Fluorescence spectroscopy of trapped molecular ions produced with matrix-assisted laser desorption/ionization

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Fluorescence Spectroscopy of Trapped Molecular Ions Produced with Matrix-Assisted Laser Desorption/Ionization

A dissertation submitted to

ETH ZURICH

for the degree of

Doctor of Sciences

presented by

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accepted on the recommendation of
Prof. Dr. R. Zenobi, examiner
Prof. Dr. M. Quack, co-examiner

December 2006
Acknowledgments

Although a final product might end up being attributed to only one name, the production of a major work is rarely achieved by a single person. That being said, I have many people to thank for their guidance and support in getting this thesis completed.

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Related Publications

M. Dashtiev, E. Wafler, M. Gorshkov, F. Hillenkamp and R. Zenobi “Ion yield in MALDI TOF mass spectrometry”, manuscript in preparation


Conferences


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M. Dashtiev and R. Zenobi. Conformation of (bio)molecules in the gas phase studied by fluorescence resonance energy transfer. ASMS conference on mass spectrometry, Seattle, Washington, USA. May 27-June 1, 2006

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Abstract

Mass spectrometry is now the key technology to study the structure of biomolecules in the gas phase. Gas-phase measurements of proteins, when compared with solution-phase measurements, can provide deep insight into the effects of the solvent environment on the native molecular structure and dynamics. This field has attracted great attention since the advent of electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI), two soft ionization techniques that can generate intact gas-phase ions of fragile high molecular weight molecules. Because MALDI and ESI typically generate unsolvated ions with few or no adducts, their structure also provides an ideal model system for theoretical calculations of conformations. A major question today is whether singly or multiply charged biomolecular ions produced by soft ionization methods retain their native (or at least a "native-like"), active conformation in the gas phase. This is often assumed but needs to be proven rigorously.

The present thesis is divided into two parts. Part A (Chapters 3-5) deals with fluorescence spectroscopy of ions trapped in a Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer. In Chapter 3 a novel scheme for laser-induced fluorescence measurements of such trapped ions is introduced. It is based on an open FT ICR cell design, continuous wave axial excitation of the fluorescence, orthogonal photon collection by fiber optics, and single photon counting detection. Chapter 4 describes a correlation between solution-phase and gas-phase fluorescence measurements for rhodamine dyes. For the first time, efficient fluorescence resonance energy transfer in the gas phase between two fluorophores is unambiguously shown. In Chapter 5 we investigate the influence of three different gases, helium, argon, and nitrogen on the fluorescence signal intensity of rhodamine 6G cations in the gas phase.

Part B (Chapters 6-8) is devoted to MALDI mechanistic studies that involve electron emission. In Chapter 6 the influence of the addition of CuCl₂ to the matrix/analyte solution on the analyte ion signal is analyzed. Chapter 7 deals with fundamental phenomena such as the
number of photons needed to produce electrons, the influence of the substrate material, and the kinetic energy of the electrons on photoelectron emission. In Chapter 8 the ratios of positive to negative ion yields for MALDI TOF mass spectrometry were measured and the dependence of the ion yield on acid/base properties of the molecules was examined.
Zusammenfassung


Teil B (Kapitel 6-8) umfasst Studien über MALDI-Mechanismen bei welchen Elektronenemission eine Rolle spielt. In Kapitel 6 wird der Einfluss der Zugabe von CuCl₂
1 Introduction

The study of biological molecules in the gas phase has rapidly turned into a new area in chemistry. In particular, mass spectrometry has allowed us to begin to interrogate large molecules of biological significance in a solvent free environment. The goal of this chapter is to provide a basic treatise plus an overview over the latest research from the leading groups across the entire field, mainly including fluorescence spectroscopy combined with mass spectrometry.
1.1 General introduction

Structural and dynamic information of proteins or other biological molecules is important for understanding their functions. The knowledge about the physical properties of biomolecules and their resulting behavior in biological processes can be obtained by different approaches. The most obvious method is to investigate molecules in their natural environment, for example, by nuclear magnetic resonance (NMR). Although this approach is the most direct, the systems under investigation are often too complex to reveal the underlying molecular processes or even which molecule or charge state is investigated. In some cases, another approach is preferred, namely to separate the molecule from the environment in a first step and characterize and study the isolated biomolecule in the gas phase. The environment can then be reconstituted in a stepwise fashion.

Mass spectrometry has many attractive features for studying biomolecules in the gas phase, including the capability of isolation of ions and elimination of unwanted species prior to detection, positive identification of the molecule from the exact mass or from MS/MS data, possibility to compare gas-phase and solution-phase structures, as well as to understand the effect of solvents on a molecule. Perhaps, the main relevance of using mass spectrometry is in gaining complementary information, which may not (or only partially) be accessible through the established techniques. For example, in addition to molecular weight one can also get information about elemental and isotopic compositions [1], structure or sequence of the molecule [2], reactivity in the gas phase or even thermochemical data (proton affinities, gas-phase basicities) [3]. Furthermore, mass spectrometry is an excellent technique in terms of sensitivity. It has a huge advantage over NMR in terms of sample amounts needed for typical study. Typical mass spectrometric experiments require as little material as few nanograms (or even less) while in NMR milligram quantities are needed!

This field has attracted great attention since the advent of electrospray ionization (ESI) [4] and matrix-assisted laser desorption/ionization (MALDI) [5,6], two soft ionization techniques that can generate intact gas-phase ions of fragile high molecular weight molecules. Because MALDI and ESI typically generate unsolvated ions without any adducts, their structure also provides an ideal model system for theoretical calculations of conformations. A major question today is whether singly or multiply charged biomolecular ions produced by soft
ionization methods retain their native (or at least a "native-like"), active conformation in the gas phase [7]. This is often assumed but needs to be proven rigorously.

1.2 Available methods

There are mass spectrometric methods available for obtaining conformational information of molecules in the gas-phase: blackbody infrared radiative dissociation (BIRD) where ions undergo unimolecular dissociation by exchanging their energy with the surroundings by absorption and emission of infrared photons [8], collision-induced dissociation where ions are dissociated as a result of interaction with a target neutral species [9], hydrogen-deuterium exchange where hydrogen atoms of the protein are exchanged with the deuterium atoms from a solution [10], covalent or non-covalent tagging of biomolecules based on the surface accessibility of specific moieties [11,12], and ion mobility spectrometry where the measured cross section of the molecule is compared to a theoretically calculated cross section [13]. However, these methods generally yield only indirect information about the gas-phase conformation, which is often derived from either the overall cross section of the molecule, from a fragmentation pattern, or from a dissociation rate.

Ideally, a method completely “orthogonal” to mass spectrometry is desirable to study gas-phase conformation of (bio)macromolecular ions. A method that is often applied in biology is fluorescence, in particular fluorescence resonance energy transfer (FRET) [14,15]. FRET is a distance sensitive method that correlates changes in fluorescence intensity with changes in distance and orientation of specific spectroscopically active (donor, acceptor, quencher) moieties. The distance between fluorophores is defined as the distance between the centers of the donor and acceptor chromophores; their relative orientation has been found to play a fairly minor role. Distance information from 10–100 Å can be obtained, which makes the method suited for studying many biological systems. FRET spectroscopy owes its origins to Förster, who first defined the spatial relationship between donor and acceptor chromophore in Annalen der Physik, which was originally published in German [16] and later translated. Stryer and Haugland’s paper “Energy transfer: a spectroscopic ruler” is widely considered as the fundamental paper, and the methods described there are still used nowadays [15]. They
used a series of synthetic poly-Pro peptides containing up to 12 residues with the termini labeled with a naphthyl and a dansyl probe as the donor-acceptor pair. The authors showed experimentally what Förster had predicted, namely, there is an inverse sixth power relationship between the FRET efficiency versus distance (12-46 (±3) Å between the probes). Stryer's subsequent review demonstrated the wider application of FRET spectroscopy to the analysis of protein structures. FRET measurements can thus provide fairly direct, quantitative information of molecular conformation.

1.3 Previous work

Little has been reported on fluorescence of gas-phase ions. In the earlier stage of its appearance the idea of laser-induced fluorescence (LIF) experiments was mainly to acquire LIF spectra of atomic ions in order to prove the validity of the concept [17-31]. Later this idea was extended onto the Penning-trapped organic ions [32] and only recently this concept was applied to study conformation of biomolecules in the gas phase [33-40]. A brief introduction to the previous work is presented below.

The first laser-induced fluorescence (LIF) spectrum of a molecular ion was reported for N$_2^+$ in 1975 [17], quickly followed by CO$^+$ [18] and CO$_2^+$ [19], and some years later by various fluorinated benzene cations [20-22]. In these experiments, molecular cations were typically produced by Penning ionization, namely, collisions of neutral analyte molecules with metastable inert gas ions (He$^+$, Ar$^+$, etc.) generated by a dc discharge. LIF excitation spectra of mass-selected CD$^+$ [23], BrCN$^+$ [24] and N$_2^+$ [25] ions confined by simultaneous rf and dc potentials in a three-dimensional quadrupole (Paul) ion trap were reported in 1980-1982. LIF of atomic ions, such as Mg$^+$, Be$^+$, Hg$^+$, and Ba$^+$ confined in a Penning trap have since been reported [26-31].

In a recent publication of Wang et al. [32], the laser-induced fluorescence excitation spectrum of gas-phase hexafluorobenzene cations (C$_6$F$_6^+$) has been measured. Electronic excitation of ions was provided by a tunable, pulsed dye laser pumped at the third harmonic wavelength (355 nm) of a pulsed Nd:YAG laser. Electron impact ionization was used to
generate abundant molecular ions (> 10^6 ions for a 10 s ion accumulation period). An LIF excitation spectrum was obtained by a scanning dye laser stepwise in the range of 450-464 nm with 0.1-0.2 nm resolution. At each laser frequency, ions were trapped for more than 100 s for fluorescence detection, allowing 1000 laser shots using the 10Hz repetition rate of the laser. The average fluorescence intensity from each laser shot was a little less than 2 photons, resulting in a summed signal-to-noise (S/N) ratio of ~75:1. To keep the ions on axis for several seconds, quadrupolar axialization (QE) in the presence of a He buffer gas at the pressure of 10^-5 torr was used. The ion abundance was highly reproducible (± 10%) from one step to the next. This set-up, however, comprised a number of optical elements (mirrors, prisms) inside the vacuum chamber, which complicated alignment procedures.

In subsequent work of the same group [39], the fluorescence lifetimes of penta- and trifluorobenzene radical cations were measured. The experimental setup was similar to that in the previous work, although some improvements had been achieved. The average fluorescence intensity from each laser shot was already 75 photons/laser pulse compared to 2 photons before. The signal-to-noise ratio was also improved and found to be ~94:1. The measurement period lasted ~ 30 min, yielding fluorescence lifetimes (t) for C_5F_5H^+ (44 ± 2 ns) and for C_6F_5H_3^+ (51 ± 2 ns). The same experiment has been performed with an equimolar mixture of C_5F_5H^+ and C_6F_5H_3^+. For simultaneous confinement of both cations, a stored inverse Fourier transform (SWIFT) produced QE at two bands was employed. The effective lifetime of the mixture, t_{eff}, was 47±2 ns.

Cage et al. [38] measured the wavelength resolved fluorescence LIF spectrum of trifluorobenzene radical cations. The trapped ions were excited for 10 seconds at the excitation wavelength λ_{exc} = 448 nm (3mJ/pulse). The minimum number of ions was estimated to be ~ 10^6. The net fluorescence spectrum was accumulated for ~80 h. Electron-induced fluorescence (EIF) emission spectra were acquired for comparison. During the EIF experiment, the ions were not trapped but rather produced and excited by impact of a continuous electron beam. The excitation wavelength corresponds to the transition from the ground vibrational level of the electronic ground state to the ground vibrational level of the second electronically excited state. Only one hot band was observed. Emission peaks were determined with an accuracy ranging from ± 0.2 nm to ± 0.5 nm.
Friedrich et al. [40] described the construction of linear quadrupole ion trap, designed for the measurement of laser-induced fluorescence (LIF). The fluorescence lifetime of gas-phase Rhodamine 640 radical cations was determined. The value obtained (6 ns) is only slightly above the highest reported solution phase value. In this work, ions were created by electrospray and then trapped for 0.1-120 seconds. After the filling period, the ion beam was switched off and the trapped ions were probed by the 2nd harmonic of Nd:YAG laser (λ=532 nm) with pulse width of 3-5 ns for 10 s at 10 Hz. Since the pulse width of the laser was in the range of the mean excited state lifetime, the fluorescence lifetime (τ) was extracted from the signal by using linear response theory, i.e. assuming that the signal can be modelled as a convolution of the system's δ-pulse response and the excitation pulse of the laser.

Khoury et al. [37] have developed sensitive methods to measure LIF from trapped ions by reducing the detection of background scattering to near zero levels during the laser excitation pulse. Measurements were performed on Rhodamine 640 and Alexa Fluor 350 cations in an rf quadrupole ion trap. Rhodamine 640 and Alexa Fluor 350 cations were generated in the gas phase by electrospray ionization and injected into a radiofrequency Paul trap where they were stored and exposed to Nd:YAG laser pulses at 532 and 355 nm for times up to 10 min. LIF was detected perpendicularly to the axis of the laser beam through a small hole in one of end caps. Photons were focused by lenses onto a photomultiplier tube (PMT), which is positioned inside the vacuum chamber. The number of trapped ions was varied over the range $10^3 \leq N \leq 6 \times 10^4$. Helium was admitted directly into the trap to maintain a constant chamber pressure of $5 \times 10^{-6}$ torr during ion storage. Both trap assembly and background gas were temperature controlled in the range of 140 K to 423 K. Analysis of the system response (LIF) to various changes of the system's parameters including temperature, potentials, laser energy, and ion number was described. The ion density was calculated self-consistently within a mean field approximation and these calculations accurately reproduce the ion number dependence exhibited by the fluorescence data and ion kinetic temperatures. Compare to solution-phase data, a strong blue shift of the emitted fluorescence light was observed.

Subsequent work of Danell and Parks [34] reports on FRET of trapped oligonucleotide duplexes. Double-stranded oligonucleotide anions were prepared with FRET donor/acceptor (tetramethylrhodamine (TMR) and texas red (TR) from the BODIPY line of dyes) pairs
attached. Gas-phase ions were generated by ESI and injected into a heated quadrupole ion trap where they were stored and exposed to Nd:YAG laser pulses at 532 nm. The experiments were conducted on an ensemble of ions (~7300 ions) of which only ~350 were within the laser-ion interaction volume. The ions were irradiated for 60 s at a laser pulse repetition rate of 100 Hz before mass analysis took place to confirm that no complete dissociation occurs. At room temperature the fluorescence from the donor was decreased by the proximity of TR. By increasing the temperature up to 120 °C, the authors were able to observe the dynamics of dissociation of the duplex by an increase of the donor fluorescence. However, only a 10% increase of the donor fluorescence was observed, which may also be interpreted as being a temperature induced effect, i.e. not due to partial “melting” of the double stranded DNA. Furthermore, acceptor fluorescence, a common diagnostic to identify a FRET process, was not detected.

Iavarone and Parks [36] introduced another approach to study gas-phase conformational changes. A dye-labeled, 20 residue protein called Trp-cage was used in this case. The native structure of this protein includes a Trp residue “caged” by three Pro residues. As the protein is heated to induce conformational changes, the Trp will be released from its cage and become more exposed to intramolecular collisions with the dye moiety. These collisions quench the fluorescence of the dye, so that conformational changes can be directly correlated with the fluorescence intensity from the dye. Protein ions were generated by nanoelectrospray and injected into a quadrupole ion trap, which can be heated to a maximum temperature ~445 K. To thermalize ions background He gas was used at a pressure of 3mtorr. The fluorescence emission intensity was monitored as a function of the background gas temperature between 300 K and 445 K. Fluorescence intensity decrease was observed for 2+ and 3+charge states, although for 3+ state the effect was much more pronounced.

The most recent work of Iavarone and Parks [35] deals with fluorescence measurements of polypeptides labeled with a fluorescent dye (BODIPY TMR). The conformational dynamics was studied as a function of temperature and charge state. Measurements of (BODIPY TMR) - [Pro]ₙ - Arg - Trp and (BODIPY TMR) - [Gly-Ser]ₘ - Arg - Trp have been performed for charge states 1+ and 2+ of n = 4 and 10 and m = 2 and 5. Experiments were performed on a quadrupole ion trap mass spectrometer that was described elsewhere. The 2+ charge states of both of these polypeptides exhibited similar temperature dependences for equal chain lengths.
(n = 4, m = 2 and n = 10, m = 5) and suggested conformations dominated by Coulomb repulsion. The blue shift of the dye emission/excitation bands was observed. The authors attributed this effect to the interaction of the dye with a charged residue that perturbs the electronic levels of the dye. The change in fluorescence as a function of temperature in the gas phase was two orders of magnitude greater than that in solution. This dramatic result was attributed to the restrictions on intramolecular dynamics imposed by diffusion-limited kinetics and the lack of shielding by the solvent.

An interesting approach of using the LIF technique for detection of trapped, ionized nanoparticles was suggested by Cai and coworkers [41]. It is well known that conventional ionization-based detectors are restricted to detection of charged particles of m/z < 10⁶[42-44]. Cai et al. developed an ion trap with a detection system based on laser-induced fluorescence of fluorescently labeled nanoparticles [41]. An Ar-ion laser was used to excite ionized dye-labeled polystyrene nanoparticles (29 nm in diameter and, corresponding to > 5 MDa in mass). Each particle was labeled with ~ 180 “fluorescein equivalents”. Ions were created inside the ion trap by MALDI, with a charge state between +1 and +10. The approximated overall detection efficiency was 10⁻³. The operating pressure was ≈ 50 mtorr. Further improvements of this instrument is described in [45,46]. The new design compromised two Paul traps, where one serves as a trapping and mass-analyzing device, while the second trap serves to capture and concentrate the ions ejected from the first trap for fluorescence detection. This allowed to analyze even bigger nanoparticles (114 nm in diameter) and also labeled antibodies (IgG, 150 kDa). Different optical detection methods of macroions have been reviewed recently [47].

1.4 Motivation

The ultimate goal of this work is to obtain gas-phase conformation of (doubly labeled) biological molecules. For this purpose it is necessary to design and construct a fluorescence detection system, which has a simple optical setup, high detection efficiency, low acquisition time, and is based on Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer rather than on an ion trap. Several advantages of FT-ICR over the Paul trap exist: (i) easier
manipulation of the trapped ion population, including ion ejection and isolation; (ii) better confinement of the ion cloud; (iii) superior capabilities for MS and MS' experiments; (iv) better control over the ion kinetic energy; and (v) lower operating pressures, allowing for well controlled ion-molecule reactions in the FT-ICR cell. FRET will be used as a probe to elucidate conformational dynamics of biomolecules. Compared to the fluorescence quenching technique used by Parks and coworkers, FRET is more general because it does not have any restrictions on the structure of the molecule (e.g. it does not require Trp or other intrinsic quenchers). For this purpose, several fluorescence dyes were investigated and a suitable FRET pair for gas-phase studies, namely rhodamine 6G covalently bound to sulforhodamine B was identified. The fluorescence detection system response for different experimental parameters such as pressure, buffer gas, etc., has also been analyzed.
1.5 References


2 Methods

This chapter gives an overview of the instruments and methods used in this work. Most of the experiments were performed on a home-built Fourier transform ion cyclotron resonance mass spectrometer with an internal MALDI source. The principles and experimental setup are described in detail. An introduction to time-of-flight instrument mass spectrometry, matrix-assisted lased desorption ionization, and fluorescence resonance energy transfer, with particular attention to FRET in the gas phase, is also presented.
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2.1 Fourier Transform Ion Cyclotron Resonance Mass Spectrometry
2.1.1 Ion motion in a magnetic field

To understand the behavior of ions in an ICR cell it is convenient to start with an ideal situation where a single ion is moving in the presence of a spatially uniform magnetic field \( \vec{B} = B_0 \hat{z} \) (Figure 2.1).

In this case, the ion is subject to a Lorentz force: \( \vec{F}_L = q(v \times \vec{B}) \), in which \( q, v \) are ionic charge and velocity, and \( \vec{B} \) is the magnetic field. This force is perpendicular to both the velocity \( v \) of the charge \( q \) and the magnetic field \( \vec{B} \) and bends the ion trajectory into a circle. According to the Newton's second law the equation for ion motion can be written as:

\[
ma = \vec{F}_L = q[v \times \vec{B}]
\]  

(2.1)

Let \( v_{xy} = \sqrt{v_x^2 + v_y^2} \) denote the ion velocity in the \( xy \) plane (i.e., the plane perpendicular to \( B \)).

Because of angular acceleration, \( \frac{dv}{dt} = \frac{v_{xy}^2}{r} \), Equation (2.1) becomes

\[
m \frac{v_{xy}^2}{r} = q v_{xy} B_0
\]

(2.2)
The angular velocity, \( \omega \) (in rad/s), about the z-axis is defined by \( \omega = \frac{v_y}{r} \), so that Equation (2.2) becomes

\[
m \omega^2 r = q B_0 \omega r
\]  

or simply:

\[
\omega_c = \frac{qB}{m} \quad \text{or} \quad v_c = \frac{qB_0}{2\pi m} = \frac{1.53561 \times 10^7 B_0}{m / z} \quad (2.4)
\]

where \( \omega_c \) is called the “unperturbed” cyclotron frequency. Thus, we obtain the result that all ions in a spatially uniform magnetic field are rotating with a cyclotron frequency, \( \omega_c \), defined by Equation (2.4). A remarkable feature of Equation (2.4) is that all ions of a given mass-to-charge ratio, \( m/q \), have the same ICR frequency, independent of their velocity. Rearrangement of Equation (2.4) yields the ion cyclotron orbital radius of an ion of velocity \( v_y \):

\[
r_c = \frac{m v_y}{q B_0} \quad (2.5)
\]

For an ion in equilibrium with its surroundings at temperature, \( T \) (in K), the velocity \( v_y \) is given by \( v_y = \sqrt{\frac{2kT}{m}} \), in which \( k \) is the Boltzmann constant. Substituting \( v_y \) in (2.5) we obtain

\[
r_c = \frac{1}{qB_0} \sqrt{2mkT} \quad (2.6)
\]

Table 2.1 summarizes typical cyclotron frequencies and radii for thermally equilibrated ions in the ICR cell at a magnetic field value of 4.7 Tesla. The value for the magnetic field strength corresponds to the actual value of the instrument’s magnet.
<table>
<thead>
<tr>
<th>Mass, Da</th>
<th>Cyclotron frequency, $v_c$</th>
<th>Cyclotron radius, $r_c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>720 kHz</td>
<td>0.05 mm</td>
</tr>
<tr>
<td>1000</td>
<td>72 kHz</td>
<td>0.15 mm</td>
</tr>
<tr>
<td>10000</td>
<td>7.2 kHz</td>
<td>0.48 mm</td>
</tr>
<tr>
<td>50000</td>
<td>1.4 kHz</td>
<td>1.09 mm</td>
</tr>
<tr>
<td>100000</td>
<td>720 Hz</td>
<td>1.54 mm</td>
</tr>
</tbody>
</table>

**Table 2.1.** Typical values for cyclotron frequencies and radii for a thermally equilibrated ion at the room temperature in the ICR cell at a magnetic field value of 4.7 Tesla.

### 2.1.2 Axial confinement of ions

To this point we have considered only idealized ion behavior in a spatially uniform magnetic field. Since the $z$-component of the Lorentz force is zero, the ions are still free to escape along $z$-axis. In order to prevent such an escape, it is common to apply a small (1 to 10 volt) electrostatic potential to each of two trapping electrodes. This electrostatic potential produces a radial force:

$$\vec{F}_r = q\vec{E}(r) = -q\nabla \Phi$$  \hspace{1cm} (2.7)

where $\Phi$ is a three-dimensional potential for axial confinement of ions, which can be defined from Laplace’s equation $\Delta \Phi(x,y,z) = 0$. A typical potential landscape for a cylindrical open ICR cell is shown in Figure 2.2. It was obtained by solving the Laplace’s equation numerically with SIMION.
Figure 2.2. Electrostatic potential surface within an open cylindrical ICR cell. Trapping potentials are set to 1 V, central electrodes are grounded.

Since this equation cannot be solved analytically for the whole volume of the ICR cell it is convenient to use a cylindrically symmetrical three-dimensional axial quadrupolar electrostatic potential of the form [1]:

$$\Phi(x, y, z) = V_{\text{trap}} \left( \gamma + \mu(2z^2 - x^2 - y^2) \right)$$  \hspace{1cm} (2.8)

or in cylindrical coordinate system

$$\Phi(x, y, z) = V_{\text{trap}} \left( \gamma + \mu(2z^2 - r^2) \right)$$  \hspace{1cm} (2.9)

in which $V_{\text{trap}}$ is the voltage applied to the trapping electrodes and $\gamma, \mu$ are defined by the cell geometry [1]. The ion motion in $z$-direction is defined as:

$$m \frac{d^2 z}{dt^2} = -q \nabla \Phi(x, y, z)$$  \hspace{1cm} (2.10)

Substituting Equation (2.9) in Equation (2.10) it is straightforward to obtain an ion’s $z$-position that oscillates sinusoidally with time,

$$z(t) = z(0) \cos(\omega t)$$  \hspace{1cm} (2.11)

where
2.1 Fourier Transform Ion Cyclotron Resonance Mass Spectrometry

\[ \omega_z = \sqrt{\frac{4qV_{mp}\mu}{m}} \]  \hspace{1cm} (2.12)

Typical trapping frequencies at a magnetic field strength of 4.7 Tesla are summarized in Table 2.2.

<table>
<thead>
<tr>
<th>Mass, Da</th>
<th>Trapping frequency, ( v_z )</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>7.3 kHz</td>
</tr>
<tr>
<td>1000</td>
<td>2.3 kHz</td>
</tr>
<tr>
<td>10000</td>
<td>720 Hz</td>
</tr>
<tr>
<td>50000</td>
<td>318 Hz</td>
</tr>
<tr>
<td>100000</td>
<td>231 Hz</td>
</tr>
</tbody>
</table>

Table 2.2. Typical trapping frequencies at a magnetic field strength value of 4.7 Tesla.

2.1.3 Ion motion in an electromagnetic field

Let us now consider an ion that is subject to both a spatially uniform magnetic field and electric field, arising from the electrostatic potential applied to the trapping electrodes of an ICR cell. To a good approximation, ion motion in an ICR cell can be described as a superposition of three different motions, namely the trapping oscillation, the cyclotron and the magnetron motion. The overall force acting on the ions is given by Equation (2.13)

\[ \vec{F} = q\vec{E} + q[\vec{v} \times \vec{B}] \]  \hspace{1cm} (2.13)

Since \( \vec{E} = -\nabla \Phi = \mu V_{mp} \begin{pmatrix} +2x \\ +2y \\ -4z \end{pmatrix} \), we can formulate these equations as:
\[ m\ddot{x} = 2\mu V_{\text{trap}} q x + q B_0 y \]
\[ m\ddot{y} = 2\mu V_{\text{trap}} q y + q B_0 x \]
\[ m\ddot{z} = -4\mu V_{\text{trap}} q z \]

The complete solution can be written as:

\[ x(t) = \rho_+ \cos(\omega_c t + \phi_+) + \rho_- \cos(\omega_c t + \phi_-) \quad (2.14) \]
\[ y(t) = -\rho_+ \sin(\omega_c t + \phi_+) - \rho_- \sin(\omega_c t + \phi_-) \quad (2.15) \]

\[ z(t) = \rho_z \cos(\omega_c t + \phi_z) \]

As can be seen from the Equations (2.14) and (2.15), the motion of an ion is a superposition of two circular orbits differing in radius, orbital frequency and phase. The circular motion with higher orbital frequency (\( \omega_+ \)) is called “cyclotron motion”. The motion with lower frequency (\( \omega_- \)) is called “magnetron motion”. The magnetron and trapping frequencies are usually much less than the cyclotron frequency, and generally are not detected. The following useful relations can be derived from Equations (2.17-2.19):

\[ \omega_+ = \frac{\omega_c}{2} + \sqrt{\left(\frac{\omega_c}{2}\right)^2 - \frac{\omega_0^2}{2}} \quad \text{reduced cyclotron frequency} \quad (2.16) \]
\[ \omega_- = \frac{\omega_c}{2} - \sqrt{\left(\frac{\omega_c}{2}\right)^2 - \frac{\omega_0^2}{2}} \quad \text{magnetron frequency} \quad (2.17) \]
\[ \omega_z = \sqrt{\frac{2qV_{\text{trap}}}{ma^2}} \quad \text{trapping frequency} \quad (2.18) \]
\[ \omega_c = \frac{qB}{m} \quad \text{unperturbed cyclotron frequency} \quad (2.19) \]
Analysis of Equations. (2.17-2.19) shows that the magnetron and reduced cyclotron frequencies converge to a common value, \( \frac{1}{2} \omega_c = \frac{gB}{2m} = \omega = \omega_2 \) at the so-called "critical" m/z, namely, when \( \omega_c^2 = 2\omega_2^2 \) or

\[
m_{\text{crit}} = \frac{B_5^2}{8V_{\text{trap}}\mu}
\]  

(2.23)

For ions with m/z > m_{\text{crit}}, cyclotron motion is no longer stable, and ions spiral outward until they are eventually lost from the trap. It is also useful to take a look at the Hamiltonian of a trapped ion. The Hamiltonian can be calculated as follows:

\[
H = E_{\text{kin}} + E_{\text{pot}} \quad \text{or} \quad H = \frac{1}{2} m \vec{v} \cdot \vec{v} + q\Phi(\rho, z)
\]  

(2.24)

Evaluating the dot product from Equations (2.14) and (2.15) and substituting Equations (2.21), (2.18), (2.9) into (2.24) we find:

\[
H = \frac{1}{2} m \left[ \omega_c^2 - \omega_2^2 / 2 \right] \rho_z^2 - \frac{1}{2} m \left[ \omega_c^2 - \omega_2^2 / 2 \right] \rho_z^2 + \frac{1}{2} m \omega_2^2 \rho_2^2
\]  

(2.25)

Inspecting the signs reveals that the energy, which is associated with the cyclotron and axial motions, is positive. Therefore, reducing the energy in either of these motions reduces their amplitude; they are stable (i.e. as ions lose cyclotron or axial energy, they relax to the bottom of their respective potential wells). In contrast, the energy that is associated with the magnetron motion is negative; it is inherently unstable (i.e. potential energy is a maximum, not a minimum at the center of the trap); thus as an ion loses energy by ion-neutral collisions, its magnetron radius increases.
2.1.4 Excitation and detection of trapped ions

It is important to recognize that pure ion cyclotron motion is not useful by itself. First, the cyclotron radius of thermal ions (Table 2.1) is too small to create a detectable electric signal (net difference between the charge induced in two opposed parallel electrodes). Second, at its instant of formation in the ion trap, the phase of each ion's orbital motion is random. Thus any charge induced on two opposed detector plates is balanced and the detected signal is zero. Therefore, ions must be coherently excited to a larger (and thus detectable) orbital radius.

Single frequency (impulse) excitation [2,3] is accomplished by applying an azimuthally spatially uniform electric field that oscillates sinusoidally with time $E(t) = E_0 \cos \omega t$. All ions with the cyclotron frequency of the excitation field will be accelerated to a larger orbital radius.

$$r = \frac{E_0 T_{exc}}{2B_0} \quad (2.26)$$

The excitation is then turned off and the coherent packet of ions start to induce a charge on either of two detection electrodes, leading to an oscillating image current between them. The frequency of this image current is then measured. It is important to note that the post-excitation ion cyclotron radius is independent of $m/z$. Thus, all ions of a given $m/z$-range can be excited to the same orbital radius. This type of excitation is usually applied when ions of interest have only a single $m/z$ ratio.

Frequency-sweep (chirp) broadband excitation [3] is normally applied to detect ions of many masses. To accomplish broadband detection, many frequencies are applied during the excitation event. The most common method for broadband excitation is to use a rapid frequency sweep, or r.f. chirp. For example, a frequency synthesizer can be programmed to sweep over frequencies from 10 kHz to 1 MHz in a 1 ms period. This will cause all ions with cyclotron frequencies in this range to be excited into large cyclotron orbits of the same radius. The image current that results from ions of several mass-to-charge ratios is a composite of sinusoids of different frequencies and amplitudes. The frequency components of the signal are obtained by applying a Fourier transform to the time domain transient. The frequency
spectrum is converted into a mass spectrum by applying a calibration formula that can be derived from the cyclotron equation.

Another approach for a broadband excitation is called “SWIFT” (Stored Waveform Inverse Fourier Transform) excitation [4-6]. The idea of this approach is based on reversing the process and beginning by defining the desired excitation profile in the mass-domain, then converting it to frequency-domain excitation spectrum, and finally performing an inverse Fourier transform to generate time-domain excitation waveform.

From the first derivative of Equation (2.4) it is straightforward to obtain the useful relation for mass resolution:

\[
\frac{m}{\Delta m_{1/2}} = \frac{\omega_c}{\Delta \omega_{1/2}}
\]  

(2.27)

where \(\Delta m_{1/2}\) and \(\Delta \omega_{1/2}\) are measured at the full width of the peak at half height. Experimental resolution is directly proportional to the time duration of the acquired transient signal and can be approximated by

\[
\frac{m}{\Delta m_{1/2}} = \frac{fT}{2}
\]  

(2.28)

where \(f\) is the cyclotron frequency of the ion in Hz and \(T\) is the amount of time the transient signal is observed in seconds. Thus achieving high resolution can be simply done by increasing the transient time. In the absence of collisions it is limited only by the amount of memory in the electronics recording the signal.
2.1.5 Quadrupolar excitation and collisional cooling

For successful fluorescence measurements in an FT ICR it is necessary to have a very tight ion packet, in order to get a maximum overlap with the excitation laser. Since in our experimental setup (see chapter 2.3) ions are created off axis by internal MALDI they have a large magnetron motion (radius) and large initial axial amplitudes. Axial motion can be reduced by introducing a buffer gas to an ICR cell, resulting in a collisional “thermalization” of ions. Of course, the initial cyclotron motion will be thermalized as well. However, as was shown in chapter 2.1.3 collisional damping causes the magnetron radius to expand since a large magnetron radius corresponds to lower (trapping) potential energy. Ions move radially toward the electrodes parallel to the magnetic field and are eventually lost from the trap.

Reduction of the magnetron motion can be achieved by applying a quadrupolar excitation field (Figure 2.3) in the presence of a buffer gas. This process is usually referred to a “quadrupolar axialization” [7-12].

![Diagrams showing quadrupolar axialization](image)

**Figure 2.3.** Left: applied voltages $V_{qe}$ for quadrupolar axialization, with $V_{qe} = V_{qe,\text{max}} \sin \omega t$, right: SIMION simulations of the equipotential lines in the xy-plane.

It was first introduced by Savard et al. [7] for ions of a single mass-to-charge ratio, and later extended by Schweikhard for ions of arbitrary $m/z$ ranges [8]. An excellent theoretical review
on quadrupolar axialization can be found in [13]. The central idea of quadrupolar axialization is the conversion of magnetron motion to cyclotron motion. Collisions then damp the cyclotron motion to zero and ion relax toward the center of the trap. The interconversion frequency is given by

\[ \omega_c = \omega_+ + \omega_- \]  

i.e. it corresponds to the unperturbed cyclotron frequency. Certainly, the magnetron expansion in the presence of collisions will still remain, but the rate of this process is much slower than the damping rate of the cyclotron and axial motions. Thus, it will result in a net reduction of ion radius.

In the presence of collisions the equation of ion motion can be rewritten as:

\[ \vec{F} = q\vec{E} + q(\vec{v} \times \vec{B}) - mk\vec{v} \]  

with the damping constant \( k \) and \( \vec{E} \) defined from Equation 2.7. The effective potential inside the cell will thus be a superposition of the trapping potential and quadrupolar excitation potential and can be written as:

\[ \Phi_{\text{eff}} = \Phi_{\text{trap}} + \Phi_{\text{qe}} \]  

where

\[ \Phi_{\text{trap}}(x, y, z) = V_{\text{trap}} \left( y + \mu(2z^2 - x^2 - y^2) \right) \] as before, and

\[ \Phi_{\text{qe}}(x, y, t) = \alpha V_{\text{qe}}(t)(x^2 - y^2) \]  

with the factor \( \alpha \) depending only on the cell geometry, \( V_{\text{qe}}(t) = V_{\text{max}} \sin(\omega_{\text{qe}}t) \). As in the case with Equation (2.32), Equation (2.33) is valid only in the center of the cell. Substituting

\[ \vec{E} = -\nabla \Phi = \mu V_{\text{trap}} \begin{pmatrix} +2x \\ +2y \\ -4z \end{pmatrix} + \alpha V_{\text{max}} \sin(\omega_{\text{qe}}t) \begin{pmatrix} +2x \\ -2y \\ 0 \end{pmatrix} \]  

in Equation (2.30) and analyzing the resulting set of differential equations, one obtains the resonant frequencies.
\[ \omega_\text{ce} = \omega_\text{e} = \omega_+ + \omega_- \quad \text{cyclotron-magnetron conversion (coupling)} \]
\[ \omega_\text{me} = 2\omega_+ \quad \text{magnetron excitation} \]
\[ \omega_\text{ce} = 2\omega_- \quad \text{cyclotron excitation} \]

As mentioned before, if \( V_\text{qe}(t) \) oscillates at \( \omega_\text{qe} = \omega_\text{e} \), cyclotron and magnetron motions are interconverted periodically.

### 2.1.6 Experimental setup

A Fourier transform ion cyclotron resonance (FT ICR) mass spectrometer with a home-built open cylindrical cell, adapted for fluorescence measurements, is employed in this work. The FT ICR mass spectrometer consists of a home-built vacuum system with a 4.7 Tesla superconducting magnet (Bruker, Fällanden, Switzerland) and commercial control electronics and data acquisition (IonSpec Corp., Lake Forest/CA, USA). A home-built open cylindrical cell was used for trapping the ions. The instrument has an internal MALDI source with a target that was positioned 2 cm from the cell. For laser desorption/ionization, the 3rd harmonic of a Nd:YAG laser (Continuum, Minilite ML-10, USA) at 355 nm with a 5 ns pulse width was used. The base pressure in the ICR cell region is in the range of 10\(^{-9}\) mbar. Figure 2.4 displays a schematic drawing of the instrument and the complete experimental setup. The Nd:YAG laser beam was directed onto the sample target through a crystal quartz view port placed on the right side of the vacuum chamber. For introducing gaseous samples, a leak valve and a pulsed valve were used.
Figure 2.4. Schematic of an FT ICR instrument with an open cylindrical cell.
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2.2 Time-of-Flight Mass Spectrometry
2.2 Time-of-Flight Mass Spectrometry

2.2.1 Basic principles

Time-of-flight mass spectrometry (TOF) was conceived and patented in 1946 by Stephens [14], first built by Cameron and Eggers in 1948 [15], and greatly improved by the addition of a two-stage extraction ion optics in 1955 by Wiley and McLaren [16]. Principles and instrumentation in TOF mass spectrometry have been extensively reviewed over the last years [17-19]. Recent developments include special designs for high resolution [20-23], multiple reflection [24], and miniaturization to a complete TOF mass spectrometer of 8 cm length [25]. With the advent of soft ionization techniques, TOF MS combined with matrix-assisted laser desorption ionization (MALDI, see chapter 2.3) and electrospray (ESI) is now the method of choice for biological analysis [17,26]. The operating principle of the time-of-flight mass spectrometer involves measuring the time for an ion to travel from the pulsed ion source to the detector. The basic idea of TOF MS is easy and illustrated in Figure 2.7.

![Figure 2.7](image)

**Figure 2.7.** A simple TOF mass spectrometer consisting of a source region s, a drift tube D, and a detector.

All ions are accelerated through the same voltage difference. Ions with the same different masses will leave the ion source with the same kinetic energy but correspondingly different velocities. In the drift region no electric field is applied, thus ions can be separated according to their velocities. Lighter and faster ions hit the detector earlier than heavier ions (Figure 2.7). Due to energy conservation, the potential energy of an ion with mass, \( m \) and number of charges, \( z \) is transformed into kinetic energy. It can be written:
\begin{equation}
\frac{1}{2}mv^2 = zeU
\end{equation}

where \( v \) is the ion velocity as it leaves the source region, \( U \) the potential difference between the acceleration electrodes, and \( e \) is the elementary charge. Substituting \( v \) in Equation (2.34) with \( D/t \), where \( D \) is the drift tube length and \( t \) is a time of flights of the ions we obtain the following equation:

\begin{equation}
t = D\sqrt{\frac{m}{2eV}}
\end{equation}

From Equation (2.35) it is straightforward to obtain mass resolution \([17]\) for a time-of-flight mass spectrometer:

\begin{equation}
\frac{m}{\Delta m} = \frac{1}{2} \frac{t}{\Delta t}
\end{equation}

where \( \Delta m \) is the full width at half-maximum (FWHM) of the mass signal, and \( \Delta t \) is FWHM of the time signal.

### 2.2.2 Continuous and delayed extraction

In a time-of-flight mass spectrometer with continuous extraction, the electrode voltages are switched on before the measurements and remain constant during the whole experiment. In continuous mode ions are extracted immediately after being generated. If ions of the same m/z are created at slightly different positions or with different velocities they arrive at the detector at slightly different times, leading to a peak broadening (low mass resolution). The initial kinetic energy distribution can be partially focused by using pulsed or delayed extraction (DE) \([16]\). By doing so, ions are allowed to drift first with their initial kinetic energies in the field-free region, before the extraction field is applied. When DE is actually applied there are the following situations that may occur: 1) ions A and B generated in the same location have different initial kinetic energies. During the field-free period, the ion with higher kinetic energy will drift faster toward the grid, thus it will experience less “push” from
the extraction pulse, which is applied after. If the delay is properly set, both ions will arrive at the same time onto the detector. 2) ions A and C have the same initial kinetic energies, but in the opposite directions. In this case, ion C will drift backward and thus will experience higher extraction field pulse and will catch up with ion A.

One disadvantage of delayed extraction is its inability to focus the ions independently of mass. In other words, only a certain mass range is well resolved. Larger and smaller ions show a resolution that is sometimes worse than with continuous extraction.

2.2.3 Detectors

An ion detector in a TOF mass spectrometer is usually a device that registers the ion impact on its active surface and converts it into an electric signal. It is actually the "eye" of the instrument. There are two major kinds of detectors employed in TOF mass spectrometry: electron multipliers (EM) and microchannel plates (MCP).

In an electron multiplier, the ion strikes an active surface, causing the emission of secondary electrons. If an electric potential is applied from one metal plate to the other, the emitted electrons will be accelerated to the next metal plate and induce emission of more electrons. These electrons can be accelerated to another metal plate and the whole process will be repeated but with more and more electrons. Finally, this avalanche of secondary electrons produces a current pulse at the output.

Figure 2.8. Principle of an electron multiplier. Arriving ions hit the first dynode, producing secondary electrons. These electrons are then accelerated (by the voltage applied across the dynodes) and amplified leading to a strong output electric signal.
A microchannel plate [27] is a specially fabricated plate that amplifies electron signal similar to an electron multiplier. Unlike an EM, a MCP has several million independent channels and each channel works as an independent electron multiplier. MCPs consist of a two-dimensional periodic array of very small diameter glass capillaries (channels) fused together and sliced into a thin plate. When an ion enters a channel it emits an electron from the channel wall. Secondary electrons are accelerated by a voltage applied across both ends of the MCP. This process is repeated many times along the channel; creating an electric signal at the output.

**Figure 2.9.** Electron micrograph of plate geometry. The diameter of one channel is approximately 2 μm. Reproduced after [28].
2.3 Matrix-Assisted Laser Desorption Ionization
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2.3 Matrix-Assisted Laser Desorption Ionization

2.3.1 Principle

Since its introduction in 1988 [29,30], matrix assisted laser desorption/ionization (MALDI) has revolutionized analytical and biological chemistry by making possible the detection of large, intact biological molecules in the gas phase. This technique also motivated improvements in commercial mass spectrometry instrumentation, resulting in significant improvements in resolution and mass accuracy for biological analysis. These accomplishments were recognized with the partial award of the 2002 Nobel Prize in Chemistry. MALDI is nowadays a prime technique for studying peptides, proteins, oligonucleotides and oligosaccharides [29-31], polymers [32-36], and many other biological molecules. MALDI is a soft ionization method, i.e., little or no fragmentation occurs, which makes detection of intact molecules straightforward.

![Figure 2.10. Schematic illustration of the matrix-assisted laser desorption process. Matrix molecules absorb the laser energy, rapidly desorb into the gas phase and liberate the embedded analytes.](image)

The basic idea of MALDI is shown in Figure 2.5. Analyte is co-crystallized with an excess of a solid matrix material, which can absorb laser photons. The mixture is then deposited onto a sample substrate, usually stainless steel. Material evaporates under pulsed laser irradiation, and creates a dense plume, containing neutral and charged particles. The ionization efficiency of MALDI was measured by Mowry and Johnston [37] to be around 1 ion per 10'000 neutral particles for laser irradiances just above threshold (threshold is the minimum laser irradiance...
necessary to “detect” ions). The fundamentals of MALDI ionization process are still a subject of active debate. Chapters (2.3.4) describe some of the main ionization models proposed.

2.3.2 Lasers

Many different laser types as well as many different wavelengths have been used in MALDI. MALDI experiments can be distinguished, depending on the wavelength of the laser, UV or IR. Due to their low cost and ease of operation, nitrogen lasers (λ = 337 nm) are the most widely used in UV MALDI. However, different laser wavelengths, such as frequency-tripled Nd:YAG (λ = 355 nm), frequency-quadrupled Nd:YAG (λ = 266 nm), and various excimer laser lines (XeCl: 308 nm, KrF: 248 nm, ArF: 193 nm) are also employed.

In IR MALDI experiments, Er:YAG (λ = 2.94 µm) and CO₂ (λ = 10.6 µm) lasers are typically used. The Er:YAG laser has been particularly employed in experiments using glycerol and water as matrices. Compared to UV MALDI, less metastable fragmentation and adduct formation, as well as greater tendency to form multiply charged ions has been reported [38-40]. Nevertheless, IR and UV MALDI mass spectra look similar [41] and many UV matrices work well for IR. For desorption/ionization of polypeptides directly from a polyacrylamide gel, a tunable mid-infrared free electron laser (FEL) has been successfully applied [42].

The important characteristic of the laser radiation is its fluence (J/cm²) and not its irradiance (fluence per unit time, in (W/cm²)); The laser pulse width was found to have little or no influence on MALDI mass spectra, at least up to lengths of tens of nanoseconds [43-47].

2.3.3 Matrix

The choice of matrix is crucial for success in MALDI experiments. In general, MALDI matrices are small organic aromatic molecules that meet several requirements. Typically they have sufficient absorption at the laser wavelength; low sublimation rate, to guarantee vacuum stability; solubility in the same solvent as analyte; and they should have suitable
(co)crystallization and (co)desorption properties. Many useful matrices have been reported since the initial work on MALDI started; an overview is given in Table 2.3.

Table 2.3. Structure of some common MALDI matrices

In UV MALDI most matrices are aromatic derivatives such as; DHB, 2,5-dihydroxybenzoic acid [48]; and cinnamic acid (3-phenyl-2-propenoic acid) derivatives including HCCA, α-cyano-hydroxycinnamic acid [49]; FA, ferulic acid (4-hydroxy-3-methoxycinnamic acid); SA, sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid) and pyridine-3-carboxylic acid (nicotinic acid, NA) [30]. DHB is a very common matrix used for mid-sized proteins up to 50 kDa. HCCA has proven to be useful for obtaining high-resolution
positive peptide signals. Sinapinic and ferulic acids are used for high mass protein ionization. Non-acidic compounds such as PNA, 4-nitroaniline and ATT, aza-thiothymine [50,51] are useful for pH neutral matrix solutions. Since the most commercial instruments are operated by nitrogen lasers, strong absorption at 337 nm is preferable for MALDI matrices. A wide range of organic molecules can be used as matrices for IR MALDI. Among them, urea, succinic acid, fumaric acid [52], water/ice, triethanolamine and glycerol are commonly used IR matrices. Some UV matrices, such as DHB, HCCA or sinapinic acid have also been used in IR MALDI [41,53].

Most matrices are solid compounds. Beside solid matrices, liquid matrices and even ionic liquids are also used for sample preparation. Using liquid matrices is advantageous, when coupled to electrophoresis or high performance liquid chromatography (HPLC) [54,55]. The homogeneity of the sample is also better with liquid matrices, while there is no “hot spot” effect, i.e. areas where good MALDI signals are observed whereas other areas yield poor signals [56]. The matrix can refresh its surface continuously, increasing shot-to-shot reproducibility 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 [57-60].

Despite many efforts, there are still no clear guidelines for matrix selection for a particular analytical problem, and better matrices are, therefore, often found only after screening a large number of candidate compounds [50,61].

2.3.4 Sample preparation

The efficiency of matrix assisted laser desorption/ionization mass spectrometry has been shown to be highly sensitive to the sample preparation procedure. Every single choice during sample preparation can affect the outcome of the MALDI measurements. The matrix/solvent system is considered one of the most important sample preparation parameters; however, other aspects such as substrate material, sample purification, and sample crystallization technique are also important.
It seems that every MALDI practitioner has a different opinion of what constitutes the best sample preparation protocol. Many experienced MALDI users refer to sample preparation as an “art”, which is mastered only through practice and experimentation. Two major techniques have been widely accepted for routine applications, generally termed the dried droplet [30] and the thin layer preparation [62,63].

The dried-droplet method is the oldest and has remained the preferred sample preparation method in the MALDI community. In this method, analyte and matrix are dissolved in the desired concentrations, typically between 1/100 to 1/10,000. Proteins and peptides are generally dissolved in a mixture of an organic solvent (methanol, ethanol) mixed with water acidified with 0.1% trifluoroacetic acid (TFA). Acidic conditions were found to enhance the signal intensities, probably due to higher proton affinity. A drop of the mixture is then deposited on the sample substrate and allowed to dry under normal conditions (room temperature).

The thin layer preparation or also referred as fast-evaporation method was introduced by Vorm et. al. in 1993 with the main goal of improving the resolution and mass accuracy of MALDI measurements. It is a simple sample preparation procedure in which matrix and sample handling are completely decoupled. First, the matrix-only solution is applied to the sample stage and then allowed to dry. Then, a drop of sample solution is applied on top of the matrix bed and allowed to dry either by itself or in a flow of nitrogen.

Sophisticated ways of applying crystals onto the sample target by spin-coating and electrospraying [64,65], condensation onto aerosol particles [66], and methods for tolerating high contents of sodium dodecyl sulfate [67] have been reported. A comprehensive review of MALDI sample preparation and purification has been given by Gross and Strupat [68].

2.3.5 Desorption/ionization mechanisms

Despite the widespread analytical applications of MALDI, the fundamental processes of ion generation and desorption are still poorly understood and are the subject of active debates.
Several physical interpretations have been proposed to rationalize desorption/ionization steps. A brief summary of some major models will be discussed below.

The ionization event is currently widely considered as a combined process of primary and secondary ionization [69,70]. During or shortly after the laser pulse, primary ions are generated. Plume expansion starts and ion-molecule reactions (e.g. proton transfer, electrons transfer, cation attachment, and others) convert primary ions to the secondary products that are finally observed at the detector [70,71]. A short summary of primary and secondary ionization mechanisms is given in Table 2.4.

<table>
<thead>
<tr>
<th>Primary ionization</th>
<th>Secondary ionization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multiphoton ionization:</td>
<td>Proton transfer:</td>
</tr>
</tbody>
</table>
| \( m \rightarrow h\nu \rightarrow m^{**} + e^- \) | \([m + H]^+ + A \rightarrow m + [A + H] \)
| \([m - H]^+ + A \rightarrow m + [A - H] \) | \([m + H]^+ + A \rightarrow m + [A + H] \) |
| Energy pooling: | Electron transfer: |
| \(2m \rightarrow h\nu \rightarrow 2m^* \rightarrow m^{**} + m \rightarrow m^{**} + m + e^- \) | \(m^{**} + A \rightarrow m + A^{**} \) |
| Excited-state proton transfer: | Cation attachment: |
| \( m \rightarrow h\nu \rightarrow m^* \) | \(m + Cat^+ \rightarrow [m + Cat]^+ \)
| \( m^* + A \rightarrow [M - H]^+ + [A + H]^+ \) | \(A + Cat^+ \rightarrow [A + Cat]^+ \) |
| \( m^* + m \rightarrow [M - H]^+ + [M + H]^+ \) | |
| Ground-state proton transfer and desorption of pre-formed ions: | m: matrix |
| \( m + A \rightarrow [M - H]^+ + [A + H]^+ \) | A: analyte |
| \( m + m \rightarrow [M - H]^+ + [M + H]^+ \) | |

Table 2.4. Schematic illustration of primary and secondary ionization mechanisms

A different approach has been suggested by Karas and coworkers [72,73]. In their “lucky survivor” model the final ion distribution is considered to be the result of several processes: formation of singly and/or multiply charged clusters (ion precursors), neutralization processes
with electrons and subsequent liberation of singly charged ions in the gas phase. Although the presence of the clusters has been confirmed both experimentally [74-76] and theoretically [77-80] it is still unclear whether this is the main process responsible for ion formation.

Among the extensive body of literature that has been reported on MALDI mechanistic studies, the choice is mentioned here: Knochenmuss and his “energy pooling” model [81-83]; Zhigilei and his molecular dynamics simulations of MALDI process [77-80]; Dreisewerd and his IR MALDI model [84,85]; Tabet and his “cluster” model [86,87];

Recently, Frankevich et. al. found that in MALDI there is a substantial number of free electrons generated by the laser pulse [88,89]. These electrons were found to be responsible for neutralization processes in the plume, leading to the positive ion yield suppression and charge reduction [90]. Ways to suppress or promote electron emission, resulting in a higher/lower positive ion yield were explored [91].

The second part of this thesis (Chapters 6-8) is devoted to the MALDI mechanistic studies that involve electron emission. In Chapter 6 the influence of the addition of CuCl₂ to the matrix/analyte solution on the analyte ion signal is analyzed. Chapter 7 deals with fundamental phenomena such as the number of photons needed to produce electrons, the influence of the substrate material, and the kinetic energy of the electrons on photoelectron emission. In Chapter 8 the ratios of positive to negative ion yields for MALDI TOF mass spectrometry were measured and the dependence of the ion yield on acid/base properties of the molecules was examined.
2.4 Gas-Phase Laser-Induced Fluorescence and Fluorescence Resonance Energy Transfer
2.4 Gas-Phase Laser-Induced Fluorescence and Fluorescence Resonance Energy Transfer

2.4.1 Basic theory of fluorescence

Fluorescence is the emission of one or more photons by a molecule or atom activated by absorption of a quantum of electromagnetic radiation. A photon of frequency $\nu$ colliding with an atom will either be absorbed or scattered. The probability of absorption is greatest when the energy of this photon, $h\nu$ (where $h$ is Planck's constant), matches one of the atom's excitation energies. If the photon is absorbed, its energy is transferred to one of the atom's electrons. The atom is now excited and therefore unstable. If the atom is relatively isolated, as in a low-pressure gas, this additional energy is dissipated by the emission of another photon of the same or different frequency in a random direction. In denser gases, liquids and solids, however, the energy is dissipated by intermolecular collisions, resulting in the loss of energy, and thus a longer emission of wavelength.

The photophysical processes that occur from absorption to emission are often shown in a so-called Jablonski diagram. Of course all possible energy routes cannot be described in a single Figure, and different forms of the diagram can be found in different contexts.

![Jablonski diagram](image)

Figure 2.11. Perrin- Jablonski diagram and illustration of the relative positions of absorption, fluorescence and phosphorescence spectra.

In the diagram the electronic singlet states $S_0$, $S_1$, $S_2$, and the triplet $T_1$ along with three vibrational energy levels are shown. In the ground state, the molecule will be in its lowest
vibrational level of $S_0$. At room temperature the higher vibrational energy levels are in general not populated (less than 1% according to Boltzmann statistics). The magnitude of the absorbed energy determines which vibrational level of $S_1$ (or $S_2$) becomes populated. On picosecond timescales vibrational relaxation to lower levels of $S_1$ (in solution) or intramolecular vibrational redistribution (in the gas phase) can happen. On longer timescales one may also have internal conversion (IC).

Internal conversion is a non-radiative transition between two electronic states of the same spin multiplicity. In solution, this process is followed by a vibrational relaxation towards the lowest vibrational level of the final electronic state. The excess vibrational energy can be indeed transferred to the solvent during collisions of the excited molecule with the surrounding solvent molecules. When a molecule is excited to an energy level higher than the lowest vibrational level of the first electronic state, vibrational relaxation (and internal conversion if the singlet excited state is higher than $S_1$) leads the excited molecule towards the 0 vibrational level of the $S_1$ singlet state within a time-scale of $10^{-13}-10^{-11}$ s. From $S_1$, internal conversion to $S_0$ is possible but is less efficient than conversion from $S_2$ to $S_1$, because of the much larger energy gap between $S_1$ and $S_0$. Therefore, internal conversion from $S_1$ to $S_0$ can compete with emission of photons (fluorescence) and intersystem crossing to the triplet state from which emission of photons (phosphorescence) can possibly be observed.

Since emission typically occurs after $10^{-9}$ s, the molecule is fully relaxed at the time of emission, hence, as a rule, emission occurs from the lowest vibrational level of $S_1$ (Kasha's rule) and the fluorescence spectrum is generally independent of the excitation wavelength. After emission the molecule returns to the ground state, possibly after vibrational relaxation. This completes the simplest case of fluorescence: excitation, internal conversion, emission and relaxation. The energy lost to the surroundings, due to vibrational relaxation and internal conversion is the reason why a Stokes shift is observed. The Stokes shift is the gap between the maximum of the first absorption band and the maximum of the fluorescence spectrum. Another possible de-excitation pathway from $S_1$ or $S_2$ is intersystem crossing toward the $T_1$ triplet state, which may subsequently relax via phosphorescence or by a secondary non-radiative relaxation step.
Intersystem crossing (ISC) is a non-radiative transition between two isoenergetic vibrational levels belonging to electronic states of different multiplicity. For example, an excited molecule in the 0 vibrational level of the $S_1$ state can move to the isoenergetic vibrational level of the $T_n$ triplet state; then vibrational relaxation brings it into the lowest vibrational level of $T_1$. Intersystem crossing may be fast enough ($10^{-7}$–$10^{-9}$ s) to compete with other pathways of de-excitation from $S_1$ (fluorescence and internal conversion $S_1 \rightarrow S_0$).

Crossing between states of different multiplicity is in principle forbidden, but spin–orbit coupling (i.e. coupling between the orbital magnetic moment and the spin magnetic moment) can be large enough to make it possible. The probability of intersystem crossing depends on the singlet and triplet states involved. If the transition $S_1 \rightarrow S_0$ is of $n \rightarrow \pi^*$ type for instance, intersystem crossing is often efficient. It should also be noted that the presence of heavy atoms (i.e. whose atomic number is large, for example Br, Pb) increases spin–orbit coupling and thus favors intersystem crossing.

The efficiency of the fluorescence process is defined by the quantum yield and the fluorescence lifetime. The quantum yield is defined as the ratio of the number of photons emitted to the number of photons absorbed

$$\Phi = \frac{\# \text{photons emitted}}{\# \text{photons absorbed}} = \frac{k_r}{k_r + k_{nr}} \quad (2.37)$$

where $k_r$ is the rate constant for radiative deactivation $S_1 \rightarrow S_0$ with emission of fluorescence and $k_{nr}$ is the overall non-radiative (such as ISC, IC, etc.) rate constant.

The fluorescent lifetime of the excited state ($\tau$) is the average time a molecule stays in the excited state before returning to ground state. It can be expressed as the inverse of the total depopulation rate:

$$\tau = \frac{1}{k_r + k_{nr}} \quad (2.38)$$

Typically fluorescence lifetimes are in the 5-15 ns range. The fluorescence lifetime is an important parameter for practical applications of fluorescence such as fluorescence resonance
energy transfer, which will be discussed later. For more details about fluorescence, its physical properties and applications, see [92-95]

2.4.2 Fluorescence resonance energy transfer

Fluorescence resonance energy transfer (or Förster resonance energy transfer) describes an energy transfer mechanism between two fluorescent molecules [96-98]. A fluorescent donor is excited at its specific fluorescence excitation wavelength. By a long-range dipole-dipole coupling mechanism, this excited state is then nonradiatively transferred to a second molecule, the acceptor. The donor returns to the electronic ground state. This energy transfer mechanism is termed "Förster resonance energy transfer" (FRET), named after the German scientist Theodor Förster. When both molecules are fluorescent, the term "fluorescence resonance energy transfer" is often used, although the energy is not actually transferred by fluorescence.

![Figure 2.12. Schematic illustration of intramolecular fluorescence resonance energy transfer.](image)

The rate constant for transfer between donor and an acceptor at a distance $r$ is:

$$k_r = \frac{1}{r^6} \left[ \frac{R_0}{r} \right]^6$$

(2.39)
where $\tau^0_d$ is the excited-state lifetime of the donor in the absence of transfer and $R_0$ is the Förster critical radius (distance at which transfer and spontaneous decay of the excited donor are equally probable, i.e. $k_T = 1/\tau^0_d$). $R_0$ (in Å) is given by

$$R_0 = 0.2108 \left[ \kappa^2 \Phi^0_d n^{-4} \int_0^\infty I_d(\lambda) \varepsilon_A(\lambda) \lambda^4 d\lambda \right]^{\frac{1}{6}} \quad (2.40)$$

where $\kappa^2$ is the orientation factor, which can take values from 0 (perpendicular transition moments) to 4 (collinear transition moments). For more details on $\kappa^2$ see [96]. $\Phi^0_d$ is the fluorescence quantum yield of the donor in the absence of transfer, $n$ is the average refractive index of the medium in the wavelength range where spectral overlap is significant, $I_d(\lambda)$ is the fluorescence spectrum of the donor normalized so that $\int_0^\infty I_d(\lambda) d\lambda = 1$, $\varepsilon_A(\lambda)$ is the molar absorption coefficient of the acceptor (in dm$^3$ mol$^{-1}$ cm$^{-1}$) and $\lambda$ is the wavelength in nanometers.

The transfer efficiency is given by

$$\Phi_T = \frac{k_T}{1/\tau^0_d + k_T} = \frac{1}{1 + (r/R_0)^6} \quad (2.41)$$

The sixth power dependence explains why resonance energy transfer is most sensitive to the donor-acceptor distance when the distance is comparable to the Förster critical radius (see Figure 2.13).
It should be emphasized that the transfer rate depends not only on distance separation but also on the mutual orientation of the fluorophores. This dependence is described by orientation factor, $\kappa^2$, which is given by

$$
\kappa^2 = \left(\cos \alpha - 3 \cos \beta \cos \gamma\right)^2
$$

where $\alpha$ is the angle between the donor and acceptor transition moments, $\beta$ is the angle between the donor moment and the line joining the centers of the donor and acceptor, and $\gamma$ is the angle between the acceptor moment and the line joining the centers of the donor and acceptor. When the fluorophores are free to rotate at a rate that is much faster than the deexcitation rate of the donor (isotropic dynamic averaging), the average value of $\kappa^2$ is $2/3$.

### 2.4.3 Prerequisites for FRET

Several criteria must be satisfied for successful FRET measurements in solution:

- The distance between the donor and acceptor molecules does not change during the lifetime of the excited donor.
- The donor and acceptor molecules must be positioned within a range of 10-100 Å of each other.
• The absorption spectrum of the acceptor must overlap the fluorescence emission spectrum of the donor.
• The quantum yield of the donor must be high enough
• Donor and acceptor dipole moments must be in the proper orientation

Let us now consider possible problems for FRET observation when going from solution to the gas phase. Evaluation of Equation (2.40) requires a knowledge of the gas-phase emission and absorption spectra of the donor and acceptor, respectively. Both are very sensitive to the environment, thus the solution measurements can therefore not be taken. Shifts in absorption and emission bands can be induced by a change in solvent; these shifts are called solvatochromic shifts (for more details see [95]). In other words, when a solute is surrounded by solvent molecules, its ground state and its excited state are more or less stabilized by solute-solvent interactions, depending on the chemical nature of both solute and solvent molecules. Furthermore, it is clear that efficient vibrational relaxation ($10^{12}$ s) during the excited state lifetime will be absent in a low pressure environment, because the collision rate is too slow (for our case at the pressure of $10^4$ Torr the rate is only about $\sim 10^{-4} - 10^{-5}$ s). Absence of the vibrational relaxation will lead to a blue shift of the emission spectrum. Therefore, a gas-phase fluorescence spectrum of the donor must be taken first before actually conducting FRET experiments.

In the absence of collisions, it is logical to assume that the fluorescence lifetime of the excited state may be longer than that in solution. Thus, the quantum yield can vary from its solution value. Coupling a FRET pair to a macromolecule can also introduce new uncertainties, especially with regard to orientation factor.

### 2.4.4 Steady state FRET (experimental approach)

Rearrangement of the Equation (2.41) yields the donor-acceptor distance:

\[
\ell = \left( \frac{1}{\Phi_r - 1} \right)^{1/6} R_0
\]

(2.43)
Introduction

where transfer efficiency, $\Phi_T$, can be determined experimentally and $R_0$ can be calculated from Equation (2.40). The transfer efficiency is given by:

$$\Phi_T = 1 - \frac{\Phi_D}{\Phi_D^0}$$

(2.44)

where $\Phi_D$ and $\Phi_D^0$ are the donor quantum yields in the absence and presence of acceptor, respectively.

Because only the relative quantum yields are to be determined, a single observation wavelength is sufficient and the latter is selected so that there is no emission from the acceptor. Then, Equation (2.44) can be rewritten in terms of absorbances at the excitation wavelength $\lambda_n$ and fluorescence intensities of the donor in the absence and presence of acceptor:

$$\Phi_T = 1 - \frac{A(\lambda_n)}{A_D(\lambda_n)} \frac{I_D(\lambda_n, \lambda_{nm}^D)}{I_D^0(\lambda_n, \lambda_{nm}^D)}$$

(2.45)

The factor $A_D/A$ arises from the contribution of the acceptor moiety to the overall absorption at the excitation wavelength $\lambda_n$.

2.4.5 Time-resolved FRET (experimental approach)

If the fluorescence decay of the donor following pulse excitation is a single exponential, the measurement of the decay time in the presence ($\tau_D^T$) and absence ($\tau_D^0$) of transfer is a straightforward method of determining the transfer rate constant, the transfer efficiency and the donor–acceptor distance, by using the following relations:

$$\frac{1}{\tau_D^T} = \frac{1}{\tau_D^0} + k_T$$

(2.46)

$$\Phi_T = 1 - \frac{\tau_D^0}{\tau_D^T}$$

(2.47)

$$r = \frac{R_0}{\left(\frac{\tau_D^0}{\tau_D} - 1\right)^6}$$

(2.48)
The advantage of this method is its ability to check whether the donor fluorescence decay in the absence and presence of acceptor is a single exponential or not. If this decay is not a single exponential in the absence of acceptor, this is likely to be due to some heterogeneity of the microenvironment of the donor. It can then be empirically modeled as a sum of exponentials:

\[ i_0(t) = \sum \alpha_i \exp(-t/\tau_i) \]

Transfer efficiency can be calculated by using the average decay times of the donor in the absence and presence of acceptor as follows:

\[ \Phi_T = 1 - \frac{\langle \tau_D \rangle}{\langle \tau_P \rangle} \]

where the amplitude-averaged decay times are defined as: \( \langle \tau \rangle = \frac{\sum \alpha_i \tau_i}{\sum \alpha_i} \). This approach is valid if the donor fluorescence decay is not too far from a single exponential, so that an average of the donor–acceptor distance can be estimated.
2.5 References


2.5 References


In this chapter, a novel scheme for laser-induced fluorescence measurements of ions trapped inside a Fourier-transform ion cyclotron resonance (FT ICR) mass spectrometer will be introduced. It is based on an open FT ICR cell design, continuous wave axial excitation of the fluorescence, orthogonal photon collection by fiber optics, and single photon counting detection. Rhodamine 6G ions generated by an internal MALDI source were used to develop and test the set-up. Due to photobleaching processes, the excitation laser power and the observation time window have to be carefully optimized. An ion tomography method was used to align the excitation laser.

Adapted from:
3.1 Introduction

Studies of biomolecular ions in the gas phase can provide new information about their structure and interactions. This field has attracted great attention since the advent of electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) [1,2], two soft ionization techniques that can generate intact gas-phase ions of fragile high molecular weight molecules [3-5]. Methods that complement mass spectrometry, such as hydrogen-deuterium exchange [6], covalent or noncovalent tagging of biomolecules [7,8], collision-induced dissociation (CID) [9], blackbody infrared radiative dissociation (BIRD) [10], and ion mobility [11] have been applied to obtain conformational information. All of these use mass spectrometry as a readout and hence exhibit good sensitivity. However, only indirect structural information can generally be obtained.

Fourier-transform ion cyclotron resonance (FT-ICR) is a very powerful mass spectrometric technique, celebrated for its extremely high mass accuracy and mass resolving power. FT-ICR uses a Penning trap which provides both temporal and spatial confinement of ions, so it is well suited for studies combining optical and mass spectroscopic techniques. For combined LIF-MS investigations, several advantages of FT-ICR over the Paul trap exist: (i) easier manipulation of the trapped ion population, including ion ejection and isolation; (ii) better confinement of the ion cloud; (iii) superior capabilities for MS and MSn experiments; (iii) better control over the ion kinetic energy; and (iv) lower operating pressures, allowing for well controlled ion-molecule reactions in the FT-ICR cell. The challenge for LIF detection inside the FT-ICR trap is the delivery of photons and optical detection in the confined space of the Penning trap, which is generally located inside the bore of a superconducting magnet.

Nevertheless, a few initial steps have been taken towards realizing an LIF-FT-ICR experiment. Marshall’s group has performed fluorescence spectroscopy of atomic and organic ions trapped and mass-selected in an FT-ICR, using a pulsed laser as an excitation source [12-15]. This set-up comprised a number of optical elements (mirrors, prisms) inside the vacuum chamber, which complicates alignment procedures. Scott et al. developed a detection scheme for obtaining fluorescence lifetimes of ions trapped in an FT-ICR, using a dual-cell set-up [16]. Their design comprises a conventional cubic FT-ICR cell for ion storage/manipulation and an second cell for optical detection using an off-axis parabolic mirror. The optical
detection efficiency of this set-up is presumably rather high, but compromises had to be made in terms of FT-ICR performance and capabilities for ion manipulation. Also, the transfer of ions between the two cells was relatively inefficient. These results showed that combining fluorescence spectroscopy with FT-ICR is feasible, although an efficient way of performing such investigations is still challenging.

In this paper we present a new, simplified FT-ICR cell designed for LIF detection that circumvents many of the problems described above. We discuss the figures of merit and show optimization procedures for maximizing the LIF signal. This set-up will be used to study the conformation of biomolecular ions and their intermolecular interaction in the gas phase.

3.2 Experimental

The FT ICR mass spectrometer consists of a home-built vacuum system with a 4.7 Tesla superconducting magnet (Bruker, Fällanden, Switzerland) and commercial control electronics and data acquisition (IonSpec Corp., Lake Forest/CA, USA).

As the ion trap is enclosed by a superconducting magnet, the main difficulty for building a fluorescence detection system is space limitation. In our initial set-up, the detection optics were arranged axially, collinear with the excitation laser beam path, which made it difficult to remove the scattered laser light from the window and the sample target. In the arrangement presented here, the detection optical configuration is perpendicular to the excitation configuration, which decreases spurious signals from scattered light considerably. Further improvements are discussed below. The optical system is shown schematically in figure 1.
An open cylindrical cell with a 3 cm internal diameter was built for this fluorescence experiment. An aspect ratio of 1 is achieved by assembling three cylinders of 3 cm length each. The central cylinder is divided into four segments for excitation and detection of the ions. In the center of one of the excitation electrodes, there is a hole that can accommodate a fiber optics collimator for photon collection. The hole is covered with a small piece of wire mesh (6 mm diam.) to minimize electrical field distortions. The inner walls of the FT ICR cell are coated with vacuum-compatible, electrically conductive paint (Aquadag E, Acheson Co., Port Huron/Ml, USA), which dramatically reduced the scattered excitation laser light. Due to the use of an internal MALDI source, the scattered excitation laser light from the sample target constitutes another major portion of the background. A special sample target was designed to alleviate this problem: this sample target functions simultaneously as a MALDI target and as a beam stopper for the excitation laser beam (figure 2).
This improvement also dramatically attenuated the intensity of the scattered laser light reaching the collimator and the fiber optics.

Rhodamine 6G (R6G; Acros Organics, New Jersey, USA) was chosen as test sample. R6G is an ionic dye and has a high fluorescence quantum efficiency in solution (>0.9); its fluorescence emission spectrum is well known. MALDI samples were prepared using the standard dried droplet method using 2,5-dihydroxybenzoic acid (DHB) as the matrix (Fluka AG, Buchs, Switzerland). Rhodamine 6G cations were generated using pulses from a Nd:YAG laser (Continuum, Minilite ML-10) operated at 355 nm. Quadrupolar axialization was employed for reducing the dimensions of the ion cloud, and N2 gas was pulsed into vacuum chamber to cool the ions. In some experiment the ions of interest were isolated using SWIFT waveform.

The trapped ions were excited by an Ar ion laser (Innova 300, the Coherent) with a wavelength of 488 nm; a filter was used to remove plasma lines from the laser tube. The fluorescence emitted from the excited ions in the cell passed through the mesh window, and were then collected by a collimator. A vacuum-compatible optical fiber was used to guide the light from the collimator to the end of optical vacuum feedthrough (Caburn Vacuum Science, Ltd., Glyndc/East Sussex, U.K.). Outside of the vacuum chamber, a 3 m optical fiber was used to reach a second collimator. A notch filter (Holographic SuperNotch-Plus with an optical density of >6 at 488 nm; Kaiser Optical Systems, Ann Arbor/MI, USA) was used to block a very large fraction of the scattered excitation laser light. After filtering by the notch filter, the fluorescence photons entered another collimator, and were guided by an optical fiber to the detection system, a single photon counting module (SPCM-ACQ-16) with maximum dark count of 25 counts/s. The converted signal was recorded by 800 MHz gated
photon counter / multiscaler (PMS 400, Becker & Hickl GmbH, Berlin, Germany), using a
discriminator threshold voltage of -0.02 V and a bin width of 0.1 s.

The sequence of events in a typical experiment (figure 3), controlled by the FT ICR
electronics, started with a Nd:YAG laser pulse for ion generation, gated trapping of the ions,
cooling/thermalization of the ion kinetic energy by admission of a ≈ 100 μs pulse of nitrogen
gas, and quadrupolar axialization. Axialization and pumpdown take a minimum of ≈ 3 s. The
Ar ion laser is normally on the entire time, but collection of photons is timed to commence
either before, during, or after axialization, by gating the photon counter. The experimental
event sequence, which last anywhere is concluded by excitation of the ions in order to record
a mass spectrum. Ion excitation eliminates the spatial overlap of the ion cloud and the Ar ion
laser beam, i.e., it represents the end of the irradiation time, which in this way can be adjusted
between 3 s and tens of seconds.

Figure 3. Experimental event sequence.
3.3 Results and Discussion

Ion tomography / laser alignment

Because the ions are produced off-axis in these experiments, there is a large magnetron motion initially, and axialization of the ions, which creates a tight package in the center of the FT ICR cell, is absolutely necessary for efficient fluorescence collection. It was found to be difficult to detect any fluorescence without axialization. It follows that it is also necessary to align the excitation laser in order to obtain maximum overlap with the ion cloud while keeping the background at a minimum. This was done by a one-dimensional tomography experiment.

The mass spectrum of Rhodamine 6G was measured while the laser beam (laser power = 10 mW) was translated horizontally. In figure 4, it can be seen that in the absence of any axialization, the intensity of ions varied randomly with the laser beam position.

![Figure 4. Detected intensity of Rhodamine 6G ions as a function of excitation laser position with dipolar excitation (filled circles) and without dipolar excitation (filled triangles). Laser energy was 10 mW, and the irradiation time was 3s per point.](image-url)
We interpret this to be due to the magnetron motion of the ions, resulting in a random overlap between laser beam and the ion cloud. With dipolar axialization present, no rhodamine 6G ions can be detected for a laser beam position between -0.5 mm to +0.5 mm with respect to the axis of the cell. This is due to complete photodissociation of the R6G ions when there is complete overlap of laser beam and ion cloud. When laser beam was shifted from 0.5 mm to 1.2 mm or from -0.5 mm to -1.2 mm, the mass spectral intensity of the ions increases gradually, because the area of overlap decreases; at a distance of more than 1.2 mm from the cell axis, no more ions were exposed to the laser beam, such that the intensity of the R6G ions is maximized because they are not depleted by the laser photons any more. From this experiment, it can also be derived that the diameter of the ion cloud is about 0.7 mm, and the laser beam diameter in the FT ICR cell is about 1.7 mm; the latter number agrees well with the known beam diameter.

**Photodissociation of the ions**

Unlike molecules in solution, gas-phase species cannot be efficiently cooled by collisions with a dense bath that dissipate excess internal energy. Thus, photodissociation becomes a more serious problem in the gas phase, especially when a continuous laser is used as the excitation source, providing rapid heating rate. The mass spectrum of photofragments at different excitation laser powers is shown in figure 5.

They both show the cationic moiety of R6G at 443.23 Da as the base peak, plus a fragment at 415 Da, most likely caused by the loss of ethyl from the protonated tertiary amine, accompanied by migration of an H atom to the nitrogen. The spectra are plotted on the same vertical scale, and the most striking difference is a strong reduction in overall ion current due to photobleaching by the laser beam at an irradiation power of 8 mW. This is accompanied by a larger relative intensity of the [M-28]^+ fragment at higher irradiation power, which is also a signature of photodissociation of the R6G parent ion.
Figure 5. Mass spectra obtained at different excitation laser powers, (a) 3 mW, and (b) 8 mW. Both spectra are plotted on the same vertical scale. RF interferences are marked with asterisks. The insert shows the structure of the Rhodamine 6G.

The dependence of the photodissociation on excitation laser power for an irradiation time of 3 s before ion detection is shown in figure 6.
Figure 6. Intensity of Rhodamine 6G ions as a function of excitation laser power (3s irradiation time).

Up to an excitation laser power of 3 mW, the intensity of the ions detected by mass spectrometry decreases only slowly with increasing laser power. Above 3 mW, the extent of dissociation increases rapidly with increasing excitation laser power; up to 8 mW, where only a small intensity of the Rhodamine 6G parent ions remains.

Optimization of the trapping potential

The trapping potential can affect the number of trapped ions and the motion of ions in the cell, so it is an important parameter that should be optimized in this experiment. As shown in figure 7, a small trapping potential led to a lower fluorescence signal.
At a low trapping voltage, the potential well is shallower, allowing the trapped ions to spread over a larger axial range in the center of the cell, which reduces the collection efficiency of the collimator, thus leading to a small fluorescence signal. When the trapping potential applied is too high, the ions are confined to a smaller trapping oscillation amplitude in the deep potential well. In this case, space charge effects will lead to a radial expansion of the ion cloud, which reduces the overlap with the excitation laser beam and thus also decreases the fluorescence intensity. In addition, there may be some contributions from an increase of magnetron motion due to a deterioration in the ion axialization. The settings for axialization were kept constant throughout this experiment; these may not be ideal for the higher range of trapping voltages employed. When the trapping potential was 15 V, the collected fluorescence intensity was maximized. It should be noted that 15 V is the voltage applied to the trapping cylinders. The effective potential at the center of the trap is much lower, about by a factor of 3. Trapping voltages between 10 and 15 V were applied to all other fluorescence experiments.
Optimization of the excitation laser power for fluorescence signal detection

The dependence of the fluorescence intensity on the excitation laser power is shown in figure 7.

![Graph showing fluorescence signal intensity as a function of excitation laser power.](image)

**Figure 8.** Fluorescence signal intensity as a function of excitation laser power (3s irradiation time).

When the laser power is small, the extent of photodissociation is minor. As expected, the fluorescence intensity increases with the increasing excitation laser power. The maximum fluorescence signal intensity was obtained when the excitation laser power was 8 mW. When the excitation laser power was larger than 8 mW, the fluorescence signal intensity decreased again due to photodissociation. At first sight, the data in figures 6 and 8 appear to be incompatible: above 8 mW, most of the Rhodamine 6G ions should have photodissociated after an irradiation time of a 3s (Figure 6), while the maximum fluorescence intensity in figure 8 appears well above 8 mW. However, figure 8 reports total numbers of fluorescence photons counted above background level for the duration of the experiment. The higher the excitation laser power, the faster the fluorescence decay, but the higher the fluorescence yield, i.e., the fluorescence signal, which was produced during the first 3s by the ions was the highest at 8 mW. The first fragments of R6G that are created by laser irradiation of the ion could are expected to be fluorescent species. This agrees with findings by the Blades group,
although the excitation laser power used here was much lower than the one used in ref probably due to the completely different set-up of the trap.

**Fluorescence signal decay**

The fluorescence signal decay was measured at two different laser power levels, 8 mW and 12 mW. In figure 9, each curve comprises 200 points, each representing a 0.1 s counting interval. When the excitation laser power was 8 mW, the characteristic fluorescence signal decay time of Rhodamine 6G ions trapped in the cell is 4.3 s. When the power was 12 mW, the decay time of the trapped parent ions is reduced to 1.85 s. With increasing excitation laser power, the background is also increased, an effect that is clearly visible in the figure.

![Fluorescence signal decay](image)

**Figure 9.** Fluorescence signal decay of trapped Rhodamine 6G ions at different excitation laser power 8 mW (solid diamonds) and 12mW (open circles). 200 bins of 0.1 s width each were recorded.

**Detection efficiency**

We can estimate the overall detection efficiency based on the geometry of our set-up. The diameter of the collimator mounted on the FT ICR cell is $d = 5$ mm, and its area $s = 19.6$ mm$^2$. 
The "emission surface" of an infinitesimally small ion package at the location of the detector (= surface of a sphere with a $r = 15$ mm, equal to the radius of cell) is $706.5 \text{ mm}^2$. In other words, the collimator collects only about 2.8 % of the emitted photons. This collection efficiency can be increased by using multiple collimators mounted around the FT ICR cell, but such a concepts has not been implemented yet. Than there are additional losses on the way to detector due to fiber optics, fiber connections, and filters along the way. We estimate the overall collection efficiency to lie between 1 and 2 %.

Figure 10 shows the fluorescence signal (total fluorescence photon counts) vs. the Rhodamine 6G ion signal intensity. We can assume the latter to be proportional to the number of stored ions. With a small number of ions in the trap (points from 0 to 10 mV on the plot), it is very difficult to get a well defined fluorescence signal because of the background level. However, above an ion signal of 10 mV, the fluorescence intensity depends linearly on the number of ions. Thus, even with many ions in the trap, we still excite all of them, i.e. the space charge limit is not reached yet. This may not be expected, because the diameter of the laser beam is only ca. 2 times larger than that of a well axialized ion cloud necessary to produce a coherent time domain signal.

![Figure 10. Rhodamine 6G signal intensity vs. mass spectral signal intensity](image-url)
3.4 Conclusions

We have developed a laser induced fluorescence detection device coupled with FT ICR mass spectrometry equipped with an internal MALDI ionization source. Rhodamine 6G was used to characterize this device. An ion tomography experiment was used to align the excitation laser beam. In the case of rhodamine 6G cations, a rather low excitation laser power, less than 10 mW, has to be employed in order to avoid photodissociation and to achieve a good fluorescence signal level. MALDI ionization can be used to create intact biomolecular ions, i.e., it should soon become possible to investigate biomolecular ions by laser induced fluorescence in our FT ICR mass spectrometer.
3.5 References


3.5 References


Fluorescence resonance energy transfer (FRET) is a distance sensitive method that correlates changes in fluorescence intensity with conformational changes, for example of biomolecules in the cellular environment. Applied to the gas phase in combination with Fourier-transform ion cyclotron resonance mass spectrometry (FT ICR MS) it opens up possibilities to define structural/conformational properties of molecular ions, in the absence of solvent, and without the need for purification of the sample. For successfully observing FRET in the gas phase it is important to find suitable fluorophores. In this study several fluorescent dyes were examined, and the correlation between solution-phase and gas-phase fluorescence data were studied. For the first time FRET in the gas phase is demonstrated unambiguously.

4.1 Introduction

Fluorescence resonance energy transfer (FRET) [1-3] is a distance sensitive method that correlates changes in fluorescence intensity with changes in distance and orientation of specific spectroscopically active (donor, acceptor, quencher) moieties. The distance between fluorophores is defined as the distance between the centers of the donor and acceptor chromophores [2]; their relative orientation has been found to play a fairly minor role. It is well established that the FRET technique can be used as a "molecular ruler" in the 10 - 60 Å range for labeled biomolecules in solution [4]. FRET measurements can thus provide fairly direct, quantitative information of molecular conformation. Pioneering work by Parks et al. has demonstrated how the dissociation of oligonucleotide duplexes labeled by FRET pairs can be followed for gas-phase ions trapped in a Paul trap [5,6]. The oligonucleotides were labeled with fluorophores, BODIPY analogs of tetramethylrhodamine (TMR, a donor) and texas red (TR, a quencher). At room temperature the fluorescence from the donor was decreased by the proximity of TR. By increasing the temperature the authors were able to observe the dynamics of dissociation of the duplex by an increase of the donor fluorescence. However, only a 10% increase of the donor fluorescence was observed, which may also be interpreted as being a temperature induced effect, i.e. not due to dissociation. Furthermore, the acceptor fluorescence, a common diagnostic to identify a FRET process, was not detected in refs. [5,6].

It is thus desirable to unambiguously demonstrate the occurrence of FRET in the gas phase. This was the goal of the present study. Also, since the behavior of fluorophores in the gas phase is largely unknown it is important to find a suitable FRET pair before actually attempting FRET. This paper presents our findings for different dye molecules trapped in ionized form in an FT-ICR mass spectrometer / Penning trap. We found that some dyes that fluoresce in solution give very little fluorescence in the gas phase or none at all. Based on that, we examined several dyes trying to understand why this discrimination in fluorescence occurs. Also, considerable shifts in absorption and emission wavelengths when going from solution to gas phase were found. A suitable FRET pair was identified, rhodamine 6G covalently bound with sulforhodamine B through a rigid linker. The occurrence of FRET in the gas phase is unambiguously demonstrated for this system.
4.2 Experimental

Rhodamine 6G, sulforhodamine B, sulforhodamine B sulfonyl chloride (= lissamine rhodamine B), and sulforhodamine 101 were purchased from Acros Organics (New Jersey, USA). The structures of the dyes and their derivatives tested are shown in figure 1.

![Chemical structures of dyes and their derivatives](image)

Figure 1. Chemical structures of dyes and their derivatives. 1,4,6,7, rhodamine 6G and its derivatives (absorption / emission maxima for 1 are at 530 / 556 nm in ethanol). 2,5, sulforhodamine B and its derivative (556 / 575 in ethanol); 3, sulforhodamine 101 (578 / 597 in ethanol), 8,9, rhodamine 6G covalently bound with sulforhodamine B, 10, bodipy (529 / 542 nm in CHCl3).

Amide derivatives 4, 6, and 7 were prepared from rhodamine 6G and the corresponding amines as described before [7]. Compound 5 was prepared by mixing sulforhodamine B sulfonyl chloride and n-butylamine in DMF, stirring the reaction mixture overnight, evaporation and flash chromatography using CHCl3/MeOH as eluent. Separation of the o- and p-isomers was possible; the predominant p-derivative was used for the gas-phase studies. Dye pairs 8 and 9 were prepared from sulforhodamine B sulfonyl chloride and the corresponding amines 6 and 7 in a similar fashion. All compounds were characterized by NMR, high-resolution MALDI-TOF, UV/VIS, and fluorescence spectroscopy. Solution
measurements were performed on a Varian Cary 500 Scan UV/VIS/NIR spectrophotometer (Palo Alto, CA) and a Jobin-Yvon Fluorolog-3 spectrofluorimeter (Munich, Germany) in absolute methanol. Absorption/emission spectra for rhodamine dyes in solution are available from various sources (e.g., Lambdachrome, Goettingen, Germany) [8] and Molecular Probes, Eugene, OR [9].

The full description of the specially adapted FT-ICR instrument is given in the previous chapter. The sequence of events in a typical experiment starts with a laser pulse (third harmonic of a Nd:YAG laser, $\lambda = 355$ nm) for ion generation by an internal MALDI source, gated trapping of the ions, buffer gas introduction via a leak valve for 10 s, and simultaneous quadrupolar axialization [10,11] of the ions for 10-20 s. The laser for exciting the fluorophores is on during the whole sequence, normally for 20 s. The sequence is concluded by chirp excitation and detection of the ion signal giving the mass spectrum. In all experiments, He was used as a buffer gas. The fluorescence is collected over the whole experiment in the following manner: the trapped ions are excited by an Ar ion laser (Innova 300, Coherent, Santa Clara, CA) with either 488 nm or 514 nm. The typical Ar-laser power used in these experiments was 120 mW. The emitted fluorescence passes through a wire mesh-covered cutout in one of the cell plates and is then collected by a collimator. The collimator focuses the light onto an optical fiber that is connected to an optical vacuum feedthrough (Caburn Vacuum Science, Ltd., Glynde/ East Sussex, UK). Outside of the vacuum, an additional optical fiber passes the light to another collimator, to a long pass filter (FEL 500; Thorlabs, Karlsfeld, Germany), and finally to the detector (SPCM- ACQ-16; Perkin-Elmer). The signal is recorded by an 800-MHz gated single photon counter/multiscaler (PMS 400; Becker & Hickl GmbH, Berlin, Germany).
4.3 Results and Discussion

Optimization of the Pressure in the ICR Cell

Figure 2 shows the dependence of the fluorescence signal intensity of rhodamine 6G ions on the background pressure in the ICR cell. The curve represents the average of five different experiments.

The same shape of the curve was obtained for other dyes studied. An increase of the fluorescence by two orders of magnitude was found as the background pressure increases. There may be several reasons for this observation. Most importantly, quadrupolar axialization works more efficiently at higher pressure, thus squeezing an ion cloud to a tighter packet, resulting in a better spatial overlap with the laser beam. Shrinking the size of the ion cloud is possible up to a limit, when Coulomb repulsion will become important. The “saturation behavior” of the curve in figure 2 is probably simply because of better and better spatial overlap of the laser beam with the ion cloud. Once the diameter of the ion cloud becomes smaller than the diameter of the excitation laser beam, the curve should become essentially flat. A second reason for the increase in fluorescence signal with pressure may be related to
the fact that more collisions lead to a higher rate of energy transfer to the surrounding molecules, thus preventing photobleaching/photodissociation. In fact, fragmentation was observed at lower operating pressures [12], and at the elevated pressure used here, it was largely absent. Third, it is also possible that if a molecule in the singlet state underwent conversion into a nonfluorescent ("dark") triplet state, collisions can bring the molecule back to the ground state, making it available for fluorescence excitation again. We found that on increasing the pressure it is possible to increase the Ar-laser power to excite molecules to a maximum extent. In the experiment shown in Figure 2, every point of the curve was taken at the optimum laser power (i.e., the photon yield was maximized). The optimum laser power for excitation is found to be in the range of 120-150 mW, depending on the pressure used. Because a turbopump maintains the pressure in the cell region, the pressure should not exceed 10^-4 mbar. All further experiments were performed at this pressure. The pumpdown time to the pressure of 10^-7 mbar was 15 s. Mass spectra were taken at the pressure of ≤ 10^-7 mbar.

**BODIPY Dye**

As a subject of investigation we first chose compound 10. It belongs to the family of BODIPY dyes and was of interest to us because its derivative can be used for labeling a dynamic molecular cavitand ("gripper") [13-16]. Studies of the singly and doubly dye labeled cavitands in the gas phase by fluorescence spectroscopy can provide valuable insight into the conformational dynamics of these flexible structures in a nonsolvated state and can be compared with the results obtained for the solution by dynamic NMR spectroscopy [14]. Furthermore, Parks and coworkers [5,6] previously used BODIPY labeled biomolecules for FRET measurements in the gas phase, which provided an additional choice. Before starting to measure fluorescence in the gas phase, the dye 10 was characterized in solution. The absorption and emission maxima in CHCl₃ were 529 and 542 nm, respectively, and the measured quantum yield was 0.7. To predict a possible shift in the emission spectrum in the nonpolar gas-phase environment, experiments in dimethylchloride, toluene, and hexane were performed. Neither a shift nor a significant fluorescence decrease was found (figure 3).
Figure 2. Emission spectra of compound 10 in dimethylchloride (blue), toluene (red), and hexane (green)

Thus, one would not expect any decrease in fluorescence intensity in the gas phase. Under MALDI conditions, compound 10 was very unstable, giving fragments of the molecular ion without fluorine. Only on the edge of the sample was it possible to produce molecular ions. Surprisingly, even intact trapped molecular ions did not show any fluorescence in the gas phase (figure 4).

Figure 4. Fluorescence signal intensity from compound 10 excited at 488 nm

Possibly, 488 nm is a poor wavelength to excite this dye in the gas phase (Parks and coworkers used 532 nm). Another possible explanation for this unexpected inactivity of the fluorophore in the gas phase is that in MALDI, the BODIPY moiety is protonated at a
location different from the amino group or the positive charge is delocalized over the entire BODIPY structure (Parks and coworkers used ESI).

**Rhodamine Dyes**

Another class of well-characterized dyes that we investigated is the dye of the rhodamine family. There are also practical reasons for this choice such as their low cost, possibility of easy chemical modification, and high quantum yield in solution. Because of their ionic nature, they can be easily ionized by MALDI. The spectral bands for different rhodamine derivatives lie within a range of 480–600 nm, which make them suitable for excitation by the Ar laser as well as the Nd:YAG laser (532 nm, second harmonic). The dependence of the fluorescence signal for rhodamine dyes on the number of ions at a pressure of $10^{-4}$ mbar was found to be linear. This is consistent with our previous results obtained at $10^{-7}$ mbar [12]. The number of ions in the ICR cell was maximized and maintained for all fluorescence experiments. Figure 5 shows the mass spectrum of rhodamine 6G and the corresponding fluorescence signal. The peak ($m/z = 443$) in the spectrum of Figure 5a corresponds to the molecular cation moiety of rhodamine 6G. The absence of isotope signals is due to the so-called peak coalescence effect [17]; this also refers to all other mass spectra shown. The rapid increase in fluorescence intensity in Figure 5b is caused by the pressure increase in the cell region. Then, 10 s later, the pressure is brought back to its initial value of about $10^{-7}$ mbar. The background level in Figure 5b corresponds to the signal collected at the pressure of $10^{-7}$ mbar. The signal represents the total photon count that reaches the detector. It was observed that the excitation of rhodamine 6G ions with a 488-nm wavelength resulted in stronger fluorescence than with 514 nm, while in solution 514 nm is more favorable. A blue shift of 30 nm on desolvation was previously observed by Blades and coworkers for rhodamine 6G, consistent with our findings [18]. Because rhodamine 6G nicely absorbs at 488 nm in the gas phase, it is a suitable donor molecule for attempting gas-phase FRET with the laser lines we have available. Figure 6 shows the mass spectrum and the corresponding fluorescence signal of sulforhodamine B ions.
The peak \((m/z = 559)\) in the spectrum of figure 6a corresponds to the protonated molecular ion. The fluorescence was measured using both 488 and 514 nm as excitation wavelengths. All
experimental conditions such as laser power, pressure in the ICR cell, and duration of the experiment were kept the same as in the previous experiment. As in the previous case, we found a shift in the optical spectra, which is thought to be around 40 nm to shorter wavelengths. Far fewer photons were obtained for excitation with 488 nm compared with 514 nm. This suggests the use of sulforhodamine B or its derivatives as an acceptor fluorophore.

Figure 7 shows the mass spectrum of sulforhodamine 101 and the corresponding fluorescence signal at 514 nm.

![Figure 7](image)

**Figure 7.** (a) Mass spectrum of sulforhodamine 101 (3) and (b) average fluorescence signal after excitation at 514 nm.

Fewer photons were observed in this experiment although the solution quantum yield of 0.91 is almost the same as for rhodamine 6G. This can be interpreted by a lower absorbance of sulforhodamine 101 ions at 514 nm. Almost no photons were observed when exciting with 488 nm. To understand the effect of derivatization of rhodamine 6G on the fluorescence
behavior, compounds 4, 6, and 7 as well as sulforhodamine B derivative 5 were investigated. The spectral data in solution as well as measurements of fluorescence signals in the gas phase showed the same fluorescence intensity as for unmodified rhodamine 6G and sulforhodamine B. Thus, we do not expect a significant decrease in fluorescence quantum yield on derivatization of rhodamine 6G.

**FRET Measurements**

Figure 8 shows the solution-phase absorption/emission spectra of compound 9 in MeOH using 488-nm excitation.

![Absorption/emission spectra of compound 9 in MeOH solution. The insert shows the absorption and emission spectra of the isolated fluorophores (red line, sulforhodamine B; blue line, rhodamine 6G).](image)

**Figure 8.** Absorption/emission spectra of compound 9 in MeOH solution. The insert shows the absorption and emission spectra of the isolated fluorophores (red line, sulforhodamine B; blue line, rhodamine 6G).

It can be deduced from figure 8 that fluorescence resonance energy transfer should occur with almost 100% efficiency in solution: the prerequisite for such a high efficiency is a good overlap between the donor’s emission and the acceptor’s absorption spectra, which is the case for compound 9. The quantum yield measured in solution was later compared with the
quantum yield of rhodamine 6G (0.96) and sulforhodamine B (0.91) and it was found to be lower by factor of 3. The mass spectrum and the corresponding fluorescence signal are shown in figure 9.

Figure 9. Mass spectrum of compound 9 and average fluorescence signal after excitation at 488 nm.

The lower overall photon yield is because of the lower quantum yield of compound 9. To be sure that this fluorescence is coming from the sulforhodamine B (acceptor) moiety ions and not from a tail in the rhodamine 6G (donor) emission, the following control experiment was performed. As shown before, the main part of fluorescence of rhodamine 6G ions is within a range of 500-530 nm. Selecting the same window by two filters we confirmed this fact (Figure 10a).
Figure 10. (a) Rhodamine 6G fluorescence signal collected from the (500 ... 530 nm) window and (b) fluorescence signal of compound 9 collected from the same window.

The lower overall photon counts compared with the fluorescence signal shown in Figure 5b is because of absorptions by the second filter, which was introduced, and to the somewhat narrow emission window selected by filters, which does not cover the entire emission band. Figure 10b represents the fluorescence signal intensity of compound 9 using the same wavelength window. No fluorescence at all was observed, indicating that no significant losses occur because of radiative processes. Based on these facts and taking into account that the fluorescence of pure sulforhodamine B ions excited with 488 nm was low, we can safely conclude that efficient fluorescence resonance energy transfer occurred between the rhodamine 6G and sulforhodamine B moieties of 9 in the gas phase. We did not get any fluorescence signal from compound 8 in the gas phase. Solution spectra also showed greatly suppressed fluorescence. Possible explanations include formation of a more complex fluorophore with vastly different fluorescence properties or (spectroscopic) self-quenching of
the fluorophores because of a much better spatial proximity, allowed by the flexible linker. In contrast, the more rigid spacer in 9 is expected to prevent close contact between the dye moieties. For completeness, we note that biomolecules, at least if they are somewhat stabilized by a folding motive, should not exhibit a very large degree of conformational flexibility and should be amendable to FRET investigations in the gas phase as well.

4.4 Conclusions

We examined and described several compounds and established a correlation between solution-phase and gas-phase fluorescence. A considerable shift of the absorption and emission spectra for rhodamine 6G and sulforhodamine B was found. For the first time, efficient fluorescence resonance energy transfer in the gas phase between two fluorophores was unambiguously shown. A dramatic decrease of fluorescence between a covalently bound FRET pair and a control (without acceptor) was demonstrated.
4.5 References


5 Effect of Buffer Gas on Fluorescence Yield of Trapped Gas-Phase Ions

We investigated the dependence of three different gases, helium, argon, and nitrogen on the fluorescence signal intensity of rhodamine 6G cations in the gas phase. The method is based on laser-induced fluorescence of ions trapped in a Fourier transform ion cyclotron mass spectrometer. We found that helium is the most efficient gas giving the highest fluorescence signal, while no fluorescence was detectable when using argon at the same conditions.

5.1 Introduction

One similar feature for all fluorescence measurements, both in ion traps and FT ICR mass spectrometers, is that the fluorescence detection is accomplished in the presence of a buffer gas. It is, thus, important to understand the effect of this buffer gas on the fluorescence signal intensity. The presence of a buffer gas is also necessary for collisional cooling and spatial confinement of the ions. In ion traps, for example, the high amplitude rf voltage applied to the ring electrode forms a pseudopotential well with the minimum energy at the center of the trap; thus, collisions help to bring ions to its center. It has been demonstrated that the most efficient gas for cooling ions in a quadrupole ion trap is a light gas such as He or H₂ [1]. For successful fluorescence measurements in an FT ICR it is necessary to have a very tight ion packet, to get a maximum overlap with the excitation laser. To achieve this in an ICR, quadrupolar axialization is used [2-7]. An excellent review about quadrupolar axialization can be found in [2]. Various gases (N₂, Ar, He) are commonly used for quadrupolar axialization in FT ICR MS. We are not aware of any work that clearly states which gas provides the best quadrupolar axialization for FT ICR, which means as tight (compact) an ion cloud as possible, right after the axialization event. It will, thus, be important to choose a buffer gas so one could satisfy both criteria, optimum quadrupolar axialization, and highest fluorescence yield. Here we report on a study on the effect of three different gases (Ar, He, N₂) on the fluorescence yield of trapped rhodamine 6G ions.

5.2 Experimental

Rhodamine 6G was purchased from Acros Organics/Chemie Brunschwig AG (Basel, Switzerland). The full description of the instrument is given elsewhere (see chapter 3). Briefly, the experiments were performed on a Fourier transform ion cyclotron resonance (FT ICR) mass spectrometer that consists of a home-built vacuum system, a 4.7 T superconducting magnet (Bruker, Fällanden, Switzerland), and commercial control electronics and data acquisition (IonSpec Corp., Lake Forest, CA). For laser desorption/ionization, the third harmonic of Nd:YAG laser (Minilite ML-10, Continuum; 5 ns pulse width; λ = 355 nm, Santa
Clara, CA) was used. The fluorescence was collected in a similar fashion as in (see chapter 4). The trapped ions were excited by an Ar ion laser (Innova 300, Coherent, Santa Clara, CA) with 488 nm. The Ar-laser diameter was 4 mm and the laser power for all experiments was 120 mW. The emitted fluorescence was focused onto a collimator through a wire mesh covered cut-out in one of the cell plates. The collimator focused the light onto an optical fiber, which is connected to an optical vacuum feedthrough (Caburn Vacuum Science, Ltd., Glynde/East Sussex, UK) followed by an additional optical fiber that focused the light to another collimator, then to a long pass filter (FEL 500, Thorlabs, Karlsfeld, Germany), and finally to the detector (SPCM - ACQ - 16, PerkinElmer, Fremont, CA). The signal is recorded by an 800 MHz gated single photon counter/multiscaler (PMS 400, Becker and Hickl GmbH, Berlin, Germany). The fluorescence signal represents the averaged fluorescence from 10 scans. Buffer gas was introduced via a leak valve for 10 s to a pressure of $10^3$ mbar with simultaneous quadrupolar axialization of the ions for 19 s, followed by chirp excitation and detection. The laser for exciting rhodamine 6G ions was on during the whole sequence (20 s). Since our vacuum gauge is calibrated for nitrogen, calibration factors for other gases were applied to maintain the same absolute pressure. MALDI samples were prepared using a “tablet” method using 2,5 - dihydroxybenzoic acid (DHB, Acros Organics) as a matrix. By “tablet” we mean the following sample preparation method: first, rhodamine 6G was dissolved in methanol at a $10^{-3}$ M concentration; then dry DHB was placed on the target and slightly pressed, and finally rhodamine 6G was dropped onto it. To determine the error bars of the data, one standard deviation from the mean was calculated. Ten measurements from different spots were used to calculate the deviation. The calculated standard deviation was about 13% for all three gases. The calculated standard deviation for the fluorescence signal was around 5%, i.e., the total error was about 14%.

5.3 Results and Discussion

Figures 1 and 2 show the mass spectrum and the corresponding fluorescence signals from rhodamine 6G, when using different buffer gases (Figure 2a) helium, (Figure 2b) nitrogen, and (Figure 2c) argon.
Figure 1. Mass spectrum of rhodamine 6G. The signal represents the average of 10 scans.

The fluorescence signal when using He is $160 \pm 22$ photons/s, for $\text{N}_2$ it is $105 \pm 15$ photons/s, and no fluorescence is observed for Ar. Let us now consider possible scenarios for these dramatically different fluorescence signal levels.
Figure 2. Dependence of the fluorescence signal intensity of rhodamine 6G ions versus time on three different gases, (a) helium, (b) nitrogen, and (c) argon. The excitation wavelength was 488 nm and the laser power was 120 mW. The rapid increase of the fluorescence is due to the pressure increase of the buffer gas in the trap region. The background signal represents the number of photons reaching the detector after the pressure is brought back to a nominal value of $\leq 10^{-7}$ mbar.
Difference in Spatial Overlap Due to Efficiency of Quadrupolar Axialization

Quadrupolar axialization can affect the dimension of the ion cloud in two ways: (1) contraction of the ion cloud in radial direction, perpendicular to the magnetic field vector, and (2) ion cloud compression in z-direction, parallel to the magnetic field vector. The ion cloud compression in z-direction is mainly due to collisional cooling by the neutral buffer gas. The collision rate and times for this cooling can be roughly estimated using Langevin model (this model is valid for thermal velocities). In this model, an ion is assumed to be a point charge and a neutral molecule is assumed to be a polarizable point particle (no permanent dipole moment). The interaction between an ion and a neutral arises from the force between the point charge and the induced dipole [8-10]. Using these assumptions, one can calculate the ion-neutral collision frequency per unit volume $Z_{AB}$, between ion A and neutrals B.

$$Z_{AB} = k_{igv} \rho_A \rho_B$$

(5.1)

where $\rho_A$ and $\rho_B$ are the number densities of the ions and neutrals respectively. The Langevin collision rate constant $k_{igv}$ is given by:

$$k_{igv} = 2\pi q \sqrt{\frac{\alpha}{\mu}}$$

with reduced mass $\mu = \frac{m_A m_B}{m_A + m_B}$

(5.2)

where $\alpha$ is the isotropic polarizability of the neutral in cgs units (cm$^3$), the elementary charge, $q$, is $4.8 \times 10^{-10}$ statcoulombs, and $\mu$ is the reduced mass in gram. For $N_2$, $\alpha = 1.74 \cdot 10^{-24}$ cm$^3$, for $He$, $\alpha = 0.20 \cdot 10^{-24}$ cm$^3$, and for $Ar$ $\alpha = 1.64 \cdot 10^{-24}$ cm$^3$ [11,12], thus the collision rate constants are:

$$k_{igv}^{N_2} = 8.4 \cdot 10^{-10} \text{ cm}^3 \text{s}^{-1}$$

$$k_{igv}^{He} = 5.3 \cdot 10^{-10} \text{ cm}^3 \text{s}^{-1}$$
\[ k_{k^A}^{Ar} = 7.2 \cdot 10^{-10} \text{cm}^3 \text{s}^{-1} \]

The collision frequency per ion \( z = \frac{Z_{AB}}{\rho_A} \) is proportional to the number density of the neutrals \( \rho_B \). Using the ideal gas law to calculate \( \rho_B \) at 298 K and pressure \( P_B = 10^{-3} \text{mbar} \), one obtains \( \rho_B = 2.4 \cdot 10^{13} \text{cm}^{-3} \). Thus, the collision frequencies for three gases are:

- \( z_{N^2}^{N^2} = 20160 \text{ s}^{-1} \)
- \( z_{He}^{He} = 12720 \text{ s}^{-1} \)
- \( z_{Ar}^{Ar} = 17280 \text{ s}^{-1} \)

The energy of the rhodamine 6G ions after ionization can be calculated as \( E = \frac{1}{2}mv^2 \), where \( m \) is the mass and \( v \) is the velocity of the ions. According to [13-15] and our own measurements, for the velocity of rhodamine 6G ions a value of 800 m/s can be taken. Therefore, we obtain \( E = 1.4 \text{ eV} \). The thermal energy of the ions is \( \sim kT \). Calculating for \( T = 298 \text{ K} \) we obtain \( E_{\text{thermal}} = 0.026 \text{ eV} \). Assuming that for one collision the energy loss is \( \frac{m_{\text{molecular}}}{M_{\text{ion}}} \), we obtain the number of collisions required to thermalize ions, \( N_{N^2}^{N^2} \approx 50 \), \( N_{He}^{He} \approx 150 \), \( N_{Ar}^{Ar} \approx 35 \). Comparing these numbers to the collision frequencies, we obtain the time of thermalization, \( t = 2 \cdot 10 \text{ ms} \), for all three gases.

The radial shrinking is due to an azimuthal radiofrequency field applied that periodically converts magnetron motion to cyclotron motion, while cyclotron motion is damped with collisions [2]. This process can require between milliseconds up to a few seconds. The strong increase in fluorescence signal during the first 2 s after starting axialization shows the effect of spatial compression of the ion cloud both in radial and in z-direction (Figure 2a, b). Thus we believe that after 2 s we obtain an ion packet of approximately the same size no matter which gas is used (N2, Ar, He). The MS amplitude of the ion signal and the resolution were the same for all three gases, which is also a clear indication that the size of the ion packet is very similar. Thus, we do not expect any difference in fluorescence signal intensity due to quadrupolar axialization geometric effects.
**Quenching**

One effect that may be responsible for fluorescence quenching is related to the polarizability of Ar. The polarizability of Ar is 1.85 Å³, which is much higher than that of He 0.22 Å³ [16]. This means that Ar atoms, which are in the vicinity of rhodamine 6G cations, are easily polarized. The charge-dipole interaction can result in the formation of noncovalent complexes of Ar with rhodamine 6G cations. This is not expected to occur for He at room temperature. One can think of several possible scenarios of how this should quench the fluorescence: (1) the quantum yield of the complex could be much lower than that for uncomplexed rhodamine 6G ions, or (2) formation of the complex may result in a shift of the optical absorption spectrum. We did not observe any MS signal from this complex, which indicates a short lifetime. The polarizability of N₂ is 1.97 [16], which is slightly higher than that of Ar. Thus, one would expect the formation of the same kind of complexes with N₂ as in case of Ar. This suggests that fluorescence-quenching with N₂ should be even more pronounced. However, this is not observed, which argues against fluorescence-quenching by the formation of a buffer gas-rhodamine 6G complex.

**Intersystem Crossing**

Another effect, the so-called “external heavy atom effect” may affect the fluorescence yield of a collision complex during the following process:

\[ M^* + Ar \rightarrow M + Ar + \text{heat} \]

It has been shown that the presence of a heavy atom increases the spin-orbit coupling, i.e., coupling between the orbital magnetic moment and the spin magnetic moment. Thanks to this interaction, there is the probability for intersystem crossing (i.e., a forbidden transition from the first singlet excited-state S₁ to the first triplet state T₁). The efficiency of spin-orbit coupling varies with the fourth power of the atomic number, which explains why intersystem crossing is favored by the presence of heavy atoms (for more details on “external heavy atom effect” see [9,17]). Thus, the probability of intersystem crossing for rhodamine 6G ions in contact with Ar is 104 times higher compared to He. Once intersystem crossing has occurred,
the main deactivation pathways are nonradiative decay, delayed fluorescence, and phosphorescence. Since we did not observe any delayed fluorescence, this pathway is not discussed here. It is known that in solution at room temperature, nonradiative decay from the triplet state T$_1$ is in general predominant over radiative de-excitation or phosphorescence [9]. The phosphorescence lifetime is in the range of $10^{-6}$ s to several seconds. To see whether we can observe any phosphorescence, we performed an additional experiment with the Ar excitation laser shut off after 100, 200, 500 ms, and 1s. No phosphorescence was observed, indicating that either the phosphorescence lifetime for rhodamine 6G is less than 100 ms or there is only a nonradiative channel of energy dissipation. Unfortunately, we do not have a possibility to go below 100 ms since we have a minimum time uncertainty of 100 ms due to the opening/closing time of the shutter, which is used to block the laser.

5.4 Conclusions

The most probable explanation for the effect of the buffer gas on the fluorescence yield of trapped rhodamine 6G ions is the external heavy atom effect manifesting itself during ion-buffer gas collisions. Clearly, He is the most appropriate gas for performing fluorescence measurements in an FT ICR ion trap. Despite the fact that all three gases provide an efficient quadrupolar axialization, it is advantageous to use He since it provides the highest fluorescence signal, while axialization is as efficient as with N$_2$ and Ar. We believe that this finding is of practical importance for performing future experiments involving fluorescence spectroscopy of ions in the gas phase both in ion traps and FT ICR traps.
5.5 References


6 Signal Enhancement in MALDI by Doping with Cu (II) Chloride

6.1 Introduction

Many recent efforts in matrix-assisted laser desorption/ionization (MALDI) [1,2] are directed towards better understanding of the ion formation mechanism [3-6], towards improved sample preparation [7-9], better sample homogeneity, and optimum sample supports [10-15]. There have also been concerted efforts to search for better matrices, including many proposals to use mixtures of compounds and matrix additives [16,17]. A particularly successful example to enhance the performance of 2,5-dihydroxy benzoic acid (DHB) is the addition of about 10% of the 2-hydroxy-5-methoxybenzoic acid, called ‘super DHB’, resulting in better mass spectral signal (higher S/N) and an improvement in mass accuracy for peptides [18]. Other approaches include the use of 5-methoxysalitic acid (MSA) or fucose as a co-matrix to give a significant enhancement in the spectra of complex peptide mixtures [19], and for improving reproducibility, signal intensity, and resolution for a wide range of peptides and heavy proteins [20]. Wilkins and co-workers showed an enhancement of resolution in Fourier transform ion cyclotron resonance mass spectrometry (FT ICRMS) by using a mixture of sugars with sample, which give an increase in the stability of protonated peptides and proteins [21,22]. The main benefits of matrix additives are thought to result from better homogeneity of the sample surface, better co-crystallization and hence better shot-to-shot reproducibility.

Recently, Frankevich et al. found that a MALDI sample target can be a source of photoelectrons, which play an important role in ion formation both in positive and negative modes [23-25]. The yield of electrons depends on the surface, the thickness of the sample, the matrix properties, and the laser power. The maximum electron yield was observed with a metal target and a thin layer of sample. A high electron yield leads to formation of negative matrix ions by the following reaction:

\[ M + e^- \rightarrow [M-H]^- + H^+ \]

\([M-H]^-\) can then react with other ions in the plume, resulting in neutralization and charge state reduction of positive ions [24]. It was found that using non-metallic sample targets as well as a ‘thick’ layer sample preparation, which means that the laser cannot penetrate through the sample, can suppress the electron yield from the target. Elimination of these
electrons from the MALDI plume allows many more positive ions [26]. It is possible that some of the improvements found when using matrix additives can be explained by their ability to scavenge electrons, thus boosting the positive ion yield. Recently, it has been shown that Cu(II), present as a cationization agent in MALDI samples, easily reduces to Cu(I) in the presence of electrons [27]. Due to the high electron affinity of CuCl₂, the main reaction in the MALDI plume is:

\[
\text{CuCl}_2 + e^- \rightarrow \text{Cu}_n\text{Cl}_{m}^- 
\]

It appears that electron transfer from anionic Cu clusters to positive matrix and analyte ions are very unfavorable, i.e., such copper salts should be able to act as an 'electron sink'. The role of electrons in MALDI, as well as their effect on CuCl₂ present in MALDI samples, have been thoroughly studied [23-25,27].

If the concepts described above are correct, they suggest the following practical consequence for MALDI: if used as a co-matrix, CuCl₂ should present a 'chemical' way to suppress electron emission. Indeed, as shown below, strong analyte signal enhancement was found in the positive mode from samples with CuCl₂ as matrix additive.

### 6.2 Experimental

In order to investigate this effect and to be sure that it is not an instrumental artifact, experiments were performed on both time-of-flight (TOF) and FT-ICR mass spectrometers. TOF experiments were performed using a commercial MALDI-TOF instrument (Bruker Reflex II, Bremen, Germany) equipped with a nitrogen laser (\(\lambda = 337\) nm), delayed extraction, and a total acceleration voltage of 20 kV. One hundred shots were usually accumulated on one spot. The FT-ICR mass spectrometer consists of a homebuilt vacuum system with a 4.7 T superconducting magnet (Bruker, Fällanden, Switzerland) and commercial control electronics and data acquisition (IonSpec Corp., Lake Forest, CA, USA). A home-built open cylindrical cell was used in order to trap ions. The instrument has an internal MALDI source, and the target is positioned 20mm from the cell. For laser desorption/ ionization, the third harmonic of
a Nd:YAG laser (Continuum, Minilite ML-10, USA; 5 ns pulse width; \(\lambda = 355 \text{ nm}\)) was used. Experiments were performed both in negative and positive mode. Bradykinin was purchased from Bachem AG (Switzerland). Copper(II) chloride (CuCl2) and 2,5-dihydroxybenzoic acid (DHB) were obtained from Fluka (Buchs, Switzerland). MALDI samples were prepared using a standard ‘dried droplet’ method. DHB was dissolved at 10mg/mL of water/ethanol (1:1) and CuCl2 in water at a concentration of 1 mg/mL. An aqueous solution of bradykinin (500 fmol) was mixed with the matrix solution in a 1:1 ratio. Analyte-Cu solution was obtained by mixing aqueous CuCl2 with DHB/bradykinin solution in molar ratio about 1:5 of the sample mixture. Then, 2 mL of the analyte/DHB/CuCl2 mixture were deposited on a stainless steel plate and dried at room temperature. The laser energy was adjusted to obtain optimum resolution and mass spectra and was 0.3 mJ per pulse, corresponding to an irradiance of \(6 \times 10^6 \text{ W/cm}^2\). The laser spot diameter was 1mm\(^2\). One hundred shots were accumulated on average on each spot. In order to check for the presence of electrons in the FT-ICR cell, SF6 gas (Aldrich, Milwaukee, WI, USA) was added to the system through a pulse valve. The typical SF6 pressure was \(10^{-6} \text{ Torr}\).

### 6.3 Results and Discussion

**FT-ICR MS data**

In order to define the effectiveness of suppression of electrons from the MALDI target by CuCl\(_2\), an experiment with SF\(_6\) gas was performed [23]. Free electrons emitted from the MALDI target cannot be directly detected in FT-ICR MS because of their low mass and high cyclotron frequency. SF\(_6\) has a high cross section for low-energy electron capture [28] and we can assume that the final number of generated SF\(_6^+\) ions is proportional to the number of electrons. Figure 1 shows a negative mode MALDI FT-ICR mass spectrum of DHB on a metal target.
Figure 1. Negative ion mode mass spectrum of DHB on a metal target, using a drift time of 100 ms. The insert is the corresponding SF$_6^-$ signal, measured at a drift time of 10 μs.

The typical drift time (time-of-flight of the ions to the cell) of the ions is 100 ms. The insert in Fig. 1 shows the corresponding electron signal measured by the appearance of SF$_6^-$ at short (10 μs) drift time. A strong SF$_6^-$ signal corresponds to a large amount of electrons being emitted from the sample support. By adding CuCl$_2$ the electron yield and thus the predominance of negative matrix ions is dramatically reduced. Figure 2 shows the negative mode mass spectrum of DHB with CuCl$_2$ added, and the insert in it shows the corresponding SF$_6^-$ signal at short drift time.
Figure 2. Negative ion mode mass spectrum of DHB with CuCl₂ on a metal target, using a drift time of 100 µs. The insert is a corresponding SF₆⁻ signal measured at a drift time of 10 µs.

A much lower intensity of SF₆⁻ can be seen, which indicates that CuCl₂ can effectively suppress electron emission. We believe that a low electron yield from a sample target can have a dramatic effect on the intensity of positive ions [24,29]. In order to check the effect of CuCl₂, two experiments were performed. Figure 3 shows the mass spectrum of bradykinin from a metal target using (a) DHB and (b) DHB with CuCl₂. To avoid ‘hot’ spots, 100 random shots were accumulated. An enhancement of analyte signal by more than 20 times was obtained from the sample containing CuCl₂ as an electron scavenger.
Figure 3. Positive mode mass spectrum of bradykinin obtained using (a) 2,5-dihydroxybenzoic acid (DHB) as a matrix and (b) with CuCl₂ as an additive to the DHB matrix. Asterisks indicate known RF interferences.

**TOFMS data**

In order to be sure that this effect is not only observed on one particular instrument, a similar experiment was performed on a TOF mass spectrometer. TOFMS experiments were carried out in linear mode with delayed extraction so that gas-phase reactions would be able to reveal the presence of photoelectrons. To avoid ‘hot’ spots, 100 random shots were accumulated.
from each sample. Figure 4 shows the positive mode MALDI-TOF spectrum of bradykinin using (a) DHB and (b) DHB with CuCl₂.

All experimental conditions such as sample preparation, laser power, number of scans, etc., were kept the same. As in the case above, strong analyte enhancement can be seen in Fig. 4(b). This suggests that CuCl₂ indeed acts as an efficient scavenger of free photoelectrons, which are normally responsible for neutralization processes.

6.4 Conclusions

This work shows significant enhancement of analyte ion signal in positive ion mode. We interpret this enhancement to be due to suppression of electrons in the MALDI plume, which leads to formation of negative matrix ions. These negative matrix ions would normally interact with positive analyte ions and neutralize many of them. Additives such as CuCl₂ as an electron scavenger are suggested to decrease the number of electrons in the MALDI plume.
6.5 References


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7 Kinetic Energy of Free Electrons Affects MALDI Positive Ion Yield via Capture Cross Section

A method for enhancing positive analyte ion signal in MALDI is described. The idea is based on influencing the kinetic energy of free electrons emitted from the organic/metal interface. It has been recently shown that free electrons in MALDI have energies around 1eV. This energy is close to the maximum capture cross section of most common MALDI matrices, leading to the efficient formation of negative matrix ions. This results in the reduction of the positive analyte ion yield. The effect can be minimized by shifting the kinetic energy of the electrons away from the maximum of the matrix capture cross section by choosing a proper substrate material.

7.1 Introduction

It is well known that the ion yield in MALDI is very low, on the order of $10^{-4}$ [1,2]. A better understanding of the ionization/desorption mechanisms is required to explain various processes responsible for ion formation in MALDI. Several models of ion formation have been proposed for MALDI [3-5], and a number of experimental approaches have been suggested toward signal enhancement, including improved sample preparation and proper choice of matrix/analyte ratio [6,7,8]. Significant signal improvements were found when various special MALDI targets were used [9-13]. In one example, a sample support coated with a thin layer of Teflon that carries an array of 200-μm gold spots was used, which provides hydrophilic anchors for the sample. The detection sensitivity for several standard peptides was increased to 100 amol [12]. In another example, a thin film of paraffin attached to a standard stainless steel sample support was employed for the analysis of DNA [10]. The authors showed a 5-fold decrease in detection limits, as well as improvement of the shot-to-shot and sample-to-sample reproducibility. A hydrophobic coating based on 3M Scotch Gard™ results in spot size reduction and increased detection sensitivity [11]. However, often the explanations for these effects are either not fully described in the literature or not based on any physical effects. In most cases, a smaller sample spot size and thus a higher local concentration of the analyte in a spot was the only reason given for the improvement in sensitivity.

Recently, the effects of the sample preparation and of the target material were interpreted in a new fashion [14]: it was found that a substantial number of photoelectrons are emitted from the metal target [15,16] under laser irradiation, when only a thin MALDI sample is present. It was already suspected by Karas and co-workers [17] that electrons play a role in MALDI, although these workers assumed that electrons are coming only from matrix photoionization in the case of positive ions and from reaction with protonated matrix species, in case of negative ions respectively. The presence of electrons may play a major role in suppressing positive ion signal of analytes as well as decreasing detection limits. In work from this laboratory [14] an increase by more than 2 orders of magnitude of positive ion signal of bovine serum albumin and bradykinin was demonstrated when electron photoemission was absent; a substantial improvement in mass resolution was also achieved.
In this work we take a closer look at the photoelectrons emitted from a MALDI target. We found that electrons with particular kinetic energies are primarily responsible for reducing the positive ion signal. A way to influence the electron energy and to increase positive analyte signal is discussed.

7.2 Experimental

The experiments were performed on a Fourier-transform ion cyclotron resonance mass spectrometer that consists of a home-built vacuum system, a 4.7 T superconducting magnet (Bruker, Fällanden, Switzerland), and commercial control electronics and data acquisition (IonSpec Corp., Lake Forest/CA, USA). A home-built open cylindrical cell was used for trapping the ions. The instrument has an internal MALDI source with a target that was positioned 2 cm from the cell. For laser desorption/ionization, the 3rd harmonic of a Nd:YAG laser (Continuum, Minilite ML-10, USA) at 355 nm with a 5 ns pulse width was used. The laser spot diameter was 900 µm. Experiments were performed both in negative and in positive modes. Bradykinin was purchased from Bachem AG (Switzerland), α-cyano-4-hydroxycinnamic acid (HCCA, purity 99%) from Sigma (Switzerland), 2,5-dihydroxybenzoic acid (DHB, purity 99%) from Acros Organics (New Jersey, USA) and sulfur hexafluoride from Aldrich (Buchs, Switzerland). HCCA was dissolved in a buffer containing 80% ACN, 0.1% TFA and 20% water, DHB was dissolved in methanol. Both HCCA and DHB were used without further treatment or purification. MALDI samples were prepared using a standard "dried droplet" method at a matrix/analyte concentration ratio of 100:1. Spectra were obtained by averaging over a large spot area while the laser was rastered continuously and randomly over the sample. Typically, 50 scans were co-added for each measurement. The mean of multiple measurements were taken for each data point. To determine the error bars of the data, one standard deviation from the mean was calculated. The standard deviation of the peak intensities was determined experimentally to be in the range 15-20% for all MALDI and electron capture experiments.

Stainless steel and nickel sample supports were machined in house and a gold support was made by vapor-depositing of a 300 nm gold layer on to a standard stainless steel support. The
deposition of gold was performed in house on a commercial coating machine (MED 020, Bal-Tec, Liechtenstein). Light microscope images were taken on a standard instrument (BH2, Olympus) with reflected light illumination.

Electrons cannot be directly measured in a FTICR cell due to their high cyclotron frequency. SF$_6$ buffer gas was therefore used to detect free electrons indirectly. It is well known that SF$_6$ is an efficient electron scavenger with a maximum cross section at an electron energy close to 0 eV [9]. Higher kinetic energy electrons are captured after inelastic scattering of electrons from SF$_6$ gas molecules. The number of SF$_6^-$ ions is thus proportional to the total number of electrons emitted from the MALDI target. The SF$_6$ was introduced into the ICR cell through a leak valve (RME 005, Pfeiffer Vacuum, Switzerland) to a pressure of 1x10$^{-6}$ Torr. Electrons produced upon laser irradiation drifted into the field-free region of the open cell and became trapped using gated trapping with a potential well of 10 V. After an appropriate delay time ions of the selected polarity were excited by chirp excitation and detected.

The experimental data in figure 2 were fitted by both linear ($y = ax$) and quadratic ($y = ax^2$) functions, using commercial software (Igor Pro V4.08, Wavemetrics, Lake Oswego / OR). The coefficients of the fitted curves were calculated based on minimizing the value of the Chi-square ($\chi^2$). $\chi^2$ is defined as $\sum_i (y_i - y) / \sigma_i^2$ where $y$ is a fitted value for a given point, $y_i$ is the measured data value for the point and $\sigma_i$ is an estimate of the standard deviation for $y_i$. All fits were restricted to go through the origin, because no photons will yield no electrons and no SF$_6$ ion signal; simply fitting the data points with linear or quadratic functions would sometimes lead to a finite intercept, which is, of course, incorrect.

7.3 Results and Discussion

Figure 1 shows microscope images of DHB deposited on stainless steel and gold substrates, respectively. The close similarity of the crystal morphology on both gold and stainless steel substrates suggests that sample morphology does not affect the ionization process of the sample. Therefore, significant changes in ion signal intensity due to the morphology of the
sample are unlikely. The spaces between the crystal needles of DHB are relatively large, and the underlying metal surface will always be exposed to the laser beam. Thus, the emission of photoelectrons from the organic/metal interface will be possible from all such samples.

Figure 1. Light microscope images of 2,5-dihydroxybenzoic acid (DHB) crystallized on (a) a stainless steel substrate and (b) a gold substrate. The typical needlelike crystal morphology of DHB is observed on both substrates. The difference in contrast is an artifact due to differences in the illumination conditions. The spaces between crystal needles result in the underlying metal surface being exposed to the laser beam, which has a diameter of 900 mm. Therefore, production of photoelectrons from the organic/metal interface should always be possible.

The work function for nickel is 5.04 eV, for stainless steel ≈ 4.6 eV, and for gold 5.1 eV. The photon energy corresponding to the 3\textsuperscript{rd} harmonic of a Nd:YAG laser is 3.49 eV. Thus, we do not expect photoelectrons from pure metals due to laser irradiation. However, it has been demonstrated that the presence of an organic layer on a metal surface leads to an intense electron emission from the metal/organic interface [14-16]. This effect can be rationalized to result from the reduction of the metal work function [18]. For a single-photon process, a linear dependence of the electron yield on laser pulse energy is expected. Alternative explanation is based on the idea that matrix-analyte complexes may exhibit ionization potentials lower than that of pure matrix, enabling 2-photon ionization by typical MALDI lasers (N\textsubscript{2} laser, 337 nm,
or tripled Nd:YAG, 355nm) [19]. In this case, a quadratic dependence on laser pulse energy would be expected.

It has been shown recently [14] that emission from a stainless steel plate covered by a thin layer of an organic matrix has a roughly linear dependence on the laser pulse energy in the range of laser energies up to 0.25 mJ (8 MW/cm²), although this has been challenged [19]. For this reason, the dependence of photoelectron emission of different metals such as nickel, stainless steel, and gold covered by a thin layer of DHB was carefully investigated in the present work. As mentioned in the experimental part, electrons can be detected by measuring the \( \text{SF}_6^- \) ion signal. Figure 2 shows the \( \text{SF}_6^- \) signal intensity versus laser energy for stainless steel (a), gold (b), and nickel (c).

As clearly seen from the fits in fig.2, the linear dependence fits better for all metals studied, thus supporting a one-photon process for photoemission. While a one-photon process is the simplest and most straightforward explanation of this data, alternative scenarios are possible: for example, deviation from quadratic behavior would be expected for a two-photon process if saturation occurs; however this usually does not lead to a clean linear power dependence. Also, a composite model including sequential 2-photon ionization of matrix in parallel with excited state energy pooling has been proposed [19], which did not yield a quadratic power dependence.
Figure 2. SF6 ion signal as a function of laser energy ($\lambda=355\text{nm}$) due to electron emission from (a) stainless steel, (b) gold, and (c) nickel. The dashed line is a quadratic function fit ($y = ax^2$), and the solid line is a linear function fit ($y = ax$). $\chi^2$ is defined as $\Sigma[(y-y_i)/\sigma_i]^2$ where $y$ is a fitted value for a given point, $y_i$ is the measured data value for the point, and $\sigma_i$ is an estimate of the standard deviation for $y_i$. In all cases, the data is better represented by a linear fit, supporting a one photon process for electron emission.

Because a metallic MALDI target can be a source of electrons, the presence of these electrons in the MALDI plume can affect the positive ion yield and can lead to a predominance of negative ions. The interaction of electrons with matrix ions in a MALDI plume has been suggested to occur by the following reaction [14]:

$$M + e^- \rightarrow [M-H]^+ + H^+ \quad (1)$$

Electron capture by $M$ has a cross section that varies with the kinetic energy of the electrons. In the work by Asfandiarov et al. [20, 21], the capture cross sections for typical MALDI matrices were measured. In their work the investigated molecules were irradiated by electrons of controlled energy (0-10 eV) in a vacuum of $10^{-4}$ Torr, and the electron capture cross section...
was plotted as a function of the incident electron energy. These authors found that the maximum cross section for DHB, sinapinic acid, nicotinic acid is around 1 eV, caffeic acid is 0.6 eV and HCCA is around 0.2 eV. As an example, the measurement of the capture cross-section for DHB vs electron energy, taken from ref 23, is shown in figure 3. The maximum of the capture cross-section is observed at around 0.85 eV. In this work, the two of the most widely used MALDI matrices, DHB and HCCA, were employed.

![Graph of 2,5-Dihydroxybenzoic acid (DHB)](image)

**Figure 3.** Plot of the electron capture cross-section of DHB, showing the total negative ion current (circles) and the ion current of deprotonated DHB, [M - H]−, vs incident electron energy. The resonance at 0.85 eV is clearly observed. Reproduced with permission from ref 23, copyright ISI publications.

Following formation of [M-H]−, negative matrix ions can further interact with positive analyte ions in the plume, leading to suppression of positive ion signal:

\[
[M-H]^- + AH^+ \rightarrow [M-H]^+ + AH
\]

(2)
In our FT-ICR setup, the sample substrate was directly connected to a voltage. We were thus able to control the kinetic energy of electrons by applying different voltages to the substrate. This, in turn, allowed us to control the number of negative matrix ions produced, affecting the rate of reaction 1. Although this is technically rather difficult to implement, and the energy of the analyte ions is also affected by changing the kinetic energy of the electrons, it is the reaction of the electrons with neutral matrix molecules, which is of primary interest here. Control of the rate of reaction 1 is possible not only by applying a voltage to the MALDI plate, but also by changing the material of the plate (work function). In the work of Gorshkov et al. [16], the electron energy distribution for stainless steel and gold was measured. It was found that the maxima for stainless steel with gold are around 1 eV and 0.55 eV respectively. Thus, by substituting the stainless steel plate with gold, the electron energy is changed from 1 eV to 0.55 eV. For DHB, this results in a shift away from the maximum capture cross section. In figure 4a the negative mode mass spectrum of DHB deposited on a stainless steel sample support is shown. The most abundant peak is 153 Da corresponding to [M-H]−, which is formed by electron-capture dissociation. Figure 4b shows mass spectrum of the same compound but on a gold plate. It can be seen that 152 Da corresponding to [M-H2]− is the predominant peak in the spectrum. This fragment has been already observed by other research groups [17,22] but the origin is not completely clear yet. It has been shown by Bourcier [22] that the 152 Da fragment appears only above a laser pulse energy of 19 mJ. A similar observation was reported by Karaset al. [17]. In normal MALDI as well as on the gold substrate, we propose that the 152 Da peak is formed by the loss of a hydrogen radical from the deprotonated DHB, resulting in a cyclic resonance structure that involves the carboxyl group and the hydroxyl group in position 2. On the stainless steel substrate, the energy of the emitted photoelectrons is in resonance with the maximum capture cross-section of DHB, i.e., electron capture rather than deprotonation will be the dominant process. Electron capture can take place on every electronegative atom of DHB, and subsequent loss of a hydrogen radical is expected to be the pathway for formation of [M - H]−. The reaction to [M - H2]− will not necessarily involve the adjacent carboxyl and 2-OH groups, i.e., the formation of the stabilized cyclic resonance structure is less probable. In many instances, the [M-H]− at m/z 153 will thus survive.

The key result obtained from the data in Figure 4 is that the negative ion yield is greatly decreased when going from stainless steel to gold, by about a factor of 2. This is interpreted to
be a direct result of a lower capture cross-section, as the kinetic energy of the electrons is shifted from its maximum at 1 eV (stainless steel) to 0.55 eV (gold). Shifting the electron energy from the maximum changes the rate of reaction 1. Our interpretation is that decreasing the number of negative matrix ions in the MALDI plume will decrease the total number of negative charges in the plume and prevent the neutralization of positive analyte signal.

**Figure 4.** Negative-mode MALDI mass spectra of 2,5-dihydroxybenzoic acid (DHB) deposited on (a) a stainless steel plate and (b) a gold plate. The decreased number of [M - H]\(^+\) ions in the case of the gold substrate slows the rate of reaction 2, thus preventing the neutralization of positive analyte ions. Standard deviation of the MALDI peak intensities was 15-20%.
In the case of HCCA, the energy of photoelectrons emitted from either stainless steel or gold does not coincide with the capture cross-section maximum of HCCA. However, it was found [21] that the formation of the main fragment \([M - \text{CO}_2]^-\) has a maximum at an electron energy of 0.5 eV in negative ion mass spectrometry. This energy corresponds almost exactly to the energy of photoelectrons emitted from gold. Figure 5 shows a negative-mode mass spectrum of HCCA deposited on stainless steel and gold. As can be clearly seen in Figure 5b, the formation of \([M - \text{CO}_2]^-\) is a predominant process on the gold plate.

Figure 5. Negative-mode MALDI mass spectra of \(\alpha\)-cyano-4-hydroxycinnamic acid (HCCA) deposited on (a) a stainless steel plate and (b) a gold plate. Standard deviation of the MALDI peak intensities was 15-20%. Since the formation of the \([M - \text{CO}_2]^-\) fragment peaks at an electron energy of 0.5 eV, and the kinetic energy of photoelectrons emitted from gold is
around 0.55 eV, the intensity of the [M - CO₂] fragment it is more pronounced on the gold substrate.

Figure 6 shows the spectrum of bradykinin on stainless steel (a) and gold (b) plates. All the experimental conditions such as laser power, sample preparation, analyte concentrations, and so forth were identical.

![Figure 6](image)

**Figure 6.** Positive-mode MALDI mass spectrum of bradykinin with DHB as a matrix, deposited on (a) a stainless steel plate and (b) a gold plate. Standard deviation of the MALDI peak intensities was 15-20%. An increase by a factor of about 2 is demonstrated on the gold substrate.

An increase by a factor of 2 in ion intensity for gold is demonstrated. On the basis of the above considerations, it is straightforward to understand this increase. As found before, the average electron energy from the gold plate is 0.55 eV; thus, reaction 1 is ineffective, and many fewer negative matrix ions are present in a MALDI plume. As a result, the rate of reaction 2 will be lowered as well. It should be noted that the signal enhancement is not as dramatic as in the case of nonmetallic targets. In the experiments presented here, we influence the charge balance of the MALDI plume only by shifting the kinetic energy of the electrons...
away from the maximum cross-section for negative ion formation. However, this does not stop reaction 1 completely.

### 7.4 Conclusions

The laser energy dependence of photoelectron emission from MALDI samples deposited on stainless steel, gold, and nickel was investigated. The results obtained support a one-photon model for photoemission, as proposed in ref 14. The substrate material was shown to play an important role in the MALDI process. By choosing different substrates, we were able to manipulate the photoelectron kinetic energy, and, by virtue of the energy-dependent capture cross-section of the matrix, decrease the number of negative matrix ions, which led to an increase in analyte signal in positive ion mode.
7.5 References


The ratios of positive to negative ion yields for matrix-assisted laser desorption ionization were studied for fibrinopeptide and bradykinin in combination with six matrices (CHCA, DHB, 4-NA, ATT, ANP, 5-AQ). The selection of these particular compounds was based on their acid/base properties. The findings are (1) in the time-of-flight measurements the detection efficiency for positive ions is higher than for negative ions by a factor of 3.5±1.2 (2) the total positive/negative ion yield ratio is independent of acid/base properties of the analyte molecules (3) higher positive ion yield was observed when using nonmetallic supports.

Adapted from: M. Dashtiev, E. Wafter, M. Gorshkov, F. Hillenkamp and R. Zenobi “Ion yield in MALDI TOF mass spectrometry”, manuscript in preparation
8.1 Introduction

Matrix-assisted laser desorption ionization (MALDI) has rapidly become a powerful technique for the analysis of peptides and proteins [1-3]. In general, MALDI MS analysis is carried out in positive mode [4], although there is only anecdotal evidence that positive ions are more abundant. In some cases, e.g. the analysis of phosphopeptides both positive and negative modes can be beneficial [5]. In collision-induced dissociation (CID) studies of peptides it has been demonstrated that negative CID spectra are generally as informative as the positive ion spectra [6]. Therefore, it is clear that a better understanding of the negative mode and an optimization of the negative ion yield are desirable for MALDI applications.

The overall yield of charged species in MALDI is rather low, on the order of $10^{-4}$ [7,8]. In negative mode, in addition to matrix and analyte signals, a signal for electrons is often detected. The origin of these electrons has recently been studied in detail by Frankevich, who showed that metal substrates emit photoelectrons under UV laser irradiation, when covered with a thin layer of organic material [9-12]. During our previous studies we have observed that in MALDI positive analyte ion yields were strongly substrate dependent under otherwise identical experimental conditions [10,13]. In the case of two widely different substrates, metal (stainless steel) and nonmetal (polyether ether ketone, PEEK), positive analyte ion yield on non-metal was two orders of magnitude higher than on a metal substrate. This effect was attributed to photoelectrons emitted upon laser irradiation, which can then react in the plume. Another example is provided by direct LDI measurements of ultrafine particles in a bipolar TOF instrument, where more positive than negative analyte ions have been observed [14]. The authors suggested the following explanation: under laser irradiation ultrafine aerosol particles yield primarily positive ions, neutrals, and electrons. Because of the small particle size, the density of the plume is low and the probabilities of electron capture by neutrals and positive-negative charge recombination in the plume are low. Thus, the positive ion yield is higher than the negative ions yield.

Investigation of ion yields in MALDI, comparison of positive to negative ion yields, can thus be very important since it provides us with a better understanding of the MALDI mechanisms. Numerous ionization mechanisms have been proposed for MALDI [15-21] but none of them compares the total yields of positive to negative ions nor provides any quantitative predictions.
We therefore decided to systematically measure and compare the yield of positive and negative ions for a given analyte. We report MALDI delayed extraction time-of-flight (TOF) data for samples that promote or suppress photoelectron emission as well as for analytes with different acid/base properties. Complementary experiments were carried out in our internal source MALDI Fourier-transform ion cyclotron resonance mass spectrometer (FT-ICR), where the MALDI source is close to the ICR trap and ions are flying freely toward the trap [22]. We compare the total positive and negative MALDI ion yields for two different peptides (bradykinin, fibrinopeptide A) in combination with six matrices (CHCA, DHB, 4-NA, ATT, ANP, 5-AQ). The selection of these particular compounds is based on their acid/base properties. DHB and CHCA are strongly acidic, ATT and 4-NA are neutral, and ANP and 5-AQ are basic. Fibrinopeptide A is very acidic (theoretical pI=3.92) and bradykinin is basic (theoretical pI=12). Ion yields were measured and compared for each combination of peptide/matrix on metallic and nonmetallic MALDI targets.

### 8.2 Experimental

#### Materials

Bradykinin and fibrinopeptide A (human) were purchased from Bachem AG (Switzerland), α-cyano-4-hydroxycinnamic acid (CHCA, purity 99%) from Sigma (Switzerland), 2,5-dihydroxybenzoic acid (DHB, purity 99%) and 2-amino-5-nitro-4-picoline (ANP, purity 98%) from Acros Organics (Switzerland), 6-aza-2-thiothymine (ATT, purity 99%), 4-nitroaniline (4-NA, purity 99%) and cesium iodide (CsI, purity 99%) from Fluka (Buchs, Switzerland), and 5-aminoquinoline (5-AQ, purity 97%) from Aldrich (Buchs, Switzerland). All compounds were used without further treatment or purification.

#### Sample preparation

CHCA was dissolved in a solvent containing 70% acetonitrile (ACN), 0.1% trifluoroacetic acid (TFA), and 30% water; DHB and CsI were dissolved in ethanol/water (1:1); ATT, 4-NA, 5-AQ, and ANP were dissolved in ACN/water (1:1). Bradykinin and fibrinopeptide were dissolved in pure water. MALDI samples were prepared using a standard “dried droplet” method at a matrix/analyte concentration ratio of about $10^{-5}$. Then, 2µl of the matrix/analyte
solution were deposited on a MALDI target. To either allow or suppress photoelectron emission the experiments were carried out on stainless steel or plastic (polyether etherketone, PEEK) target materials, respectively. In TOF experiments nonconductive surfaces were obtained by simply covering the stainless steel target with a piece of adhesive nonconductive tape (Scotch 810 Magic Tape, a commercial adhesive tape).

**Mass Spectrometry**

All MALDI-TOF mass spectra were acquired on an AXIMA CFR instrument (Kratos Analytical, Shimadzu Biotech, Manchester, UK) equipped with a drift tube of 1.2 m and a nitrogen laser (λ=337nm, 3ns pulse width). Spectra were collected in the reflectron mode with and without delayed extraction. When used, the extraction delay was 200 ns. The accelerating voltage was set to ± 20 kV depending on the polarity used. Detector bias was -2 kV. During acquisition the sample target was monitored by a monochrome video-image system (25 x magnification). Spectra were the sum of 200 profiles automatically acquired while rastering the sample spot.

Complementary experiments were performed on a Fourier-transform ion cyclotron resonance (FT-ICR) mass spectrometer that consists of a home-built vacuum system, a 4.7 T superconducting magnet (Bruker, Fallanden, Switzerland), and commercial control electronics and data acquisition (IonSpec Corp., Lake Forest/CA, USA). A home-built open cylindrical cell was used for trapping the ions. The instrument has an internal MALDI source with a target positioned at 2 cm from the ICR trap. For laser desorption/ionization, the 3rd harmonic of a Nd:YAG laser (Continuum, Minilite ML-10, USA) at 355 nm with a 5 ns pulse width was used. All experiments were performed both in negative and in positive modes. To determine the error bars of the data one standard deviation from the mean was calculated. The integration of spectra was performed using commercial software (Igor Pro V4.08, Wavemetrics, Lake Oswego/OR) In the case of fibrinopeptide with 5-AQ only very weak analyte signals were registered occasionally, which shows that this matrix is not suitable for the analysis of fibrinopeptide both on metal and non-metal substrates.
8.3 Results and Discussions

Detection efficiency

Although it is believed that there is some discrimination in detection efficiency for positive over negative ions in TOF instruments, no clear evidence of this fact has been reported so far. In order to confirm that, we first performed an experiment with (matrix-free) desorption ionization from CsI crystals. In this experiment the laser pulse breaks up the solid, which leads to equal number of positively and negatively charged particles (atomic and molecular ions, as well as ionic clusters). Figure 1 shows positive/negative mode TOF mass spectra of CsI ions and their integrals, which correspond to the total ion currents.

Figure 1. Laser desorption ionization TOF mass spectra of CsI crystals on scotch tape acquired in both positive (bottom) and negative (middle) modes. The integration of the spectra (top) corresponds the total ion yield. Asterisk indicates on factory impurities.
With the exception of polarity, both spectra were recorded under identical, typical experimental conditions. Impurities in the negative mode mass spectrum of CsI are from the scotch tape, which is used to prevent photodetachment emission. As can be seen from the figure 1(a) the integrated signal in positive mode is higher than in the negative mode, by a factor of about 4. The calculated ratio of the total ion currents obtained from five repeated measurements was found to be 3.5±1.2. A comparative study was performed on our home-built internal MALDI FT-ICR mass spectrometer because (i) no ion guides/optics are used to transmit ions; (ii) in ICR, the detection efficiency does not depend on the polarity of the ions. Figure 2 shows FT-ICR positive/negative mass spectra of CsI and the integrated signal, which shows equal response for both positive and negative modes.

Figure 2. Laser desorption FT-ICR mass spectra of CsI on PEEK target acquired in both positive (bottom) and negative (middle) modes. The integration of the spectra (top) corresponds to the total ion yield.
8.3 Results and Discussions

Repetition of measurements up to 5 times did not show any difference in the ion yield ratio. Thus it is obvious that there must be a detection discrimination in the TOF mass spectrometer. So far, we do not fully understand the reasons for such a discrimination. It may be caused by the detector voltage, which results in post-acceleration favoring detection of positive ions. On our instrument, the detector is operated at -2 kV, thus positive ions experience a total acceleration by 22 kV, while negative ions "feel" only 18 kV (using ±20 kV extraction). Also, the differences in system configurations (i.e. ion guides, lenses, etc.) may have been optimized for positive ions. We assume that this scaling factor is instrument specific and cannot be directly applied to other mass spectrometers. It is important to note that, in order to directly compare between experiments on the absolute scale one would have to take into account all of these factors. Thus, scaling by 3.5±1.2 when comparing positive and negative ion signal was applied to all TOF experiments reported here.

Ion yield measurements on stainless steel

Figure 3 shows mass spectra of bradykinin with DHB acquired in positive and negative modes. With the exception of polarity, both spectra were recorded under identical, typical experimental conditions. It can be seen that the positive analyte peak, which corresponds to a protonated bradykinin molecule has a greater relative intensity than that in negative mode. This is straightforward to understand from the gas-phase proton transfer model for MALDI [15], because the proton affinity of bradykinin is higher than that of DHB. However, the integration of the mass spectra gives roughly equal total signal for negative and positive ions because of the very intense negative matrix ion signals. We believe that this roughly equal ratio of positive over negative ions is simply a coincidence. The full set of experimental results on stainless steel is summarized in Table 1. Stronger ion signals in the negative mode are most probably due to interaction of matrix molecules with photoelectrons, emitted from the metallic target. The mechanism of this interaction has been thoroughly discussed in our previous work [13]. Briefly, it was shown that in MALDI a sample target covered with a thin layer of organic material is a source of photoelectrons and under some experimental conditions the number of these photoelectrons exceeds the number of negative and positive ions [9]. Since the most abundant species in MALDI are matrix neutrals, the most probable interaction of electrons will be the formation of matrix anions. Following their formation,
these matrix anions can further react with positive analyte ions in the plume, leading to suppression/neutralization of positive ion signal. Thus, negative ion signal becomes more pronounced.

Figure 3. Positive (bottom) and negative (middle) TOF mass spectra of bradykinin with DHB as a matrix on stainless steel target. The integration of the spectra (top) corresponds to the total ion current. With the exception of polarity both spectra were taken under identical experimental conditions.
Table 1. Summary of all repeat measurements for positive/negative analyte and total ion yield ratios on metallic and nonmetallic supports. 5 measurements were taken for each data point.

**Ion yield measurements on nonconductive surfaces**

Since in MALDI experiments the exposure of the sample target to the laser beam is often avoided, i.e. no photoelectrons are ejected, the experiments on scotch tape may better reflect typical experimental conditions. Figure 4 shows positive and negative mode mass spectra of bradykinin with DHB on scotch tape. As in the previous cases, both spectra were taken under typical identical experimental conditions. The integration of the mass spectra in both polarities shows higher response for analyte ion yields in positive mode. The weaker negative mode ion signal is probably due to the absence of photoelectrons that could later interact with matrix molecules and suppress positive signal.
Figure 4. Positive (bottom) and negative (middle) TOF mass spectra of bradykinin with DHB on scotch tape. The integration of the spectra (top) corresponds to the total ion current. With the exception of polarity both spectra were taken under identical experimental conditions.

Experimental results on scotch tape are also summarized in Table 1. In all cases it is clear that the total ion yield ratio is independent of the acid/base properties of molecules studied and that the total yield of the ions in positive and negative modes is about equal. This result is straightforward to understand. According to the charge balance law and taking into account that there is no outside charge involved, the charge in the MALDI plume should be also balanced. Figure 5 shows the trend of positive/negative analyte ion yield ratio versus matrix basicity. The equidistant spacing between the matrix basicities was chosen for direct visualization.
According to the gas-phase proton transfer model [15] the highest positive ion yield is expected when using basic analyte and acidic matrix. Thus, for bradykinin we observe the monotonous signal decrease with increasing of matrix basicity. The ANP data point falls off from the trend most probably due to low compatibility of ANP matrix with bradykinin. For fibrinopeptide, analyte positive/negative ion yield ratio is lower than for bradykinin for all data points, which was expected, because fibrinopeptide A is an acidic peptide. However, no clear trend was observed for fibrinopeptide as for bradykinin.

Figure 5. Trend of positive/negative analyte ion yield ratio versus matrix basicity on nonmetallic MALDI support. The equidistant spacing between the matrices was chosen for direct visualization.
8.4 Conclusions

Positive and negative ion yields were measured and compared for TOF and FT-ICR mass spectrometers. The results obtained on the TOF instrument suggest that there is a detection discrimination against negative ions. The scaling factor for our particular instrument has been determined to be $3.5 \pm 1.2$. The dependence of the ion yield on acid/base properties of the molecules was examined and it was found to play a minor or no role in the overall ion yield. Higher positive ion yield on the insulating sample targets was interpreted as a result of photoelectrons emitted upon laser irradiation.
8.5 References


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9 Summary and Outlook

In this chapter the main findings are evaluated and summarized. An outlook and some future potentials applications are discussed. The problems of the current setup and possible solutions will also be addressed.
9.1 Summary

This work presents a new concept for structural determination of biological molecules in the gas phase. Methods based on fluorescence detection have been successfully applied in structural studies of biomolecules in solution, and they would be a viable means for obtaining additional information about gas-phase ions. A laser-induced fluorescence detection system for ions trapped in an FT ICR mass spectrometer was developed. The system was thoroughly characterized with a test compound of rhodamine 6G. From ion tomography experiments an ion cloud size was estimated to be about 1 mm in diameter. By means of photobleaching it is now possible to align the laser beam for fluorescence excitation. The trapping potentials, laser power and other experimental parameters were also optimized. The dependence of three different gases, helium, argon, and nitrogen, on the fluorescence signal intensity of rhodamine 6G cations was investigated. It was found that the use of a helium results in the highest fluorescence yield, while no fluorescence was detected when using argon under the same conditions.

Since the behavior of fluorophores in the gas phase is largely unknown it was important to identify a suitable FRET pair. Chapter 4 presents our findings for different dye molecules trapped in ionized form in an FTICR mass spectrometer. We found that some dyes that fluoresce intensely in solution give very little fluorescence in the gas phase or none at all. Also, considerable shifts in absorption and emission wavelengths when going from solution to gas phase were found. A suitable FRET pair was identified, rhodamine 6G covalently bound with sulforhodamine B through a rigid linker. The occurrence of FRET in the gas phase was unambiguously demonstrated for our system.

In the Chapters 6-9 some mechanistic aspects of MALDI were explored. It was found that an addition of CuCl$_2$ significantly enhance the analyte ion signal in positive ion mode. This effect was attributed to ability of CuCl$_2$ to scavenge free electrons, generated in MALDI. The laser energy dependence of photoelectron emission from MALDI samples deposited on stainless steel, gold, and nickel was also investigated. The results obtained supported a one-photon model for photoemission. The substrate material was shown to play an important role in the MALDI process. By choosing different substrates, we were able to manipulate the photoelectron kinetic energy, and, by virtue of the energy-dependent capture cross-section of
the matrix, decrease the number of negative matrix ions, which led to an increase in analyte signal in positive ion mode. The ratios of positive to negative ion yields for MALDI TOF mass spectrometry were measured. It was found that in time-of-flight instruments the detection efficiency for positive ions is higher than for negative ions by a factor of 3.5±1.2. Likewise, the total positive/negative ion yield is independent of acid/base properties of the analyte molecules as well as higher positive ion yield was observed when using nonmetallic sample supports.

9.2 Outlook

Technical problems

It has been shown that in MALDI all analyte molecules fly with the same velocity independent of their mass [1,2]. Therefore the kinetic energy of molecules is proportional to their mass. Since we have an internal MALDI source, where the target is placed only 2 cm away from the cell, we are immediately faced with the trapping efficiency problem of high kinetic energy ions. Many attempts such as static cooling of the ions [3], dynamic trapping [4], collisional cooling with quadrupolar axialization [5], multiply charged ions production [6,7], were made to overcome this problem but with limited success. All these methods were found to be efficient only in the range up to 8 kDa. Also, this problem cannot be solved by simply increasing the trapping potentials of the ICR cell trapping electrodes, because there is the so-called “critical” mass limit at which the magnetron and reduced cyclotron frequencies are equal so the ion cyclotron motion is no longer stable [8]. The approximate practical m/z limit for our ICR cell at which we are still able to trap ions was found to be around 2-3 kDa. Thus, an ESI source is absolutely necessary for doing combined fluorescence / ICR measurements on larger biomolecules, both because ESI is very soft, and because of the m/z limit.
Observation of conformational change between "vase" and "kite" forms of a cavitand

The next step will be the investigation of the "vase-kite" switching mechanism (Figure 1) at the level of single molecules [9-13]. This experiment is expected to yield information whether conformational changes in the gas phase can be induced and directly observed with FRET. For this purpose we are planning to use a donor - acceptor pair of fluorophores attached to the ends of the cavitand, an open-ended molecular container that surrounds guests to a large extent. These molecules should be able to capture (by complexation) a single molecule in the "vase" form and hold it during ion formation, while releasing it upon switching to the non-bonding "kite" conformation. These switching mechanisms can be detected by FRET. These molecules were found by variable temperature (VT) $^1$H-NMR spectroscopy to exist in an open "kite" conformation at low temperatures (<213K) but to adopt a "vase" conformation, capable of guest inclusion, at elevated temperatures (>318K). Recently, it has been also discovered that protonation with common acids promotes, at room temperature, the reversible conformational change and this change can be followed not only by $^1$H-NMR spectroscopy but also by optical spectroscopy. We propose to manipulate these conformations by changing temperature in the ICR cell region. We will also investigate the ability of conformational changes by adding different cations to the cavity of the cavitand.

Figure 1. Illustration of the "vase-kite" transformation, monitored by fluorescence resonance energy transfer (adapted from [12]).

Molecular Beacons

Another system we propose to study is molecular beacons (MBs) that can report the presence of specific nucleic acids in living cells. MBs are single strand oligonucleotide probes with a hairpin structure [14,15]. Two different fluorophores F$_1$ (e.g. rhodamine 6G) and F$_2$ (e.g. sulforhodamine B) will be linked to the ends of the strand (Figure 2). Five to seven bases
at both ends of beacons are complementary to each other, forming the stem, which keeps the
two fluorophores in close proximity to each other. When the MB is in a stem-closed form and
excited at the absorption band of $F_1$, the fluorescence of $F_1$ is quenched by $F_2$, while that of $F_2$
is observed. When the probe encounters the target DNA molecule, it forms a hybrid and
forces the stem apart. Thus, the molecular beacon undergoes a conformational change that
moves the fluorophores apart, restoring the fluorescence of the fluorophore $F_1$.

Methodologically, there are two issues we have to address for this experiment: (i) the
capability to work in negative mode because of the propensity of DNA to appear as negative
ions, and (ii) the capability to add a neutral reaction partner to the MB in gaseous form.

\[ \text{Target} \]

**Figure 2.** Schematic of the operation of molecular beacons. On their own, the excitation
of $F_1$ is transferred to $F_2$ and fluorescence from $F_2$ is observed. Upon hybridization with
a complementary strand, the fluorescence of $F_1$ is no longer quenched and its
fluorescence is observed.

Solvent is generally believed to play a dominant role in the formation and stabilization of
specific interactions between biomolecules. A key question that remains unanswered is
whether these interactions, which lead to specificity in solution, must be preserved during the
ESI process, or whether removal of solvent does not destroy the noncovalent interactions
necessary for folding (proteins) and duplex formation (DNA).

**ESI-MS, trapping, and fluorescence detection of Green Fluorescence Protein**

The next candidate compound will be Green fluorescent protein (GFP). GFP is one of the
most widely studied and exploited proteins in biochemistry and cell biology [16-18]. GFP is a
rather small protein with a molecular weight of roughly 27 kDa (Figure 3). Its fluorescence
can be used as an indicator for the formation of the 11 β-sheet barrel-like structure. GFP is
fairly resistant to denaturation, but looses its fluorescent properties when unfolded. GFP can
thus serve as a very direct probe for the existence of native or near native protein conformation of gas-phase ions. Since no FRET is needed to detect the conformational change we will try to establish a correlation between conformation of the GFP and its charge state.

Figure 3. Ribbon representation of Green fluorescence protein (GFP) in its native conformation. The fluorophore of GFP is located around the inner "waist" of GFP's β-barrel structure, and is destroyed upon denaturation.
9.3 References


Appendix

List of Abbreviations
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>BIRD</td>
<td>Blackbody Infrared Radiative Dissociation</td>
</tr>
<tr>
<td>CID</td>
<td>Collision Induced Dissociation</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>DE</td>
<td>Delay Extraction</td>
</tr>
<tr>
<td>ECD</td>
<td>Electron Capture Dissociation</td>
</tr>
<tr>
<td>EIF</td>
<td>Electron Induced Fluorescence</td>
</tr>
<tr>
<td>EM</td>
<td>Electron Multiplier</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray Ionization</td>
</tr>
<tr>
<td>FEL</td>
<td>Free Electron Laser</td>
</tr>
<tr>
<td>FRET</td>
<td>Fluorescence Resonance Energy Transfer</td>
</tr>
<tr>
<td>FT ICR</td>
<td>Fourier Transform Ion Cyclotron Resonance</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>IC</td>
<td>Internal Conversion</td>
</tr>
<tr>
<td>IMS</td>
<td>Ion Mobility Spectrometry</td>
</tr>
<tr>
<td>IR</td>
<td>Infrared</td>
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<tr>
<td>ISC</td>
<td>Intersystem Crossing</td>
</tr>
<tr>
<td>LIF</td>
<td>Laser Induced Fluorescence</td>
</tr>
<tr>
<td>MALDI</td>
<td>Matrix-Assisted Laser Desorption Ionization</td>
</tr>
<tr>
<td>MCP</td>
<td>Multi Channel Plates</td>
</tr>
<tr>
<td>m/z</td>
<td>mass-to-charge ratio</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>Nd: YAG</td>
<td>Neodym: Yttrium-Aluminium-Granat</td>
</tr>
<tr>
<td>PMT</td>
<td>Photomultiplier Tube</td>
</tr>
<tr>
<td>QE</td>
<td>Quadrupolar Excitation/axialization</td>
</tr>
<tr>
<td>rf</td>
<td>radiofrequency</td>
</tr>
<tr>
<td>S/N</td>
<td>Signal-to-Noise ratio</td>
</tr>
<tr>
<td>SPCM</td>
<td>Single Photon Counting Module</td>
</tr>
<tr>
<td>SWIFT</td>
<td>Stored Waveform Inverse Fourier Transform</td>
</tr>
<tr>
<td>TOF</td>
<td>Time-of-Flight</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
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