significantly decreased MALDI signal intensity.

To summarize, the sample preparation procedure for the study of Zn-p55F-oligodeoxynucleotide includes the use of AMNP matrix solution adjusted to neutral pH with ammonium bicarbonate, drop dialysis of the oligodeoxynucleotide samples, and detection of the analytes in the positive ion mode.

5.4.2 MALDI Experiments

Figure 27A shows the MALDI spectrum of a mixture of d(TTGTT) and p55F1.

![MALDI mass spectra](image)

**Figure 27.** MALDI mass spectra of p55F1 and d(TTGTT) in a 33:1 molar ratio at pH 6.5-7. **A.** without addition of Zn$^{2+}$, **B.** with Zn$^{2+}$, Zn$^{2+}$:p55F1 molar ratio = 5:1, **C.** with Zn$^{2+}$, Zn$^{2+}$:p55F1 molar ratio = 10:1.
The protonated molecular ion signals of p55F1, [P+H]^+, and of d(TTGTT), [O+H]^+, are observed besides a weak peak of an oligodeoxynucleotide fragment (m/z = 1399) and a low intensity nonspecific adduct of both components, probably a result of electrostatic interactions. Upon Zn^{2+} addition (Figure 27B), the specific triple complex of p55F1, Zn^{2+} and d(TTGTT), which we expect from solution chemistry, is observed (m/z = 3598). Less intense p55F1-d(TTGTT) adducts without Zn^{2+} and with two Zn^{2+} ions are also observed. Furthermore, Zn^{2+}-adducts to the oligodeoxynucleotide as well as the specific Zn-p55F1 described in the previous section are detected. All signals correspond to singly charged species. The excess charge of Zn^{2+} is compensated by deprotonation of the peptide or the oligodeoxynucleotide, as confirmed by high resolution FTICR mass spectrometry. Adduct peaks at higher mass, which would result from nonspecific gas-phase aggregation, are of negligible intensity.

We also succeeded in detecting the triple complex between p55F1, Zn^{2+}, and an oligodeoxynucleotide 11-mer, d(TTTTTGTTTTT) using a similar sample preparation for MALDI MS (Figure 28).

![Figure 28. MALDI mass spectrum of Zn-p55F1-d(TTTTTGTTTTT). Molar ratio Zn^{2+}: p55F1:d(TTTTTGTTTTT) = 165:33:1, pH = 6.5-7.](image_url)

The signals of both the triple complex and the Zn-p55F1 complex are less intense than those of the individual components. The reasons in the case of Zn-p55F1 have already been discussed. Similar arguments can also be made for the triple complex. Variations in desorption, ionization, and detection efficiencies among the triple complex, the Zn-p55F1 complex, the peptide (present in excess), and d(TTGTT), partial peptide oxidation or partial dissociation of the complex ions...
upon crystallization or during the MALDI process may all contribute to the triple complex signal being less intense than that of its components. As already pointed out in the previous section, it is therefore clear that absolute complex abundances in solution and complex peak intensities in the MALDI mass spectra cannot be directly compared. Conclusions concerning complex specificity can also not be drawn purely on signal intensities. Carefully designed control experiments have to be performed, such as the variation of the pH or competition experiments. Such controls have already been done to prove the specificity of Zn-p55F1. Similar controls for Zn-p55F1-d(TTGTT) are described below.

If a larger excess of Zn$^{2+}$ is added to the sample than shown in Figure 27B, the relative intensity of the Zn-p55F1 signal increases, as expected from the previous chapter (Figure 27C). Moreover, multiple Zn$^{2+}$-adducts to d(TTGTT) are observed. In the mass range of the p55F1-d(TTGTT) adducts, the adduct with two zinc ions dominates over the triple complex. This result raises the question of whether the triple complex observed in MALDI is indeed specific or whether it is simply the result of nonspecific aggregation. The first statement is correct and is supported by the following experimental results.

**Figure 29.** Spacefilling model of Zn-p55F1-d(ACGCC). Oxygen atoms are depicted in black. The zinc ion is not visible in this view.
Figure 29 depicts the spacefilling model of the Zn-p55F1-d(ACGCC) complex in solution [68]. The structure of this complex is similar to that with d(TTGTT). The oligodeoxynucleotide binds to Zn-p55F1 through its bases, and the negatively charged phosphate groups of the phosphodiester backbone are exposed at the surface. At relatively low metal ion concentrations, these phosphate groups are hardly occupied by metal ions, but at high concentrations they nonspecifically bind to the metal ions through electrostatic interactions. In addition to the zinc ion specifically bound to p55F1, the triple complex therefore binds nonspecifically to further zinc ions through the oligodeoxynucleotide’s phosphodiester backbone. This explains the appearance in the MALDI spectrum in Figure 27C of an intense Zn$^{2+}$-adduct to the triple complex signal besides the triple complex signal, as well as the presence of Zn$^{2+}$-adducts to the oligonucleotide alone. These dominate over the protonated oligodeoxynucleotide signal. If these assumptions are valid, the nonspecifically-bound zinc ions occupying the phosphate groups should be partially displaced upon addition of an excess of sodium ions. In the MALDI mass spectra, this should lead to the presence of intense Na$^{+}$-adducts to the oligodeoxynucleotide and to the triple complex instead of the corresponding Zn$^{2+}$-adducts. The specifically-bound zinc ion should not be ejected. This is exactly what is observed experimentally. The corresponding MALDI spectra are depicted in Figure 30. The experiment supports our assumption of a specific Zn-p55F1-oligodeoxynucleotide complex.

The investigation of Zn-p55F1 with CD spectroscopy in solution showed that this complex is only stable at pH > 6. This is what we also expect for the triple complex, since the oligodeoxynucleotide only binds to the zinc-complexed p55F1. If MALDI spectra reflect solution-phase behavior, then decreasing the pH to a value below 6 should lead to a significant decrease of the MALDI signal of the specific triple complex. This is exactly what is observed experimentally (Figure 31). Besides a strong decrease of the Zn-p55F1 signal compared to that of p55F1, the triple complex signal is absent. Instead, weak signals of a nonspecific Zn$^{2+}$-adduct distribution to p55F1-d(TTGTT) and to the oligonucleotide dimer are observed. We assume that these are nonspecific adducts with the overall positively charged p55F1 and with Zn$^{2+}$ ions. This assumption fits well with the observation of multiple Zn$^{2+}$-adducts to the oligonucleotide alone in the mass spectrum in Figure 31B.
5.4 Zinc Finger Peptide-Oligodeoxynucleotide Complexes

Figure 30. MALDI mass spectra of Zn-p55F1-d(TTGTT) in a 165:33:1 molar ratio at pH 6.5-7. A. without Na+, B. addition of an excess of Na+ compared to Zn2+. Multiple Na+-adducts to d(TTGTT) (see inset) and to the triple complex are observed. At the mass of the triple complex, they cannot be resolved.

Figure 31. MALDI mass spectra of Zn-p55F1-d(TTGTT) in 165:33:1 molar ratio A. at pH 6.5-7, B. at pH 5.5-5.5.
In order to further confirm the specificity of Zn-p55F1-d(TTGTT), another peptide (luteinizing hormone releasing hormone, LHRH, m/z = 1182 Da) was added to the sample (Figure 32B). LHRH contains a histidine residue in its sequence and thus potentially binds to Zn\(^{2+}\) ions. It also contains arginine with a positively charged side chain, which may bind through electrostatic forces to the oligodeoxynucleotide backbone. Both interactions would be nonspecific. The spectrum shows that neither a Zn\(^{2+}\)-adduct to this peptide (expected at m/z = 1246) nor a triple complex with Zn\(^{2+}\) and d(TTGTT) (expected at m/z = 2730) is detected. However, the corresponding complex signals of p55F1 are as intense as in the absence of LHRH (Figure 32A). This experiment supports the interpretation that the complex formation of Zn\(^{2+}\) with p55F1 and d(TTGTT) observed with MALDI MS is specific.

![Figure 32. MALDI mass spectra of Zn-p55F1-d(TTGTT) in 165:33:1 molar ratio A. at pH 6.5-7, B. with addition of LHRH at pH 7. Note that the Zn-p55F1 signal is even more intense because of a slightly higher pH value in B. as compared to A.](image)

The study of p55F1 using different metal ions showed that an excess of Cu\(^{2+}\) compared to p55F1 oxidizes the peptide’s thiol functions to an intramolecular disulfide bond by simultaneous reduction of Cu\(^{2+}\) to Cu\(^{+}\). Cu\(^{2+}\) mimics the action of an antiviral agent in the sense that it chemically modifies the cysteine residues. As already pointed out in the introduction to this chapter, modified p55F1 neither
forms complexes with Zn\(^{2+}\) nor with oligodeoxynucleotides. This is also reflected in the MALDI spectrum: if Cu\(^{2+}\) is added to the sample, the cysteine residues are oxidized and the signal of the triple complex disappears (Figure 33). A strong p55Fl dimer is detected instead, because peptide oxidation can lead to intermolecular disulfide bonds.

**Figure 33.** MALDI mass spectra of Zn-p55Fl-d(TTGTT) in 165:33:1 molar ratio. **A.** without addition of Cu\(^{2+}\), **B.** with addition of Cu\(^{2+}\) in the same molar amount as Zn\(^{2+}\). The metal ion adduct to p55Fl (m/z = 2113) and to its dimer (m/z = 4262) stem from a nonspecific gas-phase reaction.

Besides the complexation of Zn-p55Fl with d(TTGTT) (association constant is \(\sim 10^5 \text{M}^{-1}\), [68]), other sequences, like d(TTATT) and d(ACGCC) have also been investigated. Values of the corresponding solution-phase binding constants, to which the MALDI results are compared, are controversially discussed in the literature. We therefore aimed to determine the values using electrophoretic mobility shift assays (EMSA) on native polyacrylamide gels. Unfortunately, no binding could be detected using this method. Elimination of zinc-complexing EDTA in the buffers, using an excess of zinc, or work at different electric field strengths did not improve the result. Presumably, the triple complexes were not stable on the gel. We therefore had to rely on literature data.
Lam et al. [61] found from spectroscopic measurements that solution-phase binding constants of the nucleic acid binding protein NCp7, which contains a second zinc finger besides p55F1, with homopolymers of the different nucleotide bases all lie within one order of magnitude. For a synthetically-prepared peptide sequence corresponding to the second zinc finger of NCp7, they observed even smaller differences. These authors as well as Gorelick et al. [93] conclude that not one zinc finger alone is responsible for the nucleic acid sequence specificity of large zinc finger-containing proteins, but that several zinc fingers have to act in concert. In contrast, South et al. [68] claimed that Zn-p55F1 alone is sequence specific, based on different line broadenings found in the proton NMR spectra of d(TTGTT), d(TTATT), and d(ACGCC). However, these data only provide information on interactions involving protons, such as stacking and hydrophobic interactions, and can only be considered qualitative. Our MALDI experiments with d(TTATT) instead of d(TTGTT) support the findings of Lam et al. [61] and Gorelick et al. [93]: We found that the complex of d(TTATT) with Zn-p55F1 gave signals of about the same intensity as with d(TTGTT). MALDI experiments with oligodeoxynucleotide sequences lacking thymidine are, unfortunately, not very informative. If d(ACGCC) was used as the binding partner hardly any MALDI signal could be seen at all, although the corresponding triple complex should be stable in solution [68]. It is common knowledge that oligodeoxynucleotides that do not contain thymidines are much less stable as ions and thus much more difficult to detect by MALDI MS. Also, they may crystallize less favorably with the matrix, using the same sample preparation, than those containing mostly thymidines [47, 94].

The experiments probing the sequence specificity of p55F1 reveal a current limitation of MALDI: a “MALDI window” in which the target complex is observed has to be found. The MALDI window is given by the experimental sample preparation conditions, such as the nature of the matrix or the matrix analyte ratio. Once this window is found, experiments involving, for example, pH or oligodeoxynucleotide sequence variations can be performed. However, if a change of the experimental conditions prohibits observation, no conclusions can be drawn. An example is the above described experiment involving an oligodeoxynucleotide sequence change: the experimental conditions adequate for the sequences d(TTGTT) and d(TTATT) were not adequate for d(ACGCC). No conclusion on the sequence-specificity of Zn-p55F1 can therefore be drawn. Future work has to concentrate on finding more generally usable experimental conditions.
5.5 Summary and Perspectives

We studied the complexation of p55F1 with different metal ions as well as the complexation of Zn-p55F1 with oligodeoxynucleotides using CD spectroscopy of the solution and MALDI MS of the gas phase.

As an example for peptide-metal ion and noncovalent complexes, the complexes Zn-p55F1 and Zn-p55F1-d(TTGTT) could be detected with MALDI MS using the nonacidic matrices ATT and AMNP. Spectra were obtained from consecutive laser shots. The intensity of the complexes was lower than that of their components. A specific interaction in these complexes could be proven by performing carefully designed chemical controls, such as the variation of pH or competition experiments. All of these observations confirmed those of others made upon detection of noncovalent complexes with MALDI MS [42-44, 55]. We do not have experimental evidence for supporting the specificity criteria pointed out in papers from the Hillenkamp group [40, 41]. These authors claim specificity of a noncovalent complex only if its intensity is higher than the intensity of the components, which is only observed from the first laser shot. The authors assume that the complex crystallizes on top of the matrix and is completely removed with the first laser shot.

We clearly demonstrated that MALDI spectra may reflect solution-phase chemistry of peptide-metal ion and noncovalent complexes. We showed that it is possible to qualitatively study metal-binding properties of peptides and the pH dependence of the corresponding complexes. Care has to be taken when interpreting the corresponding MALDI spectra. Comparisons with results from solution-phase chemistry can only be done qualitatively and not quantitatively. MALDI spectra are not directly taken from analyte in solution and many other processes such as crystallization, transfer into the gas phase, strengthening of some of the noncovalent forces, weakening of others, or gas-phase reactions may interfere. In this sense, stating that MALDI spectra reflect solution-phase chemistry means that changes in solution conditions lead to corresponding changes in the MALDI spectra. Absolute complex abundances in solution and complex peak intensities in the MALDI spectra can, of course, not be directly compared. Conclusions concerning complex specificity can also not be drawn purely on signal intensities. Chemical controls have to be carefully designed to extract the relevant information from the spectra.
Using cupric ions as an example of an antiviral agent, we demonstrated that MALDI MS is a potential method for rapidly screening some antiviral HIV agents. For establishing MALDI MS as a reliable method for performing this task in the future, further antiviral agents should be tested and experiments should also be performed with proteins and nucleic acids isolated from cells. In this case, low detection limits have certainly to be demonstrated.
5.6 References


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6 Investigation of Cyclodextrin Inclusion Complexes

6.1 Introduction

Cyclodextrins (CDs) are water soluble cyclic oligomers composed of six (α-CD), seven (β-CD), eight (γ-CD), or more α-1,4-linked D-glucopyranose units [1-5]. They have a slightly conical form with all the secondary hydroxyl groups located on the exterior of the larger rim and all the primary hydroxyl groups on the smaller rim. The inner surface of the cavity is lined by glycosidic oxygen and hydrogen atoms and is therefore rather hydrophobic. The depth of all cyclodextrin cavities is the same (7.5 Å), but their diameters vary with the number of glucopyranose units (α-CD ~ 5 Å, β-CD ~ 7 Å, γ-CD ~ 9 Å). Cyclodextrins can incorporate a variety of hydrophobic guest molecules with an appropriate size into their cavity, forming an inclusion complex held together by noncovalent interactions. Inclusion complex formation may change the guest’s original physical and chemical properties, resulting, for example, in an increased solubility of the apolar guest in aqueous media, increased stability of sensitive guest molecules, and reduced volatility of highly volatile guests. These effects are exploited in many industrial applications, notably in the food and pharmaceutical industries [2].

Various methods have been used to study inclusion complex formation in the condensed phase, including kinetic analyses, spectroscopic (NMR, ESR, absorption and fluorescence), electrochemical, electrophoretic, and chromatographic methods [2, 4, 6, 7]. The investigation of cyclodextrin inclusion complexes by soft ionization mass spectrometry (MS) techniques in the gas phase, especially FAB and ESI has been pursued since the 1990s. An important question for mass spectrometrists studying inclusion complexes has always been whether the MS data represent solution chemistry. While in FAB MS, it is widely assumed that the mass spectra reveal solution-phase chemistry, this assertion is still controversially discussed in ESI MS [8, 9].

Prokai and coworkers [10] investigated cyclodextrin complexes with non-aromatic and aromatic amino acids. Using collision-induced dissociation, they probed their relative gas-phase stabilities and found them to correlate with those in solution. In a subsequent study, the authors detected tryptophan and insulin
complexes of cyclodextrins with ESI MS [11]. Camilleri et al. [12] also studied cyclodextrin complexes with various amino acids and peptides with aromatic residues. No chemical control experiments were done in these studies, but ESI MS was recommended as a tool to probe cyclodextrin inclusion complexes in solution.

Bakhtiar and coworkers [13, 14] investigated cyclodextrin complexes with different drugs as guest molecules. They took the appearance of higher order host-guest adducts as an indication of gas-phase artifacts, i.e. false positives. They found that the appearance of such nonspecific clusters depended on ESI source conditions and solution concentrations. From the absence of higher order adducts, the observation of a 1:1 adduct, and the lack of complex formation with noncyclic maltohexaose as a control, they concluded that specific inclusion complexes originating from the solution phase were detected in their ESI spectra. The same conclusion was drawn by Selva et al. [15] who also investigated CD-drug complexes with ESI MS and performed a control experiment with maltohexaose.

Cunniff and Vouros [9] questioned that in many of the earlier ESI MS studies true inclusion complexes were detected. They showed that protonated amino groups can form electrostatic adducts with cyclodextrins during the electrospray process and lead to the detection of false positives. This assumption was tested by performing experiments with (i) amino acids containing aromatic side chains and nonaromatic, basic side chains, (ii) peptides with and without aromatic amino acids, and (iii) other hydrophobic compounds. The results showed that CD-guest adducts were detected whether or not hydrophobic side chains were present, provided that an amine group was present. Furthermore, CD complexes with hydrophobic compounds without amine groups were not detected. These results confirmed the authors’ hypothesis that CD-guest adducts detected in ESI MS are mainly false positives.
In contrast to ESI MS, MALDI MS has mainly been used for the analysis of noncomplexed cyclodextrins [16-19]. Until now, only two cyclodextrin complexes have been investigated by MALDI MS. Gallagher et al. [20] investigated the complexes between different cyclodextrins and the anti-inflammatory drug piroxicam using an aqueous solution of the acidic matrix sinapinic acid. An equimolar solution of α-, β-, and γ-CD with piroxicam yielded a very weak signal of the γ-CD complex, whereas α- and β-CD complexes were not observed. Mele et al. [21] detected a β-carotene/γ-CD aggregate when using DHB matrix in 2:1 (v:v) acetonitrile: aqueous 0.1 % trifluoroacetic acid. In none of these studies control experiments were carried out to check whether preformed inclusion complexes or gas-phase artifacts were detected in the MALDI mass spectra. In our opinion, the probability for the disruption of the solution-phase complexes is quite high. Formation constants (K_a) are relatively low: most cyclodextrin complexes have a logK_a between 1 and 4 [5]. Furthermore, the interaction between host and guest is hydrophobic, and may become repulsive in vacuo, in the absence of solvent. In addition, the complex may be disrupted during cocrystallization with the MALDI matrix.

We have seen in chapter 5 that a specific noncovalent complex between a zinc-complexed zinc finger peptide and an oligodeoxynucleotide with an association constant of 10^5 M^{-1} can be detected with MALDI MS. In order to find out whether noncovalent complexes with even lower formation constants and with hydrophobic instead of ionic interactions between the complexing partners could be observed with MALDI MS, we chose to investigate cyclodextrin inclusion complexes. More precisely, the aim of this study was to find out whether (i) complexes of α-, β-, and γ-CD with different aromatic peptides and hydrophobic guest molecules can be detected by MALDI MS and (ii) whether the complexes observed represent true inclusion complexes or simply adducts formed during the MALDI process. In order to answer these questions, we used guest molecules that are known to form inclusion complexes with cyclodextrins in solution. Furthermore, control experiments using nonaromatic amines and peptides instead of aromatic guest molecules as well as linear maltoheptaose instead of cyclodextrin were carried out. The hydrophobic cyclodextrin inclusion complexes were also compared to electrostatic pseudorotaxane-like complexes that have very similar formation constants in solution. We also investigated whether the nature of the interaction between host and guest molecules influences the ability of MALDI MS to detect a specific interaction.
6.2 Experimental

6.2.1 Materials

α-, β-, γ-cyclodextrins, maltolhexaose, anthracene, 4-nitrophenol, cyclohexane, hydrochloric acid, toluene, dichloromethane, NaCl, dibenzo-24-crown-8 (DB24C8) and DB18C6 were purchased from Fluka Chemie AG (Buchs, Switzerland). Ammonium bicarbonate (NH₄HCO₃), octylamine, and dithranol were obtained from Aldrich Chemie (Buchs, Switzerland). The peptides PheGlyGly, GlyGlyPhe, TrpGlyGly, GlyGlyGly, ProTyr, and PNA were from Sigma Chemie (Buchs, Switzerland). The synthesis of fullerene-containing DB24C8 (C₆₀DB24C8) and fullerene-containing dibenzyl ammonium hexafluorophosphate (C₆₀DBA.PF₆) was done in Prof. F. Diederich's group, ETH Zürich, and is reported in Ref. [22]. All materials obtained from commercial sources were of highest purity available and were used without further treatment or purification, except PNA which was recrystallized. Aqueous solutions were prepared using bidistilled water.

6.2.2 MALDI Sample Preparation

**Cyclodextrin Complexes**

Generally, a saturated PNA matrix solution in water:ethanol 4:1 (v:v) was used for MALDI sample preparation. This matrix was recently shown to be suited for the analysis of noncovalent complexes [23, 24]. 10 µL of an aqueous cyclodextrin or maltolhexaose solution (10 mM) were mixed with 20 µL of a 5 to 12.5 mM aqueous peptide or 4-nitrophenol solution to yield the molar ratios given in the figure captions. The solution was then carefully stirred and kept at 5 °C for 30 minutes. Inclusion complex solutions of toluene, cyclohexane and anthracene were prepared according to Cuniff and Vouros [9], Szejtli [4], and Andreaus et al. [25]. A volume of toluene or cyclohexane was mixed with an equal volume of the cyclodextrin solution and stirred for 2 hours. The aqueous phase was retained for MALDI analysis. For preparing samples with anthracene, crystals were added to the cyclodextrin solution and the saturated solution stirred overnight. The supernatant was used for further analysis. 10 µL of the host-guest solution were then mixed with 20 µL of the matrix solution. 2-4 µL of the resulting solution (pH 6) were deposited on the probe tip and dried under vacuum. If not mentioned otherwise in the figure captions, the final sample solution had a pH value of 6. In order to adjust the pH values to 2 and 8.5, aqueous solutions of HCl (1%) and NH₄HCO₃ were added to the sample solution. For the experiment using hydrophobic guest molecules, 2 µL of an aqueous solution of NaCl (100 mM) were
added to the matrix/analyte mixture. For another experiment, a solid mixture of cyclodextrin in PNA was thoroughly mixed with a molar excess of a solid mixture of TrpGlyGly in PNA. All components were dried before use in order to prevent dissolution on the particles’ surface and thus complex formation prior to the MALDI experiment.

**Pseudorotaxane-like Complexes**
A $10^{-5}$ M dithranol solution in dichloromethane was used as a matrix. Receptor-ligand complex (1:1) solutions (0.01 mM) were prepared in dichloromethane. 2 µL of the matrix solution were deposited on the probe tip and dried in air. Then, the same volume of the receptor-ligand solution was applied on top and also allowed to dry.
6.3 Cylodextrin Complexes

Figure 34 depicts the MALDI mass spectrum of β-CD with PNA as the matrix. It is dominated by signals of the sodiated β-CD, [CD+Na]^+ (m/z = 1158 Da), and the potassiated β-CD, [CD+K]^+ (m/z = 1174 Da). In agreement with previous work [16-18], the protonated β-CD signal was not observed. Minor signals of the sodiated and potassiated β-CD dimer were also detected. Similar ions were observed in the MALDI mass spectra of α- and γ-CD.

Figure 34. MALDI mass spectrum of β-CD.

Figure 35. MALDI mass spectra of A. β-CD and TrpGlyGly, 1:1 molar ratio, B. β-CD and PheGlyGly, 1:1 molar ratio. The spectra were normalized to the most intense signal.
Figure 35 shows the MALDI mass spectra of β-CD and the tripeptides TrpGlyGly (Figure 35A) and PheGlyGly (Figure 35B). Both peptides form an inclusion complex with β-CD in aqueous solution due to the presence of aromatic amino acids. In addition to the ions already detected in the MALDI spectrum of β-CD alone, signals corresponding to protonated adducts of β-CD and the respective peptide (m/z = 1454 Da in A, m/z = 1414 Da in B) were detected in the spectra in Figure 35. No cationized β-CD-peptide adducts were observed. Since β-CD and peptides alone are generally observed as cationized and protonated species, respectively, the ionization of the adduct obviously occurred by protonation of the peptide within the adduct rather than by cationization of β-CD. This was also suggested by Ramanathan and Prokai [10]. This observation indicates that the proton affinity of the peptide is larger than the cation affinity of β-CD.

![Diagram](image)

**Figure 36.** MALDI mass spectra of A. β-CD and GlyGlyGly, 1:1 molar ratio, B. maltoheptaose and TrpGlyGly, 1:1 molar ratio. The spectra were normalized to the most intense signal.

In order to check whether the detected 1:1 (β-CD:guest) adducts correspond to true specific inclusion complexes formed in solution or whether they should be attributed to nonspecific adducts of β-CD and the peptide, two control experiments were performed. In the first experiment, GlyGlyGly was used as a guest molecule. In the second, the linear analogue of β-CD, maltoheptaose, was used.
instead of β-CD to complex with TrpGlyGly. GlyGlyGly does not contain any aromatic residues. Maltoheptaose does not contain any cavity for guest molecules. Therefore, no inclusion complex is expected to form in solution in both cases. The corresponding MALDI spectra are depicted in Figure 36A and B. Adduct signals of β-CD and GlyGlyGly (m/z = 1325 Da), and maltoheptaose and TrpGlyGly (m/z = 1472 Da) are detected with relative intensities similar to the adduct present in Figure 35. Therefore, these experiments clearly suggest that the β-CD adducts can form by ion-dipole interactions between the NH$_3^+$ group of the protonated peptide and an OH group from β-CD. These results agree with earlier observations by Cunniff and Vouros [9], who found that cyclodextrin-peptide and -amino acid adducts detected with ESI MS represented nonspecific ion-dipole adducts and not inclusion complexes present in solution.

In order to test this further, four different hydrophobic molecules that are not capable of forming ion-dipole adducts with cyclodextrins were used as hydrophobic guest molecules. Inclusion complexes are known to form in solution between anthracene and γ-CD [4], 4-nitrophenol and α-CD [1], cyclohexane and β-CD, as well as toluene and β-CD. Cyclohexane and toluene were previously used in the ESI MS study of Cunniff and Vouros [9]. If the cyclodextrin adducts with peptides detected in the above MALDI spectra were indeed electrostatic adducts and not hydrophobic inclusion complexes, then we should not observe any cyclodextrin adduct signals with hydrophobic molecules. Figure 37 shows MALDI spectra of the corresponding samples. None of the four cyclodextrin adducts were detected in the MALDI mass spectra. It is, in principle, possible that these adducts are not detected because the hydrophobic or aromatic molecules are not amendable to protonation. However, no cationized complex was observed either, even when adding an excess of sodium salt to the sample. Therefore, these experiments support the notion that the cyclodextrin adducts with amine-containing hydrophobic molecules shown in Figure 35 are nonspecific. Based on these findings, we have to conclude that the absence of cyclodextrin adducts with hydrophobic guest molecules in the MALDI spectra is caused by the destruction of the corresponding inclusion complexes during the MALDI process.
In a further MALDI experiment, we investigated the effect of the position of the aromatic amino acid within the peptide on the adduct formation with β-CD (Figure 38). Although the relative interaction strength with β-CD in solution is not known, PheGlyGly and GlyGlyPhe may show different behavior upon formation of an inclusion complex. Both MALDI spectra were found to be identical.
The position of the aromatic residue within the peptide does not seem to play a role. This further supports the hypothesis that the peptide’s aromatic residue is not involved in adduct formation but rather the protonated amino group.

![MALDI mass spectra](image)

**Figure 38.** MALDI mass spectra of A. β-CD and PheGlyGly, 1:2.5 molar ratio, B. β-CD and GlyGlyPhe, 1:2.5 molar ratio. The spectra were normalized to the most intense signal.

To confirm this, we performed a MALDI experiment with a solid mixture of β-CD in PNA and TrpGlyGly in PNA (Figure 39). Using this sample preparation technique, no β-CD adducts can form before the desorption and ionization processes. However, an intense signal of the β-CD-TrpGlyGly adduct is detected in the MALDI spectrum. The experiment therefore confirms that β-CD-aromatic peptide adducts can form in the gas phase. The same must be true for experiments where a conventional MALDI sample preparation is used (see Figure 35).

Besides the investigation of the complex formation of β-CD with PheGlyGly and TrpGlyGly, another aromatic peptide, ProTyr, was chosen to extend our experimental basis. The MALDI spectrum of β-CD and ProTyr also shows a protonated β-CD-ProTyr adduct signal (m/z = 1414 Da) (Figure 40A). A control experiment with maltoheptaose instead of β-CD yielded a maltoheptaose-ProTyr adduct (m/z = 1432 Da) which was as intense as the corresponding β-CD adduct (Figure 40B). We can therefore conclude from this experiment that the β-CD-
ProTyr adducts detected with MALDI do not represent a specific inclusion complex but a nonspecific electrostatic adduct.

![MALDI mass spectrum of a solid mixture of β-CD and TrpGlyGly](image1)

**Figure 39.** MALDI mass spectrum of a solid mixture of β-CD in PNA and TrpGlyGly in PNA. TrpGlyGly was used in excess compared to β-CD. The origin of the signal at about 1270 Da is unknown.

![MALDI mass spectra of A. β-CD and ProTyr, 1:2 molar ratio, B. maltoheptaose and ProTyr, 1:2 molar ratio.](image2)

**Figure 40.** MALDI mass spectra of A. β-CD and ProTyr, 1:2 molar ratio, B. maltoheptaose and ProTyr, 1:2 molar ratio. The spectra were normalized to the most intense signal.
6.4 Pseudorotaxane-like Complexes

The present study reveals that cyclodextrin inclusion complexes with aromatic peptides or other hydrophobic guest molecules with solution-phase formation constants of $10^1$ to $10^4 \text{M}^{-1}$ do not survive the MALDI process, in contrast to the zinc finger peptide-oligodeoxynucleotide complex investigated in chapter 5. The reason for the different behavior can either be attributed to the difference in the association constant or to the nature of the interaction in the complex. For example, hydrophobic interactions are weakened whereas ionic interactions are stabilized upon desolvation. To further study this, we investigated another chemical system with complex formation constants very similar to those of the cyclodextrin complexes, but with different interactions in the complex. The macrocyclic polyether dibenzo-24-crown-8 (DB24C8) and its fullerene-containing analog, C$_{60}$DB24C8, were used as receptors. Fullerene-containing dibenzyl ammonium (C$_{60}$DBA) was chosen as a ligand. DB24C8 and C$_{60}$DB24C8 are known to form pseudorotaxane-like complexes with C$_{60}$DBA with formation constants of $K_a = 12500 \text{ M}^{-1}$ (in CDCl$_3$, 298 K) and $K_a = 970 \text{ M}^{-1}$ (in CDCl$_3$:CD$_3$CN (9:1, v:v), 298 K), respectively [22, 26]. In these complexes, the linear ammonium ion threads through the cavity of the macrocyclic polyether. [N$^+\cdot\cdot\cdot\cdot\cdot$H$^-$][O$^-$] and [C$^-\cdot\cdot\cdot\cdot\cdot$H$^-$][O$^-$] hydrogen bonds as well as contributions from ion-dipole and dispersive interactions stabilize the complex. Dibenzo-18-crown-6 (DB18C6) was used for the chemical control experiment. This receptor is too small to allow the ammonium ion to thread into it. Therefore, no pseudorotaxane-like complex using this polyether is found in solution.
Figure 41 shows MALDI spectra of DB24C8 and C_{60}DBA (Figure 41A), C_{60}DB24C8 and C_{60}DBA (Figure 41B), as well as DB18C6 and C_{60}DBA (Figure 41C).

In Figure 41A and B, DB24C8-C_{60}DBA as well as C_{60}DB24C8-C_{60}DBA adducts appear as very intense protonated signals (m/z = 1508 Da and 2370 Da). The receptors and the ligands are also clearly detected. In the control experiment (Figure 41C), receptor and ligand signals are observed. However, no DB18C6-C_{60}DBA adduct (m/z = 1420 Da) is visible. The result is not influenced by the matrix. Besides dithranol, ATT and DHB were successfully used as matrices for the detection of specific pseudorotaxane-like complexes. This result clearly demonstrates that the adducts shown in Figure 41A and B are the specific pseudorotaxane-like complexes formed in solution. Since these complexes have
about the same formation constants in solution as cyclodextrin-aromate inclusion complexes, the result also suggests that it is the nature of the interaction in the complex that decides whether the complex is disrupted during the MALDI process or not. Electrostatic interactions in the pseudorotaxane-like complexes survive these processes whereas the hydrophobic interactions in the cyclodextrin inclusion complexes do not.
6.5 Summary and Perspectives

We investigated whether cyclodextrin adducts with various aromatic molecules can be detected with MALDI MS and whether the detected complexes correspond to the cyclodextrin inclusion complexes formed in solution or whether they are nonspecific adducts formed by ion-dipole interactions in the gas phase. Various chemical control experiments were performed. The results were compared to pseudorotaxane-like complexes with similar formation constants but based on different noncovalent interactions.

We found that noncovalent cyclodextrin adducts with molecules containing an amine group can be detected with MALDI MS, whether aromatic residues are present or not. These nonspecific ion-dipole adducts are formed during the MALDI process. Cyclodextrin complexes with hydrophobic molecules containing no amine group could not be observed with MALDI MS. Therefore, MALDI MS is not an adequate technique for investigating inclusion complexes with hydrophobic interactions. Solution chemistry is not reflected and gas-phase artifacts may obscure the results. These findings agree with those from the ESI study by Cunniff and Vouros [9].

On the other hand, MALDI MS was shown to be a suitable tool to study specific pseudorotaxane-like complexes with similar formation constants as the cyclodextrin inclusion complexes but with electrostatic interactions as the dominant noncovalent interactions. In this case, solution chemistry is reflected in the MALDI spectra. This result supports the findings from chapter 5, where specific Zn-p55F1 and Zn-p55F1-d(TTGTT) complexes with electrostatic interactions were detected. The example of the pseudorotaxane-like complexes demonstrated that specific complexes with association constants as low as $10^3 \text{M}^{-1}$ can be observed in the MALDI spectra. It is noteworthy that spectra were obtained from consecutive laser shots. The intensity of the complexes was higher than that of their components.

Moreover, the results from the previous and the present chapter clearly stress the importance of chemical control experiments to check for the specificity of the interaction in the detected adduct when studying noncovalent complexes with MALDI MS. Whether or not MALDI spectra reflect solution-phase chemistry depends not only on the complexes' formation constants but also on the nature of the noncovalent interaction in the complex in solution.
In terms of the MALDI mechanism, we clearly demonstrated in Part A of this thesis that, besides the complexes formed in the MALDI plume after desorption, complexes formed prior to desorption (preformed complexes) are important when studying protein-metal ion and noncovalent complexes with electrostatic interactions. Both mechanisms can be distinguished by performing control experiments. The contribution of preformed complexes could be proven by the fact that under certain conditions, MALDI mass spectra reflect solution-phase chemistry. In fact, we found that MALDI spectra more accurately reflect condensed-phase chemistry, since spectra are taken from crystalline samples. Few methods for studying protein-metal ion and noncovalent complexes in the solid phase exist (e.g. IR spectrophotometry (see chapter 4) or Raman spectroscopy). Usually, they only work for simple or very specific systems, as was the case for the model systems studied in chapter 4. Complex biomolecules are not accessible to these methods since data are too complicated. Therefore, the solid phase was not investigated in chapters 5 and 6.

As an outlook, in order to be able to analyze fragile noncovalent biochemical systems using MALDI MS it is crucial that aggregates preformed in solution are the main contribution to the MALDI ion signal. They should survive crystallization as well as desorption and ionization processes. This may be achieved by an even deeper insight into the formation of both preformed ions and gas-phase ions as was gained during the work described herein. A useful direction to pursue would be to trace the fate of a noncovalent complex from solution to the solid phase to the gas phase and probing the latter by MALDI MS. Knowledge of their chemistry in solution is generally available, but knowledge on the crystallization of the complex with different matrices is not. Such experiments would further resolve some confusion on the current controversial issues in the MALDI detection of noncovalent complexes. It would give information on what sample preparation conditions in terms of solvent (aqueous or organic), pH, or matrix (basic, acidic, liquid) are optimum, whether MALDI spectra of the target complex are expected from consecutive laser shots or only during the first shot, and what the criteria are for deciding on the specificity of a complex. The selection of the matrix is of special importance. For the success in detecting noncovalent complexes consisting of molecules of different chemical classes, the capability of the matrix to simultaneously generate high quality spectra of different classes of compounds, which is currently limited, will be a prerequisite. Furthermore, as clearly shown in the present work, the nature of the noncovalent interaction will also play an important role in whether the preformed complex survives or is disrupted during the MALDI process.
6.6 References


PART B

DEVELOPMENT OF A CONTINUOUS-FLOW INTERFACE FOR MALDI MASS SPECTROMETRY
7 Development of a Continuous-Flow Interface for MALDI MS

7.1 Introduction

7.1.1 Motivation
A recent trend in ESI and MALDI mass spectrometry is to analyze real world, complex sample mixtures with low analyte content. While ESI and MALDI MS alone can easily be used for the direct analysis of simple mixtures, additional fractionation of complex mixtures by liquid separation techniques is required prior to mass analysis. The benefits of such a coupling are the extension of the current LC MS applications to high mass molecules, the reduction of sample preparation time, the on-line monitoring of reactions, and the high information content, i.e. molecular weight and structural information. Due to their low flow rate, CE, capillary electrochromatography (CEC), and μ-HPLC are well suited for introducing a liquid flow into the mass spectrometer.

ESI MS, along with FAB MS, has been most successful for the direct coupling because the eluent from the liquid separation technique can be directly introduced into the mass analyzer. The recent development of nano-ESI MS [1-3] made this technique even more attractive for coupling. Reviews and papers on LC ESI [4-9], CE ESI [9-14], CEC ESI [7, 8, 15] and CE FAB [14, 16], and LC FAB [17] are available to give an overview on this topic. Moore et al. [18] reported a very innovative example of liquid separation and ESI MS. They incorporated a porous, monolithic, poly(styrene-divinylbenzene) chromatography support in an electrospray needle. In addition to spraying the liquid into the mass spectrometer, the packed needle also allowed separation. Experiments with proteins and peptides showed that the separation efficiencies for the monolithic support were at least equal to those of conventional particulate supports.

Despite the widespread use of ESI with liquid separations, there are several drawbacks of electrospray ionization. First, an ESI mass spectrum depicts multiply charged ions. On one hand, their presence allows the detection of large ions at relatively low m/z, but on the other hand, it complicates the interpretation of the spectra of complex mixtures. Second, the sensitivity is affected by the presence of salts, impurities and organic buffers which are often required in chromatographic separations. Third, ESI solvents are generally incompatible with gradient elution common in chromatography. Finally, ESI sources are generally combined with relatively slow quadrupole analyzers which have to
scan the whole mass range. MALDI MS overcomes these disadvantages. Since it is a pulsed technique, it can be combined with a TOF analyzer resulting in a complete mass spectrum from every single laser shot. MALDI TOF MS therefore allows a high analysis speed which is especially important in a continuous-flow interface for rapidly eluting peaks. It should be pointed out that ESI TOF mass spectrometers have also been developed recently [19, 20]. As a further advantage of MALDI TOF, its sensitivity should be mentioned. It is relatively constant as a function of m/z and rather tolerant towards the presence of salts and buffer [21]. However, there is one major drawback of MALDI TOF MS. Typically a sample cocrystallized with the matrix is analyzed. This sample introduction mode is therefore highly incompatible with a liquid separation technique. The success of its coupling to MALDI MS will therefore rely on how the interface is realized.

7.1.2 Interfacing Liquid Separation Techniques with MALDI MS in Off-Line and Continuous-Flow Mode

There are several reports of off-line combinations of CE or LC separation techniques with MALDI MS [22-27]. The effluents were either collected in fractions in an array of cups [22, 23], on stainless steel pins [24], on a moving-belt-like system [26, 27] with the matrix solution being subsequently added to the sample, or they were continuously deposited on a precoated membrane target [25]. The collection of CE fractions poses a greater problem than that of LC fractions because electrical contact to the capillary must be maintained during separation and the eluent volume is as small as a few nanoliters. The analysis of synthetic polymers with broad molecular weight distributions can be performed by coupling gel permeation chromatography (GPC) with MALDI MS. In this case, GPC fractions, each with a narrow polymer distribution, are investigated by MALDI TOF MS. Using only MALDI MS, broad polymer distributions would lead to incorrect average molecular weight values due to discrimination against higher molecular weight oligomers. Off-line combinations of MALDI MS with thin layer chromatography (TLC) have also been reported [28-30].

In contrast to off-line experiments, analysis in continuous-flow (CF) mode involves fewer sample handling steps reducing the chance for sample loss. It is therefore inherently more effective in trace analysis. Moreover, speed and throughput are theoretically higher in CF mode. Coupling in CF mode can be realized in two ways: either by introducing an aerosol into the mass spectrometer or by delivered the liquid sample to the probe tip through a narrow bore capillary.
7.1 Introduction

Murray et al. and Fei et al. [31, 32] developed an aerosol MALDI interface in which an aerosol beam is formed from a mixture of protein samples and the matrix solution which is subsequently transferred into a TOF mass spectrometer at flow rates up to 1 mL/min. MALDI is performed from the aerosol particle surface. The technique has also been demonstrated for LC coupling [32].

CF interfaces for MALDI TOF MS have been proposed by the research groups of Lubman [33], Li [34-36], Yeung [37], Amster [38], Hercules [39], and Karger [40]. Their work is described below.

Lubman and coworkers [33] designed a CF interface for a hybrid ion trap/reflection TOF analyzer. This instrument combined the better resolution and high pressure capabilities of an ion trap with the high speed and large m/z range of the TOF analyzer. The interface was similar to a frit-type probe used in CF FAB: the capillary reached to the back side of a stainless steel frit housed in the center of the probe tip. A filter paper was wrapped around the probe tip to absorb excess liquid. The UV-absorbing liquid matrix 3-NBA assisted in the desorption and ionization of the analyte. According to the authors, the rate of introducing the liquid into the mass spectrometer and the rate of vaporization must be in balance in order to obtain a stable flow. Therefore, the flow rate, the temperature of the liquid, the repetition rate of the laser, the total vapor pressure of the solvent, and the pumping speed had to be optimized. Ethylene glycol was used to prevent freezing of the solvent which expands into the vacuum. The trap was heated to 60 - 80 °C with an IR lamp. The interface was tested in static mode, CF mode, and with flow injection analysis (FIA), i.e. repeated sample injections into a continuous flow of carrier solvent. Besides the liquid matrix and the anti-freezing agent, the solution in the continuous mode contained acetonitrile, water, and 0.1% TFA in water, and was introduced into the mass spectrometer with a flow rate of 2 - 4 μL/min. The authors found that the resolution and the signal intensity of various peptides in the mass range of up to 8 500 Da were worse in the CF compared to the static mode (resolution of 280 compared to 350 for gramicidin S) which was attributed to a higher pressure and solvent background in the CF experiment. When performing FIA of neurotensin, they observed peak tailing with time, i.e. the signal sharply rose at a certain time after injection and then slowly decreased. The authors proposed to solve the problem by optimizing the flow rate, the temperature of the probe tip, the desorption laser power, and the surface area of the probe tip.

A similar interface was developed by Li and coworkers [34] which was coupled to a linear TOF mass spectrometer with orthogonal ion extraction. The same liquid matrix and anti-freezing agent were used. Heating of the probe tip was
realized by putting a 100 °C point heater, made of a Nichrome 60 heating coil, three cm from the probe tip. In this set-up, heat could mainly be transferred to the probe tip by radiation. For CF experiments, a mixture of the peptide in 0.1 % TFA in water, methanol, ethylene glycol, and 3-NBA (1:1:1:1, by volume) was employed at a flow rate of 1-5 µL/min. The analysis in CF mode of myoglobin with a mass of 17.5 kDa gave a 5 to 10 times lower intensity and detection sensitivity than the analysis in static mode. This was explained by the pressure and the local sample concentration difference. For FIA, the carrier solvent consisted of a 1:1:1:1 mixture of 0.1 % TFA in water, methanol, ethylene glycol, and 3-NBA. The injected bacitracin sample (1421 Da) solution also contained the liquid matrix. The ion profile (analyte peak areas in the mass spectra are integrated and depicted as a function of elution time) showed some peak tailing but no memory effects were observed using blank injections. Injections of 20 pmole of insulin yielded a signal-to-noise ratio of 3. According to the authors, the sensitivity might be improved because the set-up allowed the sample to spread over a probe area of 5 mm in diameter, but the laser spot size was only 0.5 mm in diameter. The excess liquid was absorbed by filter paper. The major limitation of their configuration was the bad resolution of 10 for analytes with masses over 1000 Da. It is unclear why they did not observe significant peak tailing as Lubman and coworkers [33] did, since they used a similar set-up.

In a subsequent paper, Li and coworkers [35] tried to increase the sensitivity by reducing the area in which the sample can diffuse. They eliminated the frit and mounted a piece of kapton (2.8 mm diameter) on their tip. The capillary was fitted to a hole in the kapton and was placed slightly above the surface. However, they still used the filter paper to absorb the excess liquid and did not observe an increase in sensitivity when increasing the laser beam size. The authors tested their interface and coupled conventional and microbore LC with TOF MS in reflectron mode. They wanted to separate cytochrome c and lysozyme (5 to 10 pmol injected). In order not to influence the separation process, the liquid matrix was added after the column via a three-port mixing tee. In a preliminary experiment, they found the tube diameters and the whole arrangement of the tee to be critical for achieving reproducible results. No significant peak broadening and peak distortion could be detected in the ion profile compared to the UV chromatogram. Furthermore, the resolution of the cytochrome c peak was the same as in the static mode, only 16, which the authors attributed to the instrument’s poor performance.

In another follow-up study [36], the performance of the instrument was improved. Orthogonal ion extraction was replaced by parallel ion extraction and time-lag-focusing was added. These modifications resulted in a 10 fold sensitivity and a 5 to 10 fold resolution increase. However, the authors still observed a
poor resolution for proteins with masses above 6 kDa, which they attributed to the strong alkali ion adduct formation of the liquid matrix 3-NBA. They concluded that this matrix was not adequate for CF MALDI. Their test of alternative materials such as glycerol combined with laser dyes or solid matrices failed.

Yeung and coworkers [37] coupled capillary electrophoresis (CE) to a linear TOF mass spectrometer via an interface that consisted of a capillary that reached directly into the vacuum chamber. An aqueous solution of 0.5 to 1 mM CuCl₂ was used for three different purposes at once: as running buffer, UV absorbing species which assists in analyte desorption, and to prevent freezing of the expanding solvent. It is not known whether the copper salt was also involved in analyte ionization. A stable flow was maintained by optimizing the laser repetition rate and the flow rate. CF experiments with a flow rate of 150 nL/min and using serotonin (178 Da) as analyte resulted in a mass resolution of 100 and a detection limit of 10⁻⁷ M. When coupling CE to TOF MS and analyzing a mixture of serotonin and tryptamin, chromatographic peak broadening and a sensitivity worse than that in the CF mode was observed. It is noteworthy that only analytes up to a mass of 200 Da were investigated and that the method was limited to solvents which can dissolve the copper salt.

The interface of Amster and coworkers [38] included two capillaries that terminated flush with the probe surface. One of them delivered the analyte, the other a solution of α-CHCA which served as MALDI matrix. In the CF mode with a flow rate of 1.5 µL/min into the linear TOF mass spectrometer, a detection limit of 10⁻⁵ M and a resolution of 130 in the linear mode were obtained for the peptide bradykinin. Insulin could not be observed. No further results on this approach have since been published. This method did not appear very promising to us because the solid matrix used may crystallize at the end of the capillary and block the flow.

Hercules and coworkers [39] reported coupling of GPC and HPLC to a MALDI TOF mass spectrometer. The interface consisted of a porous frit which was installed at the end of a capillary. It was about the size of the capillary diameter and thus exhibited a relatively low surface area. α-CHCA was used as the matrix and was mixed with the sample solution using a mixing tee prior to entering the mass spectrometer. Memory effects were avoided by cleaning the interface with a combination of flushing the frit with solvent and UV laser ablation. Further information on the interface such as the flow rate, possible clogging of the capillary with solid matrix, or details on where the remaining sample was washed to during flushing of the capillary were not reported, but would be important for judging the interface’s performance.
Karger and coworkers [40] designed a CF interface which was meant to resemble the MALDI process in static mode as closely as possible in order to take advantage of the experience accumulated in this area. In this approach, the sample solution, premixed with α-CHCA matrix, was deposited on a rotating quartz wheel and transported to the repeller, where laser desorption took place. The solution was delivered through a 20 μm i.d. fused silica capillary with a flow driven by the pressure difference between the mass spectrometer and the atmosphere of 100 to 400 nL/min. After evaporation of the solvent on the wheel surface, a thin, ~50 μm wide, smooth trace of microcrystalline sample was formed, which yielded fairly reproducible ion signal intensities along the trace. Due to the stepwise movement of the wheel, the trace was divided in segments whose dimensions were smaller than that of the nitrogen laser spot, eliminating the need to scan the laser over the whole sample surface. The rotating wheel has two advantages compared to the above-mentioned interfaces: first, it prevents accumulation of the solvent at the capillary outlet and second, being at room temperature, it acts as a heat reservoir preventing the expanding solvent from freezing at the capillary outlet. The wheel has to be cleaned manually at atmospheric pressure after each run to avoid memory effects. For future applications, the authors plan to use laser ablation for cleaning purposes. Flushing the capillary with pure solvent between experiments and filtration of the sample solvent were found to be crucial for the lifetime of the capillary. The spectrum in CF mode of insulin was comparable to its spectrum taken in static mode although the resolution was slightly worse. The interface was also tested using CE MALDI TOF MS of a mixture of 12 peptides in the mass range from 1 to 2 kDa. Peak tailing did not seem to be significant.

An overview on coupling liquid separation methods in off-line and CF mode to MALDI is given in a review of Murray [41].

### 7.1.3 Possible Liquid Separation Techniques

As mentioned above, CE, μ-HPLC, and CEC are suitable liquid separation techniques for coupling with MALDI MS. CE provides a high-efficiency separation in a capillary tube filled with an electrolyte solution [42]. An electric field across the capillary causes electrophoretic and electro-endosmotic movements of ionic species in the sample resulting in their separation. This technique does not require sophisticated instrumentation. Care must be taken to make an electrical contact between the liquid which elutes from the capillary through the substrate and the high voltage conducting repeller plate. Moreover, the analyte and buffer solutions which are at high voltage during measurement have to be grounded when the operator is interfering.
μ-HPLC relies on the same principles as conventional HPLC [43], but is characterized by much lower flow rates, typically a few μL/min. This method requires rather sophisticated instrumentation and know-how.

CEC was first reported by Pretorius et al. [44]. It is a high resolution and high speed separation method combining the attributes of both CE (or more precisely, capillary zone electrophoresis, CZE) and capillary HPLC. Therefore, the separation of an analyte mixture is based on electrophoretic and electroosmotic movements as well as on the interactions between the chromatographic stationary phase and the species under study.

7.1.4 Purpose of this Work

The first steps in the development of a CF interface for MALDI MS relying on the two-phase MALDI methodology were addressed during this thesis work. The two-phase MALDI methodology has been developed by Dale et al. [45]. A two-phase MALDI matrix consists of solid particles that act as an energy absorber and assist in analyte desorption, and a liquid matrix for the ionization of the analyte. This is in contrast to a laser energy absorbing liquid matrix or a solid matrix which combines both properties in one chemical species. The method is very versatile because a larger number of liquid matrices are available which are not required to absorb the laser wavelength, and because the desorption laser wavelength can be chosen depending on the solid particles used. In the positive ion mode, a mixture of glycerol (liquid matrix) and graphite powder (UV absorber) has been successfully used to obtain mass spectra of proteins with masses up to 10 kDa [45]. In the negative ion mode, the two-phase matrix diethanolamine/silicon powder was successfully used to analyze various anionic analytes [46].

There are a number of additional requirements for using the two-phase MALDI methodology in a CF interface. For the UV-absorbing substrate, these are, for example, mechanical stability, chemical inertness, permeability for common solvents, and the possibility to achieve the high energy densities necessary for desorption. Among others, porous frits of various materials, such as TiN, graphite or carbon, and graphite whiskers were tested as potential substrates. In our design, a fused silica capillary of 50 μm i.d. guides the sample solution to the substrate in the mass spectrometer, where the solvent evaporates and the analyte is desorbed upon laser irradiation. The ionization of the analyte is promoted by the liquid matrix (pretreatment of the substrate or addition to the eluent). In the CF experiment, the flow of sample solution is either hydrodynamic, i.e. driven by the difference in pressure between the mass spectrometer and atmosphere, or is controlled with a syringe pump.
The following development steps for a CF interface for MALDI MS were addressed by this thesis. They are described in detail in the subsequent sections of this chapter.

1) set-up of the CF experiment and development of frit and whisker sample holders (see section 7.2)

2) study of the flow characteristics of the eluent leaving the capillary into the mass spectrometer in order to achieve a stable flow (heating, different solvents, addition of anti-freezing agent, hydrodynamic flow vs. flow controlled by a pump) (see section 7.3)

3) search for possible laser absorber substrates and first evaluation of their applicability (see section 7.4)

4) screening in static mode of laser absorber frits that were retained after the initial tests, in order to find the best liquid matrix to use as ionizing agent, the optimum way to deliver the liquid matrix, and to determine the upper mass limit of analytes to desorb (see section 7.5.1)

5) further development of the most promising frits selected from the previous test (graphite, TiN) in terms of mechanical stability, permeability for solvents, or adequate dimensions (see section 7.5.2)

6) experiments in static and CF mode with graphite whiskers (see section 7.6)

The most important parameters to address during these steps are: substrate durability (mechanical and chemical stability), capillary connection to the substrate, focusing of the laser onto the substrate area, desorption of all the analyte from the substrate, analyte concentration in the eluent, flow rate, hydrodynamic flow vs. flow controlled by a pump, external heating and repetition rate of the laser to achieve a stable flow, delivery of the liquid matrix (pretreatment of the substrate or addition to the eluent), and volume of liquid trapped inside the substrate (dead volume).
7.2 Instrumental

7.2.1 Heating of the Capillary Outlet

No exact description of a heating system and its performance to prevent freezing of the capillary outlet is described in the literature on CF interfaces for MALDI MS. Therefore we developed our own heating set-up.

Initially, we thought to heat the capillary from outside the vacuum chamber with a high power lamp, the light of which was focused on the capillary end. Unfortunately, the window of the vacuum chamber through which the light should have been entered, was absorbing a large part of the light. The resulting temperatures on the capillary outlet were not high enough. For this reason, we developed a different heating set-up (Figure 42).

A resistor (15 Ohm, 20 W) heated the repeller plate and consequently the probe and the capillary tip. As the repeller plate was at high voltage, it was not possible to mount the resistor on this plate. Therefore it was attached on a thin copper plate which was isolated from the repeller plate with a 16 mm sapphire cylinder (Saphirwerk Industrieprodukte, Brügg, Switzerland). Sapphire is characterized by low electrical and high thermal conductivity. Cylinders shorter than 16 mm produced sparks between the repeller and the copper plate. The heating process was controlled by a PID (proportional/integral/differential) controller unit (Newport Electronics, Deckenpfronn, Germany) with a thermoelement on the copper plate. The copper plate could be heated up to a given temperature with an uncertainty of a few degrees Celsius due to overshoots. The controller was protected from major damage from possible sparks between the copper and repeller plates by grounding the copper plate. The metal wire used for this purpose was rather thin to minimize heat losses.

The heat transfer between the resistor and the probe tip had to be as efficient as possible. Therefore the thermal contacts between copper plate and sapphire, sapphire and repeller, copper plate and thermoelement, as well as copper plate and resistor, were assured by a heat-conducting paste (340 silicone heat sink compound, Dow Corning, USA). Comparison of the temperatures measured by the sensor on the copper plate and a second sensor (test sensor) on the repeller plate allowed quantification of the heat transfer efficiency. We found that a temperature T-5°C on the repeller plate was reached 15 min after the copper plate had reached a desired temperature T. Temperatures up to 130 °C could be reached without difficulty on the repeller plate. Of course, temperatures at the
capillary outlet are lower due to heat losses across the repeller plate/probe tip and probe tip/capillary interfaces, which are not covered with heat sink for obvious reasons. They proved, however, to be high enough to prevent water exiting the capillary from freezing. Once this calibration was done, the second sensor was removed and the actual temperature on the repeller plate was calculated from the measured temperature on the copper plate.

The main advantage of this heating set-up compared to others reported in the literature was that the temperature of the repeller plate was known and could be controlled.

Figure 42. A. Picture of the heating set-up mounted onto the repeller plate. B. Schematic view of the repeller plate with the heating system.
7.2 Instrumental

7.2.2 Set-up of the Continuous-Flow Experiment

The set-up of a CF experiment for FIA is depicted in Figure 43.

A syringe pump (Harvard Apparatus 22, model 55-2222, Indulab AG, Gams, Switzerland) delivered the carrier solvent through a microbore fused silica capillary (uncoated, µ-sil tubing for CE, 20 to 75 µm i.d., 350 µm o.d., length 1 m, Socochim SA, Lausanne, Switzerland) to the UV-absorbing substrate in the sample holder which was screwed onto the front of the sample introduction rod and resided in the repeller electrode. A 250 µL syringe (Hamilton gas-tight, Model 1725 RN) was used in the pump and was connected through a zero-dead volume union (1/16”, Vici AG, Schenkon, Switzerland) to a Valco injection valve (model Inj-P4, internal loop with 100 and 500 nL, Omnilab, Mettmannstetten, Switzerland). A 1 meter capillary then connected the valve and the sample holder. A 10 µL syringe (Hamilton microliter, Model 701) was used with the valve to inject the sample into the carrier solvent. For CF experiments, which were the main part in the present work, the valve was removed and the capillary directly connected to the union. Stainless steel ferrules and nuts (1/16”, Vici AG, Schenkon, Switzerland) were used throughout the set-up except at the end of the capillary which was screwed into the valve or union, where a PEEK ferrule and nut (1/16” fingertight, Vici AG, Schenkon, Switzerland) were employed. At this location, stainless steel ferrules and nuts would have been too heavy and would
have pulled the capillary out of the septum which was used in the frit sample holder (see next section). The capillary was evenly cut, with a 90° angle to the capillary wall. All solutions were degassed before use and filtered (disposable syringe filters, 0.45 μm pores, Alltech, Deerfield, IL, USA).

7.2.3 Design of a Frit Sample Holder

In the initial experiments in static mode, small pieces (about 2 mm in diameter) of the UV absorber were cut and stuck onto the sample holder by means of double-sided adhesive tape or silver glue. For the CF experiments, the frit and the capillary had to be linked efficiently, i.e. the solution leaving the capillary had to enter the frit without losses. For this purpose, disks with a diameter of 2.5 mm (thickness 0.4 to 0.7 mm) were prepared and a special sample holder was designed for them (Figure 44).

![Diagram A](image1)

![Diagram B](image2)

**Figure 44.** A. Picture of the frit sample holder. B. Cross-section of a frit sample holder.

On the inner front side of the probe tip, a housing for the frit was milled out. The depth of the housing corresponds to the thickness of the frit. Furthermore, a hole
7.2 Instrumental

with a diameter of 2.3 mm was drilled into the probe tip surface. For the capillary, a hole of 1 mm diameter was drilled into the plexiglass insulator. The capillary was then inserted into the insulator and a pre-pierced septum until it reached the front of the septum. The holder was then assembled like in the case of the probe tip used in static mode, the plexiglass insulator being screwed into the probe tip. Using this set-up, the capillary is firmly pushed flush to the frit. The dimensions of the hole in the probe tip surface were optimized for a maximum of support for the frit and a minimum coverage of the frit's surface: 85% of the frit's front surface area was accessible to the laser beam. This holder was also used for experiments in static mode with the 2.5 mm frits.

7.2.4 Design of a Whisker Sample Holder

A special holder was also designed for the graphite whisker (description of the whisker see section 7.4.4). For this purpose, two holes were drilled into the face of the probe tip. The two "feet" of the whisker support fitted exactly into these holes. The whisker to which the sample was applied had to be at exactly the same position with respect to the repeller plate as compared to the sample on a normal probe tip used for static mode. Consequently, the probe tip for the whisker was shortened. Due to the depth of the two holes in the probe tip, the tip was screwed into the plexiglass insulator. A septum was put between both parts. For CF experiments with the whiskers, a hole for the capillary was drilled into the plexiglass holder, the probe tip, and the whisker support. The capillary was then pushed through the assembly until it reached into the whisker needles. A picture and a cross-section of the whisker holder are shown in Figure 45.

Using this set-up with the open capillary reaching into the whisker, the capillary diameter was too large to allow sufficiently low flow rates (see section 7.3). For this reason, a glass PicoTip (model BG10-58-4-N-10, 4 µm tip diameter without filament, New Objective, Inc, Cambridge, MA, USA) was used. It consists of a short (few cm) glass capillary with a diameter of 1 mm which had been pulled to form a tip diameter of 4 µm. This glass PicoTip was stuck into the support. The hole in the support was chosen such as to allow the PicoTip to reach into the whisker needles. The fused silica capillary was then pushed into the PicoTip and was held in place through its soft coating.
Figure 45. A. Picture of the whisker holder. B. Cross-section of a whisker holder. C. Close-up of the whisker.
Hydrodynamic flow rates (driven by the pressure difference between the mass spectrometer and atmosphere) into the mass spectrometer were determined by Vera Ivleva, a former PhD student in our group, for different solvents and using capillaries of different inner diameters. These flow rates are of the order of a few μL/min for 50 μm i.d. capillaries with a length of 1 m, which were predominantly used in the experiments. These values are in agreement with those calculated from Hagen-Poiseuille’s law for a laminar flow in a tube:

$$\frac{dV}{dt} = \frac{d^4(p_1-p_2)\pi}{128\eta l}$$

with $dV/dt$: flow rate, $d$: capillary diameter, $p_1$-$p_2$: pressure difference between atmosphere and mass spectrometer, $\eta$: viscosity, $l$: capillary length.

The hydrodynamic flow rate is dependent on the capillary’s diameter. Small particles in the eluent can change the diameter and lead to an unstable flow. For this reason, we chose to control the flow with a syringe pump (set-up see section 7.2.2). The controlled flow through a given capillary was only stable if its rate was higher than the hydrodynamic flow rate. Controlled flow rates smaller than 1 μL/min could therefore only be realized by reducing the capillary’s diameter or the capillary’s opening at the outlet. The second possibility was mainly pursued since capillaries with smaller diameters clog easily. When using the frit set-up, the capillary opening was reduced by the frit’s pores. The flow rates run in the experiments were ~1 μL/min. In the case of the graphite whisker, the capillary end was pushed into a PicoTip, a glass capillary with a tip diameter of 4 μm. Flow rates of 200 to 250 nL/min could be realized in this manner.

The stability of the flow into the mass spectrometer was checked with a capillary of 50 μm i.d. using methanol (Fluka Chemie AG, Buchs, Switzerland), acetonitrile (Merck, Darmstadt, Germany), and bidistilled water as solvents. Methanol did not freeze when expanding into the vacuum, but it formed drops, that grew, evaporated, grew again, etc. When applying a high voltage of about 25 kV on the repeller plate in which the probe tip with the capillary was fixed, this behavior led to arcing. Using water as a solvent, only small drops as well as freezing and melting processes were observed. The capillary also clogged easily due to ice formation. Sometimes, ice needles grew out of the capillary. The behavior of acetonitrile was very similar to that of water, but drops grew slightly larger and ice formation was not as accentuated. Mixtures of acetonitrile and
methanol in different volume ratios and ethylene glycol contents of up to 5% still showed some freezing. According to the literature, high ethylene glycol contents of up to 25-35% should prevent freezing [34, 47, 48]. However, we chose to heat the eluent via the probe tip to prevent freezing (heating set-up see section 7.2.1).

The pressure in the ion source chamber was \( \sim 4 \times 10^{-5} \) mbar when working in the CF mode, which was about six times higher than the pressure in the static mode. The vacuum pressure reading could also be used as an indication of the flow stability. When the flow was stable, only small oscillations around a certain pressure value were observed. This value is higher than the normal pressure value without any flow into the mass spectrometer. When the capillary was clogged with ice, the pressure reached the value observed in the static mode. An unstable flow, e.g. repeated drop formations as well as freezing and melting processes, was characterized by sudden pressure changes.

In general, a stable flow from the capillary into the mass spectrometer is obtained when the amount of volatile material delivered to the capillary tip/laser absorber equals the amount of volatile material that evaporates into the vacuum. The amount of material that is delivered depends on the flow rate and the concentration of the ionizing agent (which has a low volatility) in the solvent. The amount of material that is removed depends on the pressure in the ion source chamber, the repetition rate of the laser, and the heating temperature. Judging from the pressure reading, a stable flow into the mass spectrometer using the TiN frits was obtained under the following conditions: heating temperature of 60°C, methanol solution with 2% (v) glycerol, flow rate of \( \sim 1 \) µL/min, 2 Hz repetition rate of the laser. For the graphite whiskers, the flow rate was \( \sim 250 \) nL/min. The pressure was in either case between 2 and 5\( \times 10^{-5} \) mbar. The repetition rate of the nitrogen laser in the range of 1 to 10 Hz did not seem to influence the evaporation rate. We observed that the pressure strongly depended on the glycerol content in the solvent.
7.4 Potential Laser Absorber Materials

7.4.1 Requirements for the Laser Absorber Materials
The laser absorber for the CF interface must fulfill the following conditions:

- absorption of the laser wavelength (in our case at 337 nm in the UV)
- contain small (sub-μm to several μm) independent “units” in order to achieve high energy densities and low thermal conductivity
- mechanical stability
- chemical inertness
- no adsorption of the analyte on the material
- small dimensions
- in the case of frits: be porous and permeable for solvents

A broad range of different materials were investigated: organic materials, inorganic materials (porous ceramics, glass, carbon and graphite frits), and graphite whiskers. They are described in the following, together with a brief evaluation of their applicability. More detailed investigations of the selected materials are presented in sections 7.5 and 7.6.

7.4.2 Organic Materials
The main difficulty in obtaining a polymeric material with the specified requirements was that the companies contacted did not know (because the material was meant for other purposes) or were not allowed to tell (because of the trade secrets) about certain properties and especially the chemical structure of the polymers and additives. Depending on the material’s properties, we used it either as received, or we tried to incorporate some pores, or we modified its functional groups with UV-absorbing dyes or common MALDI matrices.

Commercial Materials
Polyacrylate CE 1171 (dissolved in toluene/xylene) from Conap (Olean, NY, USA), and Plexiglass GS 303 yellow as well as Macrolon transparent 281 (both as 3 mm thick plates) from Röhm GmbH (Brüttisellen, Switzerland) contain UV-absorbing dyes, yet it was not known whether they were porous. Very thin layers of these materials were prepared by pouring the dissolved material into a Petri dish. All the materials were glossy and had poor wetting properties indicating that the solvent cannot penetrate into the material. It was known from earlier experiments during my diploma thesis [49] that glossy materials reflect the laser beam and do not work with MALDI MS. They were therefore rejected.
Self-prepared/-modified Materials

Poly(hydroxy-ethyl-methacrylate) [poly(HEMA)], crosslinked with ethylene glycol dimethacrylate (EGDMA) and surface-modified with the UV-absorbing matrices ferulic acid, sinapinic acid and DHB, is a porous polymer. Sheets of this material with a thickness of 2 to 3 mm (5 to 10 μm pores) were used. They were obtained from Prof. B. Salih, a visiting professor in our group. Thin films of this material could not be prepared because of its insolubility in various common solvents such as dichloromethane, chloroform, or THF. Furthermore, tresyl chloride-activated agarose beads were modified in our laboratory with the MALDI matrix molecules PNA and coumarin 120 (Lambda Physik, Göttingen, Germany), a dye with a significant absorption coefficient at 337 nm (ε = 1.5 10^{-4} \text{Lmol}^{-1}\text{cm}^{-1}). The beads swell in water and were investigated in the dried and the gel form. Static-mode experiments of the agarose beads and the poly HEMA/EGDMA polymer with substance P alone and using NaCl or glycerol as ionizing agents did not yield any molecular ion signals. We assumed that the density of the UV-absorbing species on the polymeric material was not high enough to sufficiently absorb the laser energy in order to desorb the analyte. Our negative results with the modified agarose beads were, however, in contrast to experiments performed by Hutchens et al. [50]. They could successfully obtain mass spectra of myoglobin using agarose beads modified with the matrix α-CHCA.

Another way to prepare UV-absorbing thin polymer layers was by dissolving nitrocellulose from Schleicher & Schüll (Dassel, Germany) in methanol, acetone, or acetonitrile, and adding UV absorbers such as CuO powder (unknown particle size), graphite powder (1-2 μm particles), or the MALDI matrix DHB, with subsequent evaporation of the solvent. Experiments with these materials were motivated by the work of Vera Ivleva, a former PhD student in our group. She had introduced CuO and graphite particulates into the pores of nitrocellulose by filtering. The success of this method in static mode with gramicidin S was confirmed with substance P. The drawback was, however, the strong likelihood of washing the UV absorber out in CF experiments. Graphite/nitrocellulose was very homogeneous whereas CuO/nitrocellulose was not. The DHB-containing sample was carefully washed with bidistilled water to remove excess matrix. In addition to these self-prepared materials, soot-containing nitrocellulose from Schleicher & Schüll was investigated. We tried to introduce pores by adding NaCl to the dissolved polymer/UV absorber mixture, and to remove excess salt by washing the dry absorber material with bidistilled water. However, a lot of NaCl salt was incorporated in the material and could not be removed. Therefore, this modification method was not pursued further. All the nitrocellulose-containing samples gave some reasonable results with substance P in the static mode. Results in CF mode could even be obtained from the soot-containing nitrocellulose, but the experiment was not reproducible. In all the experiments,
the solvent was composed of 40 % methanol, 40 % water, and 10 % glycerol. Despite these initial results, we decided not to pursue the evaluation of the nitrocellulose-containing substrate material further because nitrocellulose is soluble in methanol, acetone, and acetonitrile, which are commonly used solvents in LC separation techniques.

In summary, none of the organic membranes yielded satisfactory results and were therefore not investigated further.

7.4.3 Inorganic Materials

Two ceramic materials were obtained from Prof. Gauckler, ETH Zürich: La_{0.8}Sr_{0.2}Co_{0.8}Fe_{0.2}O_{3}(60%)/Ce_{0.8}Gd_{0.2}O_{1.9}(40%) (LSCF/CGO) and NiO(60%)/Ce_{0.8}Gd_{0.2}O_{1.9}(40%) (Ni/CGO). LSCF and Ni are the UV-absorbing species, and CGO is a filler compound. They were prepared as follows: the oxide powders were mixed with ethanol, toluene, PEG 600, and bis(2-ethylhexyl)phthalate (bonding agents) to form a slurry. Subsequently, the slurry was casted into a mold and the solvent was evaporated. The remaining “cake” was then sintered at ~1000 °C, where the additives and the filler burned off and a porous ceramic was obtained. Ceramic sheets with a thickness of 0.1-0.2 mm were obtained in the “cake” form as well as in the sintered form. Despite the fact that the required conditions for use in CF mode were met, these materials did not yield reproducible results in the static mode using substance P alone as well as when adding glycerol as an ionizing agent. The materials were therefore rejected.

A fruitful collaboration was developed with Dr. Beat Gut from the Eidgenössische Materialprüfungs- und Forschungsanstalt, (EMPA), Dübendorf, Switzerland. He and his coworkers prepared various frits, which are described in the following. Initially, the frits had a thickness of ~1 mm and a diameter of ~1 cm, and pieces were broken from them for analysis. At a later stage, the dimensions were reduced to 2.5 mm in diameter and 0.4 to 0.7 mm in thickness. Frits were pressed with 400 bar using a special pressing tool with the required dimensions. Most of the powders used for the frits have particle size distributions. The term x/y/z is used to characterize them, with x, y, and z indicating that 10, 50 and 90 % of the particles have a smaller size than the values of x, y, and z, respectively.

Carbon frits were prepared from 2/7/14 μm Sigradur glassy carbon, meaning that 10% of the particles have a smaller size than 2 μm, 50 % of the particles have a smaller size than 7 μm, and 90 % of the particles have a smaller size than 14 μm. The powder was mixed with a bonding agent (bakelite), pressed and heated up to 400 °C. It was not possible to sinter the material, therefore it still contained some of the bonding agent. The pore diameter was ~1/10 of the average particle size.
The carbon frit worked well for substance P and bombesin. Using a similar procedure, CuO frits were prepared. As the powder’s particle size was not indicated by the manufacturer, the pore size was not known. The material was sintered at 950 °C and therefore no longer contained any additives. Despite the encouraging results using CuO added to a nitrocellulose membrane, the static-mode experiment with substance P was not successful. Low signal-to-noise ratios together with strong copper-adducts were observed. This may be due to the frit’s particle size diameter, which resulted in inappropriate energy densities.

Felts were pressed from carbon fibers at different pressures resulting in different volume densities. The material was rejected because only poor results could be obtained in experiments in static mode and its mechanical stability was very low. Graphite frits were prepared from 1-2 µm graphite powder by simply pressing. The same graphite powder had also been used by Dale et al. [45] for their two-phase MALDI experiments. The graphite frit worked well for substance P, bombesin, and insulin. Finally, a TiN frit was prepared by simply pressing the corresponding powder (particle size distribution 0.8/1.7/3.3 µm) without the addition of a bonding agent. A Si frit (particle size 21-61 µm) was prepared in the same way, but with subsequent sintering at 1100 °C under argon atmosphere. Both materials worked very well for substance P and insulin, and TiN worked even for cytochrome c, but they were rather brittle.

A 0.5 mm thick carbon frit from Le Carbone, France, with pores of 3 to 5 µm was also tested. The frit was made from fibers that were pressed and heated up to 950 °C under nitrogen atmosphere. Good results were obtained in the static mode for analytes with masses up to that of insulin.

Another part of the work concentrated on porous glass, which contains silanol groups on the surface that could be modified with UV absorbers. Vycor glass (Corning Keramik GmbH, Germany) consists of two phases, one of which easily dissolves in sulfuric acid. This allows preparation of a porous material by treatment with acid. Vycor plates with a minimum thickness of 1 mm and pores of 40 Å are available. The excess material could not be ground off without tampering its mechanical stability. The material was rejected because of its inappropriate dimensions.

Glass filter frits were also investigated. They can be obtained with pores of 1 to 100 µm and a minimal diameter of 5 mm, yet they are 3 mm thick. Unfortunately, it was not possible to reduce the thickness without clogging the pores with the dust. Therefore this material could not be used for our purposes either.
In summary, the carbon, graphite, Si, and TiN frits performed well in the preliminary static-mode experiments. Detailed investigations are presented in section 7.5.

### 7.4.4 Graphite Whiskers

We tried graphite whiskers as possible substrates for the CF interface. They were manufactured and modified according to our specification by Dr. Bernhard Linden from Linden-ChroMasSpec, Germany with whom a good collaboration has also developed. Graphite whiskers consist of bundles of graphite needles that are grown under the influence of an electrical field on a tungsten filament. The whiskers can be prepared with a diameter of 100 μm to 400 μm. The tungsten filament has a diameter of 10-25 μm. The needles can be spread along the tungsten filament or can form separate bundles. Figure 46 shows a picture of a graphite whisker.

![Graphite Whisker](image)

*Figure 46. Picture of a graphite whisker mounted on the sample holder. The displayed whisker has separate bundles of graphite needles.*

Due to their tiny dimensions, the whiskers are rather fragile. Handling and loading the sample solution had to be done extremely carefully. Experiments in static mode using the two-phase methodology were not successful due to inefficient desorption/ionization. The dimensions and shape of the whiskers, however, make them very attractive for a CF interface because dead volume is rather small. They may be used in future in combination with alternative
7.5 Investigation of Frits

7.5.1 Screening of Various Frits in Static Mode

After the preliminary selection of the available frit materials presented in the previous section, the following inorganic frits were retained for further investigations in static mode: the carbon frit from Le Carbone as well as the carbon, graphite, Si, and TiN frits from EMPA. The frits were fixed on the probe tip with double-sided adhesive tape or with silver glue. Up to 1.5 µL of sample solution were applied onto them. The sample was immediately introduced into the mass spectrometer without previous drying. The graphite frit yielded similar results as the Si frit. Data for graphite are therefore not explicitly shown in this section. The aims were to find the best liquid matrix to use as an ionizing agent, to find the optimum way to deliver the liquid matrix to the laser absorber, and to reveal the upper mass limit of analytes desorbed from the different laser absorbers.

![Figure 47. MALDI mass spectra of substance P and bombesin using A. carbon frits from EMPA and B. Le Carbone. No ionizing agent was added. The laser power in spectrum B. was much above threshold. Therefore the resolution of the analytes' signals is low. The spectra are normalized to the most intense signal.](image)
The MALDI mass spectra of both carbon frits from aqueous solution of substance P and bombesin (0.1 mM, Sigma Chemie, Buchs, Switzerland) are depicted in Figure 47. Protonated, sodiated and potassiated analyte signals are observed in about equal ratios in Figure 47A. The carbon frit from Le Carbone mainly led to potassiated analyte signals. Less intense sodiated and no protonated signals are observed. The threshold laser power for the latter spectrum was much lower than for the former one.

![Figure 48](image)

**Figure 48.** MALDI mass spectra of substance P and bombesin using the carbon frit from Le Carbone **A.** without addition of glycerol, **B.** with 10 % and **C.** with 20 % of glycerol. The spectra are normalized to the most intense signal.

The influence of the quantity of ionizing agent (glycerol) added to the same analyte solution as used before was investigated with both carbon frits. The spectra obtained with carbon (Le Carbone) when 0, 10 and 20 % (v) glycerol were added are shown in Figure 48. An increase in the ratio of the protonated to
the cationized signals with increasing glycerol content is observed. The same effect was found using the carbon (EMPA) sample. A glycerol content higher than 30 % (v) resulted in a glossy film on the frit which prevented the observation of analyte signal. The dependence of the ratio of protonated to cationized analyte signals on the glycerol content and a decrease of the signal intensity with increasing thickness of the glycerol layer was also reported by Dale et al. [45] when working with two-phase particle suspensions. Repeated washing of the Le Carbone sample with bidistilled water in order to remove sodium and potassium salts did not enhance the ratio of protonated to cationized analyte signals.

The technique of applying the ionization agent to the frit by adding it to the sample solution is problematic when using it with frit pieces of different sizes, which had been broken from the 1 cm diameter disks. The amount of the matrix, its thickness on the frit, and therefore the analyte signal intensities obtained with different frit piece sizes cannot be compared even if the liquid matrix content in the analyte solution is the same. For this reason, we were seeking a more adequate technique for applying the liquid matrix to the frit. The frit was left overnight in a well-mixed solution of the liquid matrix and a volatile solvent in the volume ratio 3:7. After this conditioning procedure, the frit was taken out of the solution, fixed on the probe tip, and the analyte was applied.

Liquid matrices and volatile solvents of different polarities were tested using this procedure. The different mixtures of these components are assumed to differ in their ability to spread on the frit material. The matrices tested were glycerol (Fluka Chemie AG, Buchs, Switzerland), 3-NBA and 2-NPOE (Aldrich Chemie, Buchs, Switzerland) (listed in order of decreasing polarity). Methanol and THF (Fluka Chemie AG, Buchs, Switzerland) (listed in order of decreasing polarity) were investigated as solvents. The mixture glycerol/THF could not be prepared because the two compounds are not miscible. The MALDI mass spectra of the carbon frit from EMPA using the five possible liquid matrix/solvent combinations are depicted in Figure 49. Signal-to-noise ratios were slightly worse using 2-NPOE as a matrix (with methanol and THF) than using the other liquid matrix/solvent mixtures. 3-NBA in THF and in methanol worked about equally well, but detrimental matrix adducts to the analyte were observed (signals around 1500 Da in Figure 49D). The results using glycerol in methanol are about the same as those of 3-NBA, but no matrix adducts were observed. The same conclusions could also be drawn when insulin was used as the analyte. For subsequent experiments, we chose a 3:7 (v:v) mixture of glycerol in methanol for conditioning the frit.
Figure 49. MALDI mass spectra of substance P and bombesin using the carbon frit from EMPA. The frit was conditioned in different liquid matrix/solvent mixtures overnight (3:7, v:v). The spectra were not normalized.
In a further step, the ability of the four different frits to generate signals of analytes with masses higher than those of substance P and bombesin was evaluated. An aqueous solution of substance P and insulin was used. The spectra of TiN and Si frits without addition of ionizing agent are shown in Figure 50. One can see that signals of substance P, but not (or no intense signals) of insulin are generated without addition of glycerol to Si and TiN. The mass spectra of the four frits after conditioning them with a 3:7 (v:v) mixture of glycerol and methanol are depicted in Figure 51. When these frits are treated with glycerol, very good signals of insulin as well as of substance P can be observed (Figure 51C and D). A discrimination of substance P compared to insulin can sometimes be observed. Under the same experimental conditions, the carbon frits hardly generate any insulin signals (Figure 51A and B). An aqueous solution of cytochrome c, with an even higher mass, was therefore only tested with pretreated Si and TiN. A mass spectrum of good quality could be obtained with the TiN frit (Figure 52). However, the cytochrome c signal was hardly visible using Si.
Figure 51. MALDI mass spectra of substance P and insulin using A. carbon frit (EMPA), B. carbon frit (Le Carbone), C. Si, and D. TiN. The frits were conditioned with a 3:7 (v:v) mixture of glycerol and methanol. The spectra are normalized to the [P+H]^+ signal.

Figure 52. MALDI mass spectrum of cytochrome c using a TiN frit from EMPA. The frit was conditioned in a 3:7 (v:v) mixture of glycerol and methanol.
In conclusion, we can say that low mass analyte signals, e.g. from substance P, can be generated with carbon frits from Le Carbone and EMPA, and with graphite, Si, and TiN from EMPA. Pretreatment of the frits with a mixture of glycerol and methanol in the volume ratio 3:7 generally increased the ratio the protonated to the cationized analyte signals. Insulin signals could be observed with graphite, Si, and TiN. At these masses, pretreatment of the frits with the ionizing agent was necessary for signal observation. TiN proved to be the best material as it even generated signals for cytochrome c.

The properties of the graphite and TiN frits were further optimized for use in a CF interface. The experiments are described in the following section.

### 7.5.2 Further Optimization of Graphite and TiN Frits

Frits with a diameter of 2.5 mm and a thickness of 0.4 to 0.7 mm were used in the following experiments. For the CF experiments we did not further condition the frits, but delivered glycerol with the solvent. The reason for this change is that, if the frit were conditioned, more glycerol would be present on the frit than could evaporate into the vacuum. This would lead to memory effects since the analyte is dissolved in glycerol and analyte signals persist as long as glycerol is present. A methanol solution of the analyte with 2% (v) glycerol was used as a standard. For experiments in static mode, 2 µL of this solution were applied to the sample. For all experiments, 0.1 mM solutions of substance P and LHRH (Sigma Chemie, Buchs, Switzerland) were used.

CF experiments were performed as follows. The capillary was pushed to the back side of the frit in the frit sample holder. Then the frit was tested in static mode using a LHRH solution. Subsequently, a substance P solution was delivered via the capillary for the CF experiment. The advantage of using a LHRH solution in static mode and a substance P solution in CF mode instead of only one solution in both modes was to avoid contamination and the need to clean the frit after its test in static mode. Before performing a CF experiment, the permeability of the frit was tested by forcing a solution of 2% (v) glycerol in methanol with a syringe via the capillary through the frit which is mounted in the frit sample holder. We checked whether the liquid penetrated through the frit’s pores or whether it leaked out between the frit and the sample holder. Before using a frit in the CF mode, it is necessary that the analyte signal is stable in static mode until glycerol or the analyte are completely exhausted.

Figure 53 shows a picture of some of the graphite and TiN frits discussed in this work.
Graphite frits

Based on the good results in static mode with the graphite frit, its permeability was checked for a possible use in CF mode. We found that the material was not permeable enough for the solvent and assumed that its porosity was too small. Therefore we fabricated frits with greater porosities. For this purpose, the graphite powder was mixed with the porosity-creating agent Porlat K85, an acrylic resin (particulate size distribution 61/150/290). After pressing the frit, Porlat K85 was burned off at temperatures between 250 and 350 °C which left the pores of the frit the size of the porosity-creating agent’s particulates (or smaller if the graphite material shrunk upon heating). Frits with 16, 20, 30 and 50 % (v) of Porlat K85 were prepared. A picture of one of these frits is shown in Figure 53A. The frits’ surfaces had a metallic luster which may reflect the laser light and may lead to inefficient desorption. The surfaces were therefore roughened using SiC abrasive paper. Although the frits showed excellent results in static mode, they were still not permeable for the solvent. It leaked out between the frit and the holder rather than from the frit’s pores. We presume the poor wetting properties of graphite for methanol as the reason for this behavior. The experience of researchers at EMPA also supported this assumption and the graphite frits in the present form had to be rejected as possible substrate materials for a CF interface.
TiN frits

The TiN frits performed the best of all the laser absorber materials tested and were therefore further investigated. TiN frits were either fabricated by pressing TiN powder or by pressing Ti powder with subsequent nitridation of Ti. Optimization of the properties of the TiN material was done in both cases.

A mechanically stable TiN frit was prepared by mixing the TiN powder (particle size distribution 0.8/1.7/3.3) with the bonding agent bakélite (9% (w)), pressing the powder, and heating the frit up to 250 °C. Bakélite did not deteriorate the MALDI spectral quality of the TiN frit since the bakélite signals appear in the low mass range. Due to the small particle size of the TiN powder, the frits were hardly permeable. Carbon powder (particle size distribution 2/7/14) was added as a porosity-creating agent. However, this method was not successful. When burning off the carbon at temperatures up to 1000 °C under oxidative conditions, TiN was oxidized to TiO₂. When working under reducing conditions, carbon was not completely burned off. This is shown in a scanning electron microscopy (SEM) image in Figure 54. MALDI results in static mode for these frits were also not satisfactory.

![Figure 54. SEM image of a frit prepared from 86 % TiN, 9 % bakélite, and 5 % carbon (by weight). The large spheres on the picture are the carbon particulates which have not been burned off.](image)

For these reasons, Porlat K85 was used as the porosity-creating agent. Frits with 47 % TiN, 28 % bakélite, 25 % Porlat K85 (by volume) were prepared (for a picture of the frit see Figure 53B). Investigations under the microscope showed that their pores were not evenly distributed. Furthermore, the frit still proved to
have a low permeability. This may be due to the presence of separate holes instead of a channel structure. Interestingly, we found that the results were not very reproducible after several weeks. This suggested that a passivation, i.e. chemical alteration of the frits occurred. However, no indication was found in the literature that TiN was reactive if exposed to air, where the frits were stored. Before dealing with this issue in more detail, the experiments with the TiN frits prepared from Ti (denoted with TiN(Ti)) are presented.

The Ti particulates were larger than the TiN particulates (particle size distribution 12/26/43 compared to 0.8/1.7/3.3 for TiN). Therefore, the TiN(Ti) frits had larger pores than the TiN frits. TiN(Ti) frits were prepared by converting Ti into TiN at 1450 °C for 2 hours under an ammonia atmosphere. First, a frit prepared with 9 % (w) bakelite yielded a very good result in static mode, comparable to that reported in Figure 51D (for a picture of the frit see Figure 53C). A SEM image of the frit is shown in Figure 55. Unsatisfactory results in CF mode however, suggested that the porosity of the frits was not high enough. For this reason frits with 50 % Ti and 50 % Porlat K85 (by volume) were prepared, among other compositions. The frit performed very well in experiments in static mode, however, using a new batch of the frit which was prepared under identical conditions, the MALDI results could not be reproduced. The color of the frits from the different batches was also different (for example grey, olive green, gold brown). These observations suggested that the protocol for preparing the TiN frits was not reproducible. We therefore investigated the issue further.

Figure 55. SEM image of a frit prepared from 91 % Ti and 9 % bakelite (by weight) with subsequent nitridation at 1450°C under ammonia atmosphere. Please note the different particle size in this frit compared to that in a frit prepared from TiN powder (see Figure 54). Note that the scales in Figure 54 and Figure 55 are different.
Frits were prepared under different atmospheres (vacuum, nitrogen, argon), at different heating temperatures (1450 to 1600 °C) and on different supports in the oven (Al₂O₃, Zr₂O₃, soot, SiO₂). A phase analysis of these frits was performed by X-ray diffraction (XRD). Since XRD signals of the frits were too small due to the frit’s small surface area, they were milled before analysis. Surprisingly, the results revealed that all frits were composed of TiN. In order to check the validity of this result we investigated whether the composition on the surface was different from the composition of the bulk material for the various frits. After removing the upper layer with abrasive paper and ultrasonicallying the frit, the same MALDI results were observed as before treatment. Furthermore, the color of the frits were the same on the frit’s surface and in the bulk. This suggests that surface and bulk composition are the same. The XRD result of the milled sample should therefore be valid. MALDI results were, however, quite different for the various frits but did not correlate with the preparation conditions. Together with the observation that different batches of the same frits yielded different MALDI results, this indicated that the preparation was not reproducible. Additionally, we observed passivation of the frits with time, as was the case for the TiN frits described in the previous paragraph. In an attempt to study the passivation process in more detail, a batch of TiN(Ti) frits was freshly prepared. One part of it was stored as usual, the other under vacuum. MALDI experiments were done after two weeks. The results were generally slightly better when the frits were kept under vacuum. However, they were even different for frits stored under the same conditions.

In conclusion for the TiN frits one can say that their preparation is not reproducible. Furthermore, some passivation of the frit surface seems to occur with time. We could not pinpoint the reason for these observations. The MALDI TOF mass spectrometer worked correctly. Therefore, we had to reject the material.
7.6 Investigation of Graphite Whiskers

For testing the whiskers in static mode, 2 µL of a methanol solution of substance P (0.1 mM) with 2 % (v) glycerol was used as a sample, like in the case of the TiN frits. The loading was done stepwise, each new load was added after waiting for the previous load to dry. This was done under a microscope to avoid damage of the whiskers. The whisker was fixed on a specially designed sample holder (see section 7.2.4). The diameter of the laser spot was about the same size as that of the whisker used. Laser focusing was rather difficult, but could be facilitated by using a DHB-covered whisker which fluoresced when irradiated with the UV laser. Since the dimensions of all whisker supports were the same, we could also use this laser adjustment for the whiskers that were not covered with DHB.

A typical MALDI mass spectrum is shown in Figure 56. A rather small, moderately resolved signal of substance P is observed. Sodiated and potassiated, but hardly any protonated substance P signals are detected. The shot-to-shot reproducibility of the signals was low. Low mass ions are very intense. They stem from graphite and glycerol at the relatively high laser powers used in the experiment (> 40 µJ).

![Figure 56. MALDI mass spectrum of substance P using a graphite whisker.](image)

Possible reasons for the poor performance are a too low substance P concentration and inefficient desorption/ionization. The first point was checked by increasing the substance P concentration by a factor of 100. Nevertheless, no improvement in the quality of the substance P signal was detected. Addition of the matrix DHB led to a high quality spectrum, indicating that the substance P concentration was not the reason for the poor performance.
Ionization was investigated next. Glycerol was added to the sample solution for analyte protonation. Nevertheless, sodiated and potassiated analyte as well as glycerol signals dominated the spectrum. To check whether the sample solution glycerol content of 2 % (v) was reasonable, the optimum percentage was calculated based on the experience that a 30 % (v) ratio of liquid matrix/graphite powder yields good results in the two-phase MALDI methodology [45]. Calculating from the whisker's length (3.5 mm) and radius (100 μm), we obtain a volume of 0.11 μL for a whisker. With an optimum amount of glycerol of 30% (v) of this volume, it yields a concentration of 1.6 % in 2 μL of solvent. The calculation assumes that the surface/volume ratio of a whisker is about the same as for graphite powder. The result indicates that 2 % (v) glycerol in the sample solution is theoretically a good choice. The poor quality of the spectrum may, however, indicate, that it is not optimum. For this reason, the glycerol content in the sample solution was increased stepwise up to 4 %, since it is known that the ratio of protonated to cationized signals increases with the volume ratio of glycerol to graphite. At 4 % glycerol, no signals were detected in the spectrum at all. They slowly reappeared within several minutes in the vacuum but their quality did not exceed that of the sample with 2 % glycerol. This observed time behavior is in agreement with the well-known fact that no signal is detected at too high glycerol/graphite ratios, and that the signal increases with time due to glycerol evaporation [45]. Samples with glycerol contents lower than 2 % and without any glycerol also did not change the quality of the analyte signal. These experiments indicated that glycerol, in the presence of alkali ions, is not important for analyte ionization.

Sodium and potassium ions seemed to be the main contributors in analyte ionization (see Figure 56). Although present in large amounts (very intense signals of Na+, K+, [glycerol+Na]+, [glycerol+K]+) they did not appear to be very effective, given the low quality of the substance P spectrum in Figure 56. Therefore, Cs+ which is generally known to be a powerful cationizing agent for peptides, was added. 2 μL of a 10^{-5} M solution of CsI (Fluka Chemie AG, Buchs, Switzerland) were added to the sample solution. A large Cs+ signal was observed, but the Cs-cationized substance P signal was even lower in intensity than the sodiated and potassiated signals. In a next step, we tried to pinpoint the source of the sodium and potassium ions in order to eliminate it and control ionization by adding glycerol. A new sample solution was prepared from methanol, glycerol, and substance P stemming from freshly opened bottles. The glassware in which the solution was prepared was also carefully rinsed with bidistilled water before use. However, only potassiated and sodiated P signals were detected using this solution, indicating that the alkali ions originated from the whisker itself. Using the drop dialysis method described in chapter 4, we tried to exchange them with ammonium ions. The whisker was immersed for about 30 min. in a drop of water
on a dialysis membrane which was floating on an ammonium citrate solution. Judging from the MALDI spectrum, the procedure was not successful.

Since the means for enhancing the ionization yield, either with glycerol or with metal ions, were exhausted, the low desorption efficiency of the whiskers was likely to be at the origin of the problem. In the two-phase MALDI methodology, thermal desorption is believed to be the main mechanism for analyte desorption. In contrast to the graphite powder, graphite whiskers do not contain separate particles but needles that are connected to each other. This leads to stronger laser energy dissipation, lower energy densities and hence lower temperatures that may not be sufficient for an adequate analyte thermal desorption. Due to their lower diameter, the tips of the graphite needles are hotter than their bases and they would therefore be more effective for desorption. However, it is neither possible to solely cover the needle tips with sample solution nor to solely irradiate this part of the needle. It is therefore concluded that graphite whiskers are not adequate substrates to use in a CF interface.

![MALDI spectrum of substance P using DHB as a matrix. (Figure 57)](image)

Due to their dimensions and shape, the whiskers are characterized by a low dead volume. They are thus very attractive interface substrate candidates. For this reason, another approach for analyte desorption was taken, relying on the use of a conventional MALDI matrix, DHB. The whisker was covered with a thick layer of DHB by stepwise evaporation of a saturated methanol DHB solution. 2 μL of a methanol substance P solution were applied. The corresponding spectrum is shown in Figure 57. A high quality spectrum of the protonated substance P ion was observed, comparable to one which is taken using a conventional stainless steel tip. The DHB-whisker was then tested for its compatibility to be used in CF mode.
We did not have the possibility to visually observe the flow into the mass spectrometer under the microscope. In a first approach, we did experiments at atmospheric pressure. First, we tried to find the optimum flow rate. 200 nL/min of methanol appeared to be the optimum flow rate at that pressure: no drop formation was observed, the volume of solvent delivered also evaporated. After the CF experiment had been run for a short time, we saw that the amount of DHB in the region where the PicoTip touched the needles was decreased but large DHB crystals were seen at both ends of the whisker. This indicated that the solvent exiting the PicoTip propagated parallel to the tungsten filament and then evaporated at both ends of the whisker. It was clear from these experiments that the lateral extension of the needle bundles has to be equal to or smaller than the laser beam size in the MALDI CF experiment. Such whiskers were, unfortunately, not available.

In the vacuum, the pressure reading was a good way to check for flow stability. A flow rate of 200 to 250 nL/min was also found to be adequate. Using whisker bundles with extensions larger than that of the laser beam the signal was comparable to that in static mode. However, the signal disappeared after 1-2 minutes and reappeared when the laser position on the whisker was slightly changed. Another problem was that DHB had evaporated to some extent after working some minutes in CF mode at a repetition rate of the laser of several Hz. This could in principle be solved by constantly delivering a small amount of DHB with the sample solution. Since Hercules et al. have already reported a similar approach for realizing a CF interface (see section 7.1.2) the experiment was not pursued.

In summary, the presented whisker set-up would be useful for the CF coupling. First, due to their large surface, drops leaving the capillary are absorbed by the whisker which leads to a stable flow. Second, whiskers are compatible with rather low flow rates of 250 nL/min, which allows low pressures in the mass spectrometer to be maintained, resulting in high signal intensities. Third, the whiskers, especially those with one single bundle of needles are characterized by a defined, low volume of about the dimensions of the laser beam and may therefore have low dead volumes. Despite these advantages of the whisker set-up, a compatible desorption/ionization method has not been found yet in order to use the whiskers in a CF interface for UV MALDI.
7.7 Continuous-Flow Interface without Memory Effects

An ideal interface would have no memory effects. This would be realized if, at a given time, the amount of analyte and glycerol delivered to the laser absorber is completely desorbed/evaporated. Glycerol has to be included in this statement since in our experiments the analyte is dissolved in glycerol. The analyte MALDI signal will therefore persist as long as glycerol is present. In addition to the parameters important for a stable flow into the mass spectrometer (see section 7.3), the analyte concentration in the eluent, the laser spot size, the adsorption of the analyte on the laser absorber and the volume of the laser absorber play an important role for the dead volume of the interface. Ideal experimental conditions with the frit or whisker set-up could not be established yet. In the following, the influence of some of the parameters on the dead volume of the interface is discussed.

In an ideal interface, the volume of the laser absorber should be negligible compared to that of the capillary. In our experiment, the volume of the 2.5 mm frit is about the same as that of the 50 μm i.d. capillary used (2 μL). The volume of a graphite whisker is only ~5 % of that volume. If frits were used in a future CF set-up, their volume should definitely be reduced, while that of the whiskers seems promising. Designs for a pressing tool for frits with a diameter of 0.5 mm are already available at EMPA.

The laser spot size should cover the complete surface of the laser absorber in order to remove all of the analyte material. The standard laser spot size used in our MALDI TOF mass spectrometer is 0.13 mm² (0.4 mm diameter). The energy required for our MALDI experiments is ~20 μJ per pulse for 0.13 mm². With a maximum energy per pulse of 160 μJ from the nitrogen laser, the laser spot can at most cover an area that is 8 times the present laser spot size. This would correspond to a diameter of 0.9 mm. The frits’ diameter should therefore not exceed this value. This is not fulfilled with the 2.5 mm frits. As far as the whiskers are concerned, their diameter is within the actual spot size of the laser.

The laser absorber may adsorb some analyte molecules on its surface. These may be replaced with other analyte molecules eluted later from the capillary, leading to a memory effect. Therefore, analyte adsorption has to be investigated in future experiments if memory effects are observed.
7.8 Summary and Perspectives

The initial steps of the development of a CF interface for MALDI MS relying on the two-phase MALDI methodology were addressed during the work presented in this chapter. A functioning CF interface is not available yet. However, experience, knowledge, and know-how crucial for further development were obtained.

A large part of the work dealt with the search for and the modification of a broad range of different laser absorber materials, such as organic and inorganic materials, and graphite whiskers, which were tested in static and CF mode. Organic materials had to be rejected very early since their properties, e.g. porosity or absorption of the laser wavelength, were not adequate. Among the various inorganic materials tested, graphite, carbon, Si, and TiN frits were studied further. Glycerol was found to be the best ionizing agent. We also determined the upper mass limit of analytes that can be desorbed from these materials. From the results of these experiments, graphite and TiN frits appeared to be the most promising and they were investigated in more detail. These frits’ properties (mechanical stability, permeability for solvents, dimensions) were modified in order to match the requirements for the use in a CF interface. Despite the good results in static mode, graphite frits had poor wetting properties and therefore were not adequate for our purposes. We also found that the preparation of TiN frits was not reproducible, and that some passivation of their surface occurred with time.

Whiskers were found to be a promising candidates for use in a CF interface. Their dimensions allowed work at low flow rates and hence low pressures in the mass spectrometer, and were compatible with low dead volumes. Unfortunately, a suitable UV desorption/ionization method could not be found.

Another part of the work concentrated on obtaining a stable liquid flow into the mass spectrometer. Important parameters were the heating of the probe tip, the amount of glycerol in the solvent, the nature of the solvent, and the flow rate. Optimum experimental conditions for the frits and the whiskers were determined. A heating system was developed that allowed a good temperature control, in contrast to other set-ups reported in the literature. We found flow rates controlled by a syringe pump to be much more stable than those controlled by the pressure difference between mass spectrometer and atmosphere (hydrodynamic flow). A CF set-up using a syringe pump was therefore designed. In addition, sample holders for the frits and the graphite whiskers were constructed to allow an
efficient connection between the capillary and the frit or the whiskers, respectively.

Finally, critical parameters for a low dead volume interface were identified and are discussed in this work. These are the volume of the laser absorber compared to that of the capillary, the size of the laser spot compared to that of the laser absorber, and the adsorption of analyte molecules on the laser absorber material.

Besides the laser absorbers investigated during the present work, other materials or set-ups are possibly useful:
An alternative to the home-made titanium frits are commercially available titanium frits with a diameter of 1.5 mm, a thickness of 1 mm and 2 μm pores (Vici, Schenkon, Switzerland). In analogy to the home-made frits, they could be transformed into TiN frits, if a reproducible preparation protocol for nitridation is found. The dead volume should be reduced by slicing the frits into several parts with decreased thickness.
Another possibility would be to fix the laser absorber onto the front side of the capillary. This could be realized by dissolving a polymer in an appropriate solvent, addition of a laser-absorbing powder, e.g. graphite or TiN, dipping the capillary end into the mixture and evaporating the solvent. The amount of polymer should be rather low in order to allow the properties of the particles to be retained. A quick evaluation of this method was done. Absorber/polymer films were prepared and stuck to the stainless steel holder used in static mode. Macrolon transparent 281 and Conap CE 1171 were used as polymer components. Although these preliminary experiments with substance P solutions containing different glycerol contents were not very successful, further optimization potential for this methodology certainly exists. Evidently, precautions have to be taken in order not to plug the capillary.
A further potential UV absorber for the CF interface would be a CuO grid stuck onto the front side of the capillary. The grid could be prepared by oxidation of commercially available copper grids with 1500 or 300 lines/inch, i.e. with distances between the copper wires of 17 and 85 μm, respectively (Buckbee-Mears, St. Paul, MN, USA). Partial oxidation maintaining a copper support for the CuO is preferable to total oxidation in order to provide enough mechanical stability.
The approach with a laser absorber made from pressed silicon powder may also be pursued further. Alternatively, the use of porous silicon from silicon wafers, which have been investigated by Wei et al. [51] may be another promising path. The authors successfully performed UV laser desorption/ionization experiments
in static mode on this material without the addition of a matrix. The surface was chemically modified to optimize its ionization characteristics. The frit might also be fabricated using microchip technologies.

An approach with graphite whiskers in a CF interface for IR MALDI might also be promising. In IR MALDI, an IR laser and a corresponding IR-absorbing liquid matrix, e.g. glycerol, is used. The present work demonstrated a stable flow into the vacuum of the mass spectrometer when adding glycerol to the sample solution. The use of an IR laser should therefore rather quickly lead to a successful CF interface. The most critical point with this approach would be to check whether the fragile whiskers withstand the shots from powerful IR lasers.

If an adequate laser absorber is found, the CF interface has to be tested with flow injection analysis to check for memory effects. A program which allows one to take ion profiles (the analyte peak area in the mass spectra is integrated and depicted as a function of elution time), and is necessary for this investigation, was developed in our group by Dr. Richard Knochenmuss. Subsequently, the interface can be coupled to one of the liquid separation techniques discussed in section 7.1.3. The working interface for MALDI MS could eventually be applied to various biological, pharmaceutical, and environmental problems.
7.9 References


<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>AMNP</td>
<td>2-amino-4-methyl-5-nitropyridine</td>
</tr>
<tr>
<td>ANP</td>
<td>2-amino-5-nitropyridine</td>
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<tr>
<td>ATT</td>
<td>6-aza-2-thiothymine</td>
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<tr>
<td>bpy</td>
<td>2,2’-bipyridine</td>
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<tr>
<td>CE</td>
<td>capillary electrophoresis</td>
</tr>
<tr>
<td>CF</td>
<td>continuous flow</td>
</tr>
<tr>
<td>CEC</td>
<td>capillary electrochromatography</td>
</tr>
<tr>
<td>CD</td>
<td>circular dichroism (chapter 5) and cyclodextrin (chapter 6)</td>
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<tr>
<td>α-CHCA</td>
<td>α-cyano-4-hydroxycinnamic acid</td>
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<tr>
<td>Da</td>
<td>Daltons</td>
</tr>
<tr>
<td>DHB</td>
<td>2,5-dihydroxybenzoic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>ESI</td>
<td>electrospray ionization</td>
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<tr>
<td>ESPT</td>
<td>excited state proton transfer</td>
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<tr>
<td>ESR</td>
<td>electron spin resonance</td>
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<tr>
<td>FAB</td>
<td>fast atom bombardment</td>
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<tr>
<td>FIA</td>
<td>flow injection analysis</td>
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<tr>
<td>FTICR</td>
<td>Fourier-transform ion cyclotron resonance</td>
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<tr>
<td>FWHM</td>
<td>full width at half maximum</td>
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<td>GPC</td>
<td>gel permeation chromatography</td>
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<tr>
<td>HIV-1</td>
<td>human immunodeficiency virus type 1</td>
</tr>
<tr>
<td>3-HPA</td>
<td>3-hydroxypicolinic acid</td>
</tr>
<tr>
<td>HSAB</td>
<td>hard and soft acids and bases</td>
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<tr>
<td>i.d.</td>
<td>inner diameter</td>
</tr>
<tr>
<td>IR</td>
<td>infrared</td>
</tr>
<tr>
<td>kV</td>
<td>kilovolts</td>
</tr>
<tr>
<td>LC</td>
<td>liquid chromatography</td>
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<tr>
<td>LD</td>
<td>laser desorption</td>
</tr>
<tr>
<td>LHRH</td>
<td>luteinizing hormone releasing hormone</td>
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<tr>
<td>MS</td>
<td>mass spectrometry</td>
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<tr>
<td>MALDI</td>
<td>matrix-assisted laser desorption/ionization</td>
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<tr>
<td>m/z</td>
<td>mass-to-charge ratio</td>
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<tr>
<td>3-NBA</td>
<td>3-nitrobenzyl alcohol</td>
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<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
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<td>2-NPOE</td>
<td>2-nitrophenyl octyl ether</td>
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<tr>
<td>o.d.</td>
<td>outer diameter</td>
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<tr>
<td>ophen</td>
<td>ortho-phenanthroline</td>
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<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>---------</td>
<td>------------------------------------------------</td>
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<tr>
<td>PEEK</td>
<td>polyether-etherketone</td>
</tr>
<tr>
<td>PNA</td>
<td>para-nitroaniline</td>
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<td>POPOP</td>
<td>1,4-bis(5-phenyloxazol-2-yl)benzene</td>
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<tr>
<td>PS</td>
<td>polystyrene</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinyliden fluoride</td>
</tr>
<tr>
<td>reTOF</td>
<td>reflectron TOF</td>
</tr>
<tr>
<td>SEM</td>
<td>scanning electron microscopy</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
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<tr>
<td>2,3,4-THAP</td>
<td>2,3,4-trihydroxyacetophenone</td>
</tr>
<tr>
<td>2,4,6-THAP</td>
<td>2,4,6-trihydroxyacetophenone</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
</tr>
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<td>TiN</td>
<td>titanium nitride</td>
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<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
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<tr>
<td>TOF</td>
<td>time of flight</td>
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<tr>
<td>UV</td>
<td>ultraviolet</td>
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<td>XRD</td>
<td>X-ray diffraction</td>
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</table>


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Zürich, September 1999

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