SPECTROSCOPY OF GAS-PHASE (BIO)-MOLECULAR IONS

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Abstract

The introduction of soft ionization techniques such as electrospray (ESI) and matrix-assisted laser desorption/ionization (MALDI) enabled the generation of intact gas-phase ions for a wide range of nonvolatile compounds such as nucleotides, polysaccharides, synthetic polymers, proteins, and protein complexes. Using modern mass spectrometers, ions of interest can be selectively isolated and mass analyzed with very high accuracy. In recent years, the attention has shifted from merely observing intact gas-phase ions to studying their structure and conformation. The structure of a biological molecule in vacuum provides an absolute reference point to account for matrix effects imposed by any environment. The key question to answer is to what extent the properties of gas-phase biomolecules are different from those in their native environment and, in this respect, whether unhydrated ions can yield information on the molecular structure in solution.

The present thesis is devoted to a combination of ESI, Fourier-transform ion cyclotron resonance mass spectrometry (FTICR-MS), and laser spectroscopy to probe the properties of isolated molecular ions. A unique home-built optical setup was constructed that allows laser-induced fluorescence/dissociation experiments on trapped ions in the gas phase. Fluorescence spectra of trapped ions were used as a reference to identify the presence of unsolvated gas-phase ions inside the ESI plume. This allows studies of isolated biomolecules without resorting to sophisticated and expensive instrumentation and, in fact, sets up a novel method to probe the properties of gas-phase molecular ions.

Laser-induced fluorescence/dissociation spectroscopy was applied to address structural and optical properties of Rhodamine 19 (R19) in the gas phase. A number of molecular structures have been observed that do not occur in solution. The differences between the optical properties of R19 established in solution and in the gas phase were discussed in view of its use as a fluorescent probe to address structural properties of unsolvated biological molecules. Based on our experimental data, it was proposed that an intramolecular relaxation mechanism can operate in the excited electronic state of rhodamine cations.
Finally, photoabsorption of the Green Fluorescent Protein chromophore anion (HBDI\textsuperscript{-}) in vacuum was studied by laser-induced dissociation action spectroscopy. Our data raise doubt about the interpretation of earlier action spectroscopy experiments and point to a considerable bathochromic shift in the absorption of HBDI\textsuperscript{-} induced by the protein interior.
Zusammenfassung


Laser-induzierte Fluoreszenz-/Dissoziationspektroskopie wurde verwendet, um strukturelle und optische Eigenschaften von Rhodamin 19 (R19) in der Gasphase zu untersuchen. Zahlreiche
Chapter 1

Introduction
1.1 Biological molecules in the gas phase

For the last two decades mass spectrometry (MS) has become a very powerful tool to characterize biological samples on the molecular level (1). This progress is mainly due to the introduction of electrospray ionization (ESI) (2) and matrix-assisted laser desorption/ionization (MALDI) (3) for MS analysis in the late 1980s. In ESI/MALDI-MS, large molecular species such as proteins or polysaccharides are transferred into the vacuum of a mass spectrometer as isolated ions. ESI and MALDI are referred to as soft ionization methods, i.e. covalent bonds are not disrupted during the transfer and therefore the intact ions are accessible in these protocols. MS detection is advantageous for several reasons. First, the ions mass measured in MS are one of the most direct characteristics of a species, and therefore one does not have to resort to laborious interpretation of experimental spectra. Most powerful mass analyzers such as Fourier transform ion cyclotron resonance (FTICR) (4) or Orbitrap® MS (5) allow very accurate mass determination at the sub ppm level. In many cases, analyte elemental composition can be directly determined from a highly accurate mass spectrum. Moreover, amino acid sequences can be determined using tandem techniques such as MS<sup>n</sup> detection of daughter ions produced by collision-induced dissociation (CID) (6) or electron-capture dissociation (ECD) (7). Furthermore, noncovalent interactions can even be preserved in ESI-MS, which opens great prospects for mass spectrometry as a tool to characterize the stoichiometry of supramolecular assemblies (8,9).

Recently, the attention has shifted from merely observing/identifying unsolvated molecules by MS to studying their intrinsic properties (10-21). Numerous interactions of a molecule with its native environment, such as solvent matrix or a crystal lattice, are eliminated in the gas phase, which allows deeper insight into the underlying mechanisms of molecular processes. For instance, the tertiary structure of a protein adopted in the gas phase can be significantly different from the native conformation in solution, where both intramolecular forces and external influences from the solvent are essential. The knowledge of a protein structure and dynamics in the absence of solvent would provide a key to assess the contribution of external and internal influences to the net molecular behavior in condensed phase (17). The secondary structure of polymers, such as polypeptides or oligonucleotides, can dramatically change from a random coil in solution to an elongated helix in the gas phase (22,23) or the other way round (24). Even small molecules, such as single amino acids, possess qualitatively different structural (25) and
photophysical (26) properties in solution and in the gas phase. Besides probing fully unsolvated species, modern experimental methods allow studies of partially solvated biological molecules, bridging the gap between the gas and solution phase (19,26,27). Importantly, the experimental data obtained in the gas phase can be complemented with theoretical calculations, the accuracy of which largely depends on the absence of chemical environment. A combination of experimental and theoretical approaches can greatly facilitate the structure assignment process (28).

1.2 Overview of the current research

Probing intrinsic properties of biological molecules in the gas phase is a challenging task, which is mainly due to the limited analyte concentration that can be experimentally accessed. The use of analytical methods common for structural analysis in condensed phase, such as nuclear magnetic resonance (NMR), X-ray crystallography, fluorescence, infrared (IR), or Raman spectroscopy, is usually not applicable in the gas phase or associated with great instrumental difficulties (16). The experimental challenge is getting much more severe as the size of the molecule under investigation increases. For these reasons, methods are often preferred that provide higher sensitivity of signal detection although they may yield relatively little or indirect information on the structure of isolated species (12,19). This section gives an introduction to the experimental strategies currently employed in order to study intrinsic properties of biological molecules. This “minireview” will focus on techniques that have shown prospects to provide information on the properties of large isolated biomolecular systems, such as proteins and protein complexes, which are of highest interest but also most challenging to address experimentally.

1.2.1 Mass Spectrometry

Mass Spectrometry in combination with ESI or MALDI is by far the best tool available to experimentally observe large biomolecular ions in the absence of solvent. Intact ions in the megadalton molecular range can be trapped and detected by MS (29), giving direct access to
unsolvated proteins, polysaccharides or supramolecular assemblies. MS detection by itself provides information on the molecular weight but says nothing or very little about the structure of analytes, and therefore needs to be combined with other techniques for structure-sensitive analysis. The number of ions that can be accessed in MS experiment is limited by the capacity of trapping devices to $\approx 10^6$-$10^7$ ions. This is the reason why instead of direct optical spectroscopy, e.g. absorption or fluorescence measurements, scientists often resort to the MS observation of fragments induced by the dissociation of parent ions. This type of analysis is referred to as consequence or action dissociation spectroscopy. The source of dissociation can be collisions with neutrals, electrons, absorption of light etc. Fragment ions or fragmentation yield, as a function of the laser excitation frequency or collision energy, can be characteristic of the parent ion structure. Besides dissociation, the products of gas-phase chemical reactions, e.g. in hydrogen/deuterium exchange, can also be a source of structural information (30).

1.2.1.1 Infrared multiple-photon dissociation (IRMPD) spectroscopy

The emergence of high-intensity tunable lasers, such as free electron lasers (FELs), has now made it possible to routinely record IR spectra of gas-phase ions. In IRMPD many photons are absorbed resonantly and their energy is redistributed along the vibrational modes of the ion, leading to vibrational heating and ultimately to unimolecular dissociation (Figure 1.1). The IR spectrum of a trapped ion is obtained as a dependence of the photofragmentation yield on the excitation frequency.

IR spectroscopy is well-suited to answer some specific questions about biomolecular ions for which other gas-phase techniques are arguably less direct probes of the structure (19). Thus, IR spectroscopy has been very successful in identifying charge-solvated versus zwitterionic structures of amino acid and small peptide cations in the gas phase by virtue of the difference in stretching frequency between the carboxylate (COO-) and carboxylic acid (C=O) groups (31-34). IRMPD-MS has also been shown to be a powerful tool to determine the site of proton attachment in gas-phase peptides (35,36) and structure of fragment ions (37,38). Recently the focus has shifted from bare biomolecular ions to partially solvated species with a known number of solvent
molecules, which serves to bridge the gap between the observations in the gas-phase and in the native biological environment. A complete series of tryptophan–water and tryptophan–methanol clusters with up to nine solvent molecules was recently studied by IR spectroscopy (39). The results suggest that 5 – 6 water molecules are required for the zwitterionic structure to become competitive in energy with the non-zwitterionic form, which is dominant in the gas phase. IR spectroscopy of gas-phase metal cation-water clusters yields information on the primary and secondary shells around the cations (40).

Figure 1.1 Mechanism of unimolecular dissociation in IRMPD for polyatomic molecules. The energy is pumped into a specific vibrational mode and is then quickly redistributed over the bath of background states by virtue of internal vibrational redistribution (IVR). The molecule can thus sequentially absorb many photons on the same transition, while the energy is stored in the bath. Once the internal energy reaches the dissociation threshold (red mark), the molecule can undergo unimolecular dissociation. Adapted from (19).

The application of IRMPD spectroscopy is currently limited to amino acids, small peptides and clusters. Larger molecular systems, e.g. proteins, require higher energies to be deposited for bond cleavage. As a result, low fragmentation yield and significant peak broadening in IRMPD spectrum due to vibrational heating are observed, which makes data interpretation ambiguous (18,41). Also, in IRMPD spectroscopy the conformers are not preselected and probed all at the same time. This is particularly critical for large biomolecules having a variety of stable conformers. Finally, it can be extremely difficult to induce dissociation of large and stable proteins by IRMPD (42).
1.2.1.2 Ultraviolet photodissociation (UVPD) spectroscopy

While vibrational spectroscopy is sensitive to the structure/conformation of ions, UVPD spectroscopy characterizes electronic transitions. Unlike IRMPD, which requires absorption of multiple photons to induce dissociation of parent species, the higher photon energy of ultraviolet light may in some cases enable single-photon fragmentation processes (43). This is advantageous for several reasons. First, due to the higher energy of UV irradiation, more abundant fragmentation is generated in UVPD than in IRMPD, which enables access to larger biological molecules. This is the reason why UVPD has found broad application to identify primary sequence of macro ions (43). Another advantage of a single-photon fragmentation is that it does not introduce substantial heating of analyte species and therefore reflects the analyte structure at ambient temperature. UVPD can therefore allow spectroscopic interrogation of cold gas-phase ions. Trapped gas-phase ions can be cooled down to temperatures of 10-15 K, which enables recording electronic spectra with extremely high resolution (20). The UV action spectrum can then be used to identify the number of stable conformers, since these often have slightly different UV spectra that can be resolved if the molecules are internally cold. Protonated aromatic amino acids in the gas-phase have recently been investigated by UV spectroscopy (20,26). It was found that while phenylalanine and tyrosine ions revealed well-resolved electronic transitions, UV spectrum of protonated tryptophan was very broad, due to the short lifetime of the excited state (26). The reason for the fast decay from the excited electronic state of TrpH⁺ compared to PheH⁺ and TyrH⁺ was identified to be due to a low crossing barrier from the π–π* state to a π–σ* state that is dissociative in the NH coordinate (44). It has been shown that the addition of just one water molecule to TrpH⁺ resulted in considerable narrowing of the main band in the UV spectrum, while upon the complexation with a second water molecule, spectral features became as narrow as those of PheH⁺ and TyrH⁺, implying a change in life time of up to two orders of magnitude (Figure 1.2). This change resulted from the shift between the π–π* and π–σ* states upon solvation (26).

Electron spectroscopy has also been applied to larger gas-phase ions. UVPD spectra of angiotensin II anions were recorded to reveal a clear dependence of the spectral fingerprint on the ionization state of the tyrosyl group (45). The spectral shifts observed were analogous to...
those observed in solution. Besides that, the formation of tyrosylate radicals upon electron photodetachment was demonstrated (45).

Independent UV-Vis photodissociation spectroscopy experiments performed on the anions of a green fluorescent protein (GFP) model chromophore stored in a quadrupole ion trap and in electrostatic ion storage ring (ELISA) (46) allowed the measurements of their UV-Vis electronic spectra in the gas phase (15,47). It was found that the light absorption of the model chromophore in vacuum is very similar to that of the GFP protein in its native environment. This result led to the conclusion that the GFP protein provides an almost vacuum-like surrounding for the chromophore, with only a minor influence on the $S_0 \rightarrow S_1$ transition in the ground state. Besides that, photoelectron detachment was identified to be the dominant deactivation mechanism of the chromophore anion in the optical range below 400 nm in these studies (15).

![Figure 1.2: Electronic photofragment excitation spectra of TrpH$^+$ with 0, 1 and 2 attached water molecules. Adapted from (26).](image-url)
1.2.1.3 UV-IR double resonance spectroscopy

A combination of IR and UV spectroscopy is a nice example of the synergy of analytical methods to address structural properties of biological molecules in the gas phase (20,48,49). While vibrational spectroscopy provides information on the structure of analytes, UV spectroscopy is sensitive to the conformation and allows rapid unimolecular dissociation without substantial heat transfer. Double-resonance UV-IR experiments on cold ions trapped by MS are carried out at EPFL by Rizzo and coworkers (20).

Figure 1.3. Spectroscopic schemes applied to cold biomolecular ions for measuring (a) electronic spectra of cold biomolecular ions via photofragment detection and (b) conformation-specific infrared spectra by detecting the depletion of the UV-induced photofragment signal. Adapted from (20).

In their experiments mass-selected ions produced by ESI are interrogated spectroscopically inside a 22-pole ion trap, which is cooled down to 6 K. The ions are equilibrated to this low temperature due to collisions with helium gas pulsed into the chamber. In addition to the classical UVPD scheme, an infrared pulse is introduced before the UV pulse in order to deplete the ground state population each time the infrared frequency coincides with a vibrational transition of the parent ion. The IR transition is then detected as a dip in the UV photofragmentation signal as the IR frequency is scanned through a vibrational band (Figure 1.3)
(20). Using this setup, vibrational spectra for two conformers of gas-phase PheH⁺ and four conformers of TyrH⁺ were recorded and interpreted (50). UV-IR spectroscopy has also been performed on gas-phase peptide ions containing aromatic amino acids (22). The high-resolution conformer-specific spectra obtained for Ac-Phe-Ala₆-Lys-H⁺ peptides allowed the authors to distinguish two types of conformers, while the structure assignment was not successful, at least at the current level of theory (22).

It is the great instrumentation challenges that limit the practical use of UV-IR-MS. Also, it is currently an open question whether it can provide useful, interpretable information on the structural properties of larger biological molecules (20).

### 1.2.1.4 Electron Capture Dissociation (ECD) Spectroscopy

In ECD, precursor ions are irradiated with low-energy electrons (< 0.2 eV) (7). The electron capture cross-section is proportional to the square of the ion charge (51), which makes ECD well suited for the analysis of multiply-charged ions produced by ESI-MS. In ECD c and z fragments are produced (Figure 1.4) due to the cleavage of the backbone bond, which is unique for tandem MS analyses and is extensively exploited for identification purposes (7).

ECD is a very fast process (<10⁻¹² s), so that the relatively small amount of energy released upon the electron capture (~6 eV) does not have time to be redistributed across the vibrational modes of a molecule and is therefore sufficient to induce bond cleavage. Upon randomization of the remaining energy along the numerous degrees of freedom of a molecule, other bonds are not significantly energized (52). Therefore, even if the c and z ions to be produced by ECD are linked by a non-covalent bond, they will not be cleaved. Fragmentation patterns induced by ECD are therefore indicative of noncovalent intramolecular interactions. Typically, a precursor ion is thermally pre-activated prior to ECD analysis, and the ECD fragmentation pattern is then monitored as a function of ion temperature. The higher the temperature the more noncovalent bonds get disrupted, resulting in a denser MS/MS spectrum. Importantly, the kinetics of protein
folding/unfolding can be explored in this fashion.

ECD-MS has been applied to explore folding of multiply charged proteins in the gas phase, such as cytochrome c and ubiquitin, to reveal a multiplicity of intermediates (13,18,52). A variety of stable gas-phase conformers were established for ubiquitin ions, in contrast to the singular native form in solution (18). Also, rearrangement of noncovalent bonds was observed upon protein refolding in the gas phase (13). Altogether, ECD spectroscopy shows that the assumption that the electrospray process and vacuum transport do not alter noncovalent bonding is not always true (13).

![Diagram](image-url)

**Figure 1.4.** The accepted nomenclature for fragment ions in an MS/MS spectrum. Fragments can be detected if they carry at least one charge. If this charge is retained on the N terminal fragment, the ion is classified as either a, b or c. If the charge is retained on the C terminal, the ion type is either x, y or z. A subscript indicates the number of residues in the fragment. Adapted from (7).

### 1.2.1.5 Hydrogen/Deuterium (H/D) Exchange

H/D exchange has been extensively used to deduce structural information in solution, in particular to probe protein folding (53,54). Hydrogens buried in the core of a folded protein exchange much less readily than do those on the surface, which can be employed for structural characterization. It was shown that H/D exchange could also be applied to examine proteins in the gas phase (17,21,30,55,56). Typically in H/D exchange experiments, gas-phase proteins are trapped in an FTICR mass spectrometer for extended time intervals (up to 1 hour) while being exposed to a small partial pressure of D₂O vapors. The number of labile hydrogens is then obtained from the high-resolution FTICR mass spectrum. Several H/D exchange studies of
protonated cytochrome c ions have been reported (17,21,55). A systematic decrease in the exchange level was observed as the net charge of cytochrome c increased. This was a surprising result since higher charge states of a protein in the gas phase are generally attributed to less folded conformation and should therefore exhibit more extensive H/D exchange. The contrary experimental trend was explained by the existence of a self-solvation shell around each charge occurring in the gas phase that protects sites that would otherwise undergo exchange (10).

Even for simpler systems, such as small peptides and amino acids, the interpretation of H/D exchange experiments in the gas phase is not straightforward (57,58). For example, Gly$_2$H$^+$ exchanges all labile hydrogens with D$_2$O, whereas GlyH$^+$ and Gly$_n$H$^+$ ($n > 3$) do not undergo facile exchange. On the other hand, Gly$_n$H$^+$ ($n = 1–5$) exchanges all labile hydrogens with ND$_3$ (57). A number of exchange mechanisms were considered. For example, in the relay mechanism proposed for exchange with D$_2$O (57) two basic sites with similar gas-phase basicities (GB) need to be in close proximity, the more basic site being protonated. H$^+$ and D$^+$ are shuttled between the sites via the water molecule to affect the exchange. Mechanisms involving simple H-bond formation or salt-bridge intermediates are unfavorable due to the low gas-phase basicity of D$_2$O. H/D exchange in the gas phase, although of fundamental mechanistic interest, is nowadays rarely applied as a tool to probe the properties of unsolvated biomolecules, because it is being outperformed by other methods offering more direct structural information, such as ECD or ion-mobility (IMS) spectroscopy (12,18).

1.2.1.6 Laser-Induced Fluorescence (LIF) Spectroscopy

While consequence dissociation spectroscopy methods discussed above rely on the detection of daughter ions produced upon exposure to light (or electron beam in ECD) by MS, which can be extremely sensitive, the observation of fluorescence from trapped ions is technically considerably more challenging (15,59-65). Nevertheless, the use of direct fluorescence spectroscopy to probe the properties of gas-phase ions is of increasing interest since it is able to provide very detailed structural information on the molecular level. In particular, Förster resonance energy transfer (FRET) (66,67) and photoinduced electron transfer (PET) (68,69) have shown great power to address protein folding dynamics in solution.
Despite a number of reports on the proof-of-principle detection of fluorescence from trapped gas-phase ions (60-65), structure elucidation by LIF has been only moderately successful so far (15,16,70-72). This is largely because of the limitation on the maximal number of ions that can be trapped in MS (≈ 10^6-10^7), compromising the sensitivity of the method. Moreover, the capacity of trapping devices substantially decreases for heavier ions, which introduces additional technical challenges to probe the properties of large biomolecular ions (> 10 kDa) by LIF spectroscopy.

In a series of experiments by Iavarone et al., fluorescence spectroscopy was used to monitor conformational changes of a gas-phase 20-residue miniprotein, Trp-cage, as a function of internal temperature and net charge (70,73). The native structure of this protein includes a Trp residue “caged” by three Pro residues. The local conformational dynamics of the protein was probed through interactions of a covalently attached fluorescent reporter (BODIPY TMR) with the Trp. Upon heating up to 445 K, the Trp is released from its cage and becomes more exposed to intramolecular collisions with the dye moiety. These collisions quench the fluorescence of the dye by PET, so that conformational changes can be directly correlated with the fluorescence intensity from the dye. It was shown that the compact structure of doubly charged Trp-cage ions in the gas phase is more resistant to heat-induced unfolding than that of triply charged ions, probably due to a weaker electrostatic repulsive force. This observation was supported by theoretical calculations (Figure 1.5) (70).

The same methodology was applied to address conformational dynamics of short polypeptides in the gas phase (16,72). Again, fluorescence quenching and shorter lifetimes of a fluorescent reporter (BODIPY TMR) at elevated temperatures were attributed to efficient collisional quenching by Trp located at the other end of the polymer, as a result of increased peptide flexibility (16). The change in fluorescence of the BODIPY TMR moiety as a function of temperature in the gas phase was two orders of magnitude greater than that in solution. Strong electrostatic fields in the absence of solvent environment were shown to significantly affect the PET transfer from BODIPY TMR to Trp as well as to induce considerable shifts in fluorescence spectra compared to solution (16).

Recent observations of FRET in the gas phase for model systems (71,74) give us some hope that,
in addition to PET, this powerful method will soon be applied to measure intramolecular distances (75) in large unsolvated biomolecular ions.

![Figure 1.5: Normalized fluorescence intensity per ion vs temperature for 2+ and 3+ ions of Trp-cage-BoTMR. Adapted from (70).](image.png)

1.2.2 Ion Mobility Spectroscopy (IMS)

In IMS, a weak electric field (E) induces a drift of gas-phase ions through a buffer gas resulting in a linear velocity $v_D = K \cdot E$, which is proportional to the mobility constant (K) of the ions in the buffer gas. Different components of an ion packet can thus be spatially separated based on the difference in their mobility. The value of K is derived from the time ($t_D$) required for ions to drift through a specified distance (12). The principle of IMS separation is a way similar to that of gas chromatography (GC), where the separation is further promoted by interaction of gaseous molecules with stationary phase. This is the reason why IMS is sometimes referred to as gas-phase ion chromatography (76). Typically, MS is employed as an ion detector in IMS analysis, so, besides ion mobility, at the same time one obtains complementary information on the molecular weight and charge state of a component (77).

IMS has been established as a very powerful tool to estimate the shape of gas-phase ions. Methods have been developed to calculate cross-sections based on the experimentally measured
ion mobility, mass and charge state. Calculated cross-sections are compared to those of trial geometries in order to estimate a conformation type. There is now a substantial body of evidence for polypeptide sequences that form compact globular, helical or helical-coil conformations. For example, it has been demonstrated by IMS that Ac-Ala-n-Lys peptides tend to adopt helical structure in the gas phase, while being a random coil in solution. Recently, independent IMS-MS analyses revealed that the overall geometry of large noncovalent molecular complexes could be preserved when transferred from solution into the gas phase by ESI. Robinson and coworkers demonstrated that the 11-subunit trp-RNA binding protein complex maintains its quaternary ring-like structure in the gas phase. In a similar experiment, Loo and coworkers showed that the noncovalent 28-subunit 20S proteasome complex preserves its native stoichiometry in IMS-MS, and its dimensions are similar to those measured by crystallographic methods. These findings suggest that many elements of large protein complexes stay intact upon desolvation by ESI, and IMS and MS analyses can therefore reveal structural details of the solution complexes.

1.2.3 Optical Spectroscopy in Molecular Beams

Cold molecular beams of analyte produced by heat-induced evaporation or laser desorption can be formed by supersonic expansion into high vacuum. Jet-cooled gas-phase molecules and their clusters can be probed by optical spectroscopy, e.g., by resonance-enhanced multiphoton ionization (REMPI), LIF, or double-resonance methods, to yield high-resolution spectra. These spectra can provide very detailed structural information on small biological molecules in the gas phase, such as amino acids or nucleobases and their hydrated clusters.

It is the low efficiency of neutral vapor generation that prevents gas-phase molecular beam optical spectroscopy from being applied to larger biological molecules such as proteins, protein complexes, polysaccharides, lipids etc. These can be delivered into the gas phase intact only by soft ionization methods, such as ESI, MALDI and related techniques. This is the major reason why the attention of the spectroscopy community is shifting towards trapped biomolecular ions. Another important factor is the extremely rapid progress in computational
power. For small molecules, theoretical predictions are sometimes trusted better than experimental data. The behavior of proteins, on the other hand, is essentially terra incognita for theory due to the system complexity, highlighting the importance of experimental examination.

### 1.3 Scope of this thesis

One of the principal targets in the Zenobi group is to look at the properties of biological molecules in the gas phase. The key question to answer is to what extent the properties of gas-phase proteins are different from those in their native environment and, in this respect, whether unhydrated protein ions can give any information on the protein structure in solution. A combination of electrospray ionization, Fourier-transform ion cyclotron resonance mass spectrometry (ESI-FTICR-MS), and laser spectroscopy was chosen as an experimental platform to probe the properties of isolated molecular ions. Both laser-induced fluorescence and fragmentation of gas-phase ions are used to extract structural information.

Chapter 2 describes the methods and instrumentation employed in this study: electrospray ionization (ESI), electrosonic spray ionization (ESSI), FTICR mass spectrometry, and fluorescence spectroscopy.

Chapter 3 describes a unique home-built optical setup that allows laser-induced fluorescence/dissociation experiments on trapped ions. The advantages of this setup for structural analysis as well as its performance characteristics are discussed.

Fluorescence spectra, being sensitive to the solvent environment, can be used as an alternative to MS in order to detect gas-phase ions. Chapter 4 describes the discovery that completely unsolvated ions are present in abundance inside an ESI plume already at ambient conditions. This was done by comparing fluorescence spectra obtained in different regions of the ESI plume with those from isolated ions trapped in FTICR. This discovery allows studies of isolated biomolecules without resorting to sophisticated and expensive instrumentation and, in fact, sets up a novel method to probe the properties of gas-phase molecular ions, along with those summarized above.
Fluorescence spectroscopy relies on labels as messengers of molecular dynamics. Understanding the behavior of fluorophores in the gas phase is therefore a key prerequisite for the correct structural assignments of unsolvated biological molecules by LIF. Chapters 5 and 6 describe the structural and optical properties of a widely used fluorophore, Rhodamine 19 (R19), in the gas phase and compare them with those in the condensed phase. A number of molecular structures have been observed that do not occur in solution. The differences between the optical properties of R19 established in solution and in the gas phase were discussed in view of its use as a fluorescent probe to address structural properties of unsolvated biological molecules. Based on the experimental data, it was proposed that intramolecular relaxation mechanism could operate in the excited electronic state of rhodamines.

Chapter 7 describes dissociation action spectroscopy experiments to probe the photoabsorption of the Green Fluorescent Protein (GFP) chromophore in vacuum. Our data raise doubt about the interpretation of action spectra made in earlier studies. While the absorption maximum of the chromophore anions has been suggested earlier to be nearly equal in the protein and in the gas phase, we predict a considerable bathochromic shift induced by the protein, which is in good agreement with recent theoretical studies and inference from solution-phase data.

Chapter 8 highlights the projects that are currently underway in the laboratory, such as FRET spectroscopy of polyproline helices, fluorescence spectroscopy of the GFP protein and its chromophore analogs in the gas phase.

1.4 References


This chapter presents the experimental methods used in this work: electrospray ionization/electrosonic spray ionization, Fourier-transform ion cyclotron resonance mass spectrometry, and fluorescence spectroscopy.
2.1 Ion sources

2.1.1 Internal matrix-assisted laser desorption/ionization (MALDI)

In earlier LIF spectroscopy experiments in the Zenobi group, gas-phase ions were produced by an internal MALDI source (1). In internal MALDI, ions are generated in high vacuum rather than in the ambient atmosphere, such as in atmospheric pressure ionization (API) mass spectrometers (2). Gas-phase ions formed in high vacuum can be directly detected by time-of-flight mass spectrometry (TOF-MS) without the need for ion transport (3). Alternatively, ions produced by internal MALDI can be trapped in an FTICR cell for complementary structural analysis by LIF spectroscopy (Figure 2.1) (1).

Figure 2.1: Schematic of our FTICR instrument with an open cylindrical cell. Adapted from (4).
The major limitation of internal MALDI-FTICR-MS is a poor trapping capacity of heavy ions provided by the instrument. It has been shown that analyte species desorbed in MALDI have the same initial velocity (close to the initial velocity of matrix) independent of their mass (5,6). The kinetic energy of ions produced is therefore proportional to their mass. Since the target in internal MALDI is located only $\approx 2$ cm away from the entrance of the cell (Figure 2.1), trapping of high molecular weight species becomes a great challenge. The most direct approach to address this problem is using elevated trapping potentials of the ICR cell in order to increase the trapping capacity. Unfortunately, one can only increase potentials up to a certain level, over which the ion cyclotron motion becomes unstable (7). Many other strategies have been attempted in order to increase the trapping efficiency, such as static ion cooling (8), dynamic trapping (9), collisional or sympathetic cooling (10-13) although with limited success. The approximate practical $m/z$ limit of our internal MALDI-FTICR-MS setup at which it is still possible to trap ions was found to be around 4-5 kDa (4). Since our ultimate goal is to explore large biological molecules such as proteins, a completely new setup for optical spectroscopy was designed. It is compatible with both the internal MALDI source discussed above and external ion sources, such as electrospray ionization (ESI) (14) and electrosonic spray ionization (ESSI) (15), which allow access to macromolecular ions. The detailed description of the optical setup will follow in Chapter 3. Although its performance was first tested with the internal MALDI ion source, all the important results reported in this thesis were obtained using ESI and ESSI sources. For this reason, we focus here on the important aspects of ESI and ESSI, while a detailed overview of MALDI mechanisms can be found elsewhere (16).

2.1.2 Electrospray Ionization (ESI)

In ESI, analyte solution is continuously pumped through a thin metal capillary. The capillary is held at a high voltage of ca. 3 - 5 kV, while its counter-electrode at a distance of several centimeters is grounded. It is the high electrostatic field at the meniscus of the liquid of $\approx 10^6$ V/m (17) that is responsible for the charge separation within the capillary, subsequent formation of a Taylor cone (18,19) and ultimate emergence of charged microdroplets (20) (Figure 2.2). ESI
is often assisted by an auxiliary gas to achieve better nebulization and desolvation, as well as to discriminate charged droplets against neutrals (14).

**Figure 2.2.** Processes in the atmospheric pressure region of an ESI ion source run in the positive ion mode. Penetration of the imposed electric field into the liquid leads to an enrichment near the meniscus of positive ions present in the solution. This causes a destabilization of the meniscus and formation of a cone and a jet charged by an excess of positive ions. The jet splits into droplets charged with an excess of positive ions. Evaporation of the droplets brings the charges closer together. The increasing Coulombic repulsion destablizes the droplets that emit jets of charged progeny droplets. Evaporation of progeny droplets leads to the emission of second-generation progeny droplets, and so on, until free gas-phase ions form at some point. Adapted from (17).

The droplets are positively charged due to an excess of positive electrolyte ions at the surface of the cone and the cone jet. Solvent evaporation leads to droplet shrinkage and an increase of the electric field normal to the surface of the droplets. At a given radius the increasing repulsion between the charges overcomes the surface tension at the droplet surface, causing Coulomb fission of the droplet. Further evaporation of the parent droplet leads to repeated fissions. The progeny droplets also evaporate and undergo fission (17). The resulting very small charged droplets ultimately lead to gas-phase ions. Two mechanisms were proposed to be responsible for the ultimate gas-phase ion formation (Figure 2.3). According to the “charged residue model” (CRM), the final gas-phase ions are produced by the complete evaporation of last solvent
molecules from the ion (21,22). In the competing “ion evaporation model” (IEM), gas-phase ions are generated directly from highly charged droplets when the electrostatic forces within the droplet are sufficient to expel the ion from the surface (23,24). Experimental support for both models has been found, suggesting that the operating ion production mode depends on the analyte, however no rigorous proof of these mechanisms has yet been obtained (17).

![Diagram of ion formation mechanisms](image)

**Figure 2.3.** A schematic representation of the possible pathways for ion formation from a charged liquid droplet. The upper and the lower parts of the diagram illustrate the ion formation mechanisms depicted in the CRM and the IEM model, respectively. The major difference between these two models is that the final ion in the latter model is produced by desorption, whereas the ion in the former model is produced by evaporation of solvent comprising the droplet.

In a recent study, Nguyen and Fenn found that the relative abundances of the desolvated ions in the mass spectrum were substantially higher when the nitrogen bath gas contained vapor of a polar solvent species than when no such solvent vapor was present, at least for amino acids and short peptides (25). The extent of the observed increases directly correlated with the number of charges on the solute ions. According to the CRM mechanism, adding solvent vapor to the background bath gas should rather decrease the rate of solvent evaporation from the charged droplets. The observed enhancement of ion formation by the presence of solvent vapor in the bath gas was therefore attributed to be indicative of an IEM mechanism operating, promoted by condensation of solvent molecules on the surface of the liquid droplets. According to the authors, the condensation enthalpy released by the adhesion of solvent molecules to the droplet surface is sufficient, as it were, to “sputter” solute ions from that surface into the ambient gas (25).
Contrary to small molecules, it is generally assumed that gas-phase ions of globular proteins are produced via the CRM mechanism (26). This model assumes that the charged macro-ions are produced from very small droplets that contain one macromolecule. As this droplet evaporates completely, the charges on the droplet are transferred to the macromolecule. The experimental support for the CRM mechanism is based on the observation of protein dimers and multimers at high concentrations as a result of more than one protein molecule being present in the final droplets (17). Based on the CRM model, the charge state distribution of gas-phase protein ions can be estimated as \( Z = 0.078 M^{1/2} \), where \( Z \) and \( M \) are the charge and the mass of a protein, respectively (27). A good agreement of this analytical expression with experimental observations is considered strong evidence that globular proteins and their complexes are transferred into the gas phase by the CRM mechanism (27).

### 2.1.3 Electrosonic Spray Ionization (ESSI)

ESSI (15) is a variation of ESI that employs a supersonic nebulizing gas (Figure 2.4).

![Diagram of ESSI source](image)

**Figure 2.4.** Schematic of ESSI source; SS stands for stainless steel, FS – fused silica, ID – inner diameter.

The major mechanistic difference between ESSI and ESI is the process of spray formation. In ESI, both charge separation and nebulization of a liquid sample are achieved by applying high voltage. Alternatively, in ESSI the voltage is only needed to pre-separate charged species in the
capillary, while the nebulization is fully controlled by the gas. Besides that, the supersonic flow affects the temperature of the spray via adiabatic expansion of the gas and allows more efficient solvent evaporation (15). As a result, narrower peaks are observed in ESSI mass spectra compared to other techniques, such as ESI and nanoelectrospray (nanospray) (28). Also, the fact that the abundant charge state is normally as low or lower than that recorded by ESI or nanospray together with the extremely narrow charge-state distribution observed in ESSI-MS suggests that protein ions are maintaining their folded conformation if sprayed by ESSI (15). Moreover, based on the weak dependence of the charge-state distribution on interface settings of a mass spectrometer, it has been suggested that the ultimate formation of gaseous ions in ESSI-MS is accomplished already at atmospheric conditions (15). In contrast, in ESI the formation of detected macromolecular ions is believed to take place in the interface-ion guide region of the instrument. The possibility to generate gas-phase ions outside a mass spectrometer allows studies of ion-molecule reactions at atmospheric pressure (29,30). For example, Cooks and coworkers transferred peptide/protein ions generated by ESSI through a heated coiled metal tube to induce fragmentation (29). The most important feature of this method is that it allowed characterization of the neutral fragments by use of corona discharge ionization. This neutral re-ionization was followed by MS analysis to yield increased structural information from the resulting mass spectra in both the positive and the negative ion modes (29). Similarly, ESSI has been used to generate ionic reaction intermediates whose conversion into products was promoted by atmospheric-pressure thermal activation (30). A number of reactions have been performed under these conditions, including Borsche-Drechsel cyclization, Fischer indole synthesis, and pinacol rearrangement (30).

The assumption that ESSI is capable of producing unsolvated ions at ambient conditions has been used to experimentally measure the gas-phase basicity (GB) of multiply charged ions (31,32). Deprotonation reactions of peptide and protein ions have been promoted by introducing volatile reference bases with a range of GBs at atmospheric pressure between an ESSI source and the inlet of a quadrupole time-of-flight mass spectrometer, thereby “bracketing” the GB of the peptide or protein. A good agreement has been observed with reference values obtained by FTICR-MS.

Owing to the supersonic gas flow, ESSI-MS tolerates high sample introduction rates, up to 50 µL min⁻¹. This makes it a potentially useful tool for the applications where high sample flows are
unavoidable or desirable. For example, ESSI-MS can be conveniently coupled with high-pressure liquid chromatography (HPLC), which utilizes flows that are only partially compatible with conventional ESI (33). ESSI-MS can also be applied to study kinetics of rapid reactions by mass spectrometry, where high flow rates are required to minimize “dead time” as well as to avoid the laminar regime of reaction sampling by transfer capillaries (34).

2.2 Fourier-transform ion cyclotron resonance mass spectrometry (FTICR-MS)

Below a brief theory of ion motion in FTICR-MS is given. For detailed overview refer to (7).

2.2.1 Radial confinement of ions: 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 2.5).

![Figure 2.5: Sketch of an open cylindrical ICR cell. Adapted from (4).](image)
In this case, the ion is subject to a Lorentz force: $\vec{F}_L = q[\vec{v} \times \vec{B}]$, in which $q$ and $v$ 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 spiral. According to Newton’s second law the equation for ion motion can be written as:

$$m\ddot{\vec{r}} = \vec{F}_L = q[\vec{v} \times \vec{B}]$$

(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 $\vec{B}$). Because of angular acceleration, $\frac{|d\vec{v}|}{dt} = \frac{v_{xy}^2}{r}$, Equation (2.1) becomes

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

(2.2)

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

$$m \omega^2 r = q B_0 \omega r$$

(2.3)

or simply:

$$\omega_c = \frac{q B_0}{m} \quad \text{or} \quad v_c = \frac{q B_0}{2 \pi m} = \frac{1.535611 \times 10^7 B_0}{m/z}$$

(2.4)

where $\omega_c$ is called the “unperturbed” cyclotron frequency. In the equation (2.4), $B_0$ is in Tesla, $m/z$ – in Thompsons. 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 (2.2) yields the ion cyclotron orbital radius of an ion of velocity $v_{xy}$:

$$r_c = \frac{m v_{xy}}{q B_0}$$

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

\[
r_c = \frac{1}{qB_0} \sqrt{2mkT}
\]  

(2.6)

Table 2.1 summarizes typical cyclotron frequencies and radii for thermally equilibrated ions in the ICR cell at a magnetic field strength of 4.7 Tesla. The value for the magnetic field strength corresponds to the actual value of our instrument’s magnet.

<table>
<thead>
<tr>
<th>Mass, Da</th>
<th>Cyclotron frequency, ( \nu_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.

2.2.2 Axial confinement of ions: trapping potential

To this point we have only considered 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 (1-30 volt) electrostatic potential to each of two trapping electrodes. This electrostatic potential produces a radial force:
\[ \vec{F}_R = q\vec{E}(r) = -q\nabla\Phi \] (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 2.6.

Figure 2.6: Electrostatic potential surface within an open cylindrical ICR cell. Trapping potentials are 1 V, central electrodes are grounded. Reprinted from (4).

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 (35):

\[ \Phi(x, y, z) = V_{\text{trap}} \left( \gamma + \mu (2z^2 - x^2 - y^2) \right) \] (2.8)

or in cylindrical coordinates

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

in which \( V_{\text{trap}} \) is the voltage applied to the trapping electrodes and \( \gamma, \mu \) are defined by the cell geometry (35). The ion motion in \( z \)-direction is defined as:

\[ m \frac{d^2 z}{dt^2} = -q \nabla \Phi(x, y, z) \] (2.10)
By substituting Equation (2.9) in Equation (2.10), it is straightforward to obtain an ion’s z-position that oscillates sinusoidally with time,

\[ z(t) = z(0) \cos(\omega_z t) \]  

(2.11)

where

\[ \omega_z = \sqrt{\frac{4qV_{\text{trap}}}{m}} \mu \]  

(2.12)

Typical trapping frequencies at a magnetic field strength of 4.7 Tesla are summarized in Table 2.2. Note that for ions with \( m/z \) values below 10000 cyclotron frequency \( \omega_c \) is more than an order of magnitude larger than \( \omega_z \). This is the main reason why cyclotron motion is usually recorded in ICR rather than trapping oscillations.

<table>
<thead>
<tr>
<th>Mass, Da</th>
<th>Trapping frequency, ( \nu_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.

### 2.2.3 Net ion motion in ICR

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 $\vec{E} = -\nabla \Phi = \mu V_{\text{trap}} \begin{pmatrix} +2x \\ +2y \\ -4z \end{pmatrix}$, we can formulate these equations as:

$$m\ddot{x} = 2\mu V_{\text{trap}} qx + qB_0 \dot{y}$$

$$m\ddot{y} = 2\mu V_{\text{trap}} qy + qB_0 \dot{x}$$

$$m\ddot{z} = -4\mu V_{\text{trap}} qz$$

The complete solution can be written as:

$$x(t) = \rho_+ \cos(\omega_+ t + \varphi_+) + \rho_- \cos(\omega_- t + \varphi_-) \hspace{1cm} (2.14)$$

$$y(t) = \rho_+ \cos(\omega_+ t + \varphi_+) + \rho_- \cos(\omega_- t + \varphi_-) \hspace{1cm} (2.15)$$

$$z(t) = \rho_\zeta \cos(\omega_\zeta t + \varphi_\zeta), \text{ where}$$

$$\omega_\zeta = \frac{\omega_c}{2} + \sqrt{ \left( \frac{\omega_c}{2} \right)^2 - \frac{\omega_z^2}{2} } \hspace{1cm} \text{reduced cyclotron frequency} \hspace{1cm} (2.16)$$
\[ \omega_+ = \frac{\omega_c}{2} - \sqrt{\left(\frac{\omega_c}{2}\right)^2 - \frac{\omega_z^2}{2}} \] magnetron frequency \hspace{1cm} (2.17)

\[ \omega_z = \sqrt{\frac{2qV_{\text{trap}}}{ma^2}} \] trapping frequency \hspace{1cm} (2.18)

\[ \omega_c = \frac{qB}{m} \] unperturbed cyclotron frequency \hspace{1cm} (2.19)

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):

\[ \omega_c = \omega_+ + \omega_- \] \hspace{1cm} (2.20)

\[ \omega_z^2 = 2\omega_+ \omega_- \] \hspace{1cm} (2.21)

\[ \omega_+^2 + \omega_-^2 + \omega_z^2 = \omega_c^2 \] \hspace{1cm} (2.22)

Analysis of Equations (2.17-2.19) shows that the magnetron and reduced cyclotron frequencies converge to a common value, \( \frac{1}{2} \omega_c = \frac{qB_0}{2m} = \omega_+ = \omega_- \) at the so-called “critical” \( m/z \), namely, when \( \omega_c^2 = 2\omega_z^2 \) or

\[ m_{\text{critical}} = \frac{B_0^2}{8V_{\text{trap}}\mu} \] \hspace{1cm} (2.23)

For ions with \( m/z > m_{\text{critical}} \), cyclotron motion is no longer stable, and ions spiral outward until they are eventually lost from the trap.
2.2.4 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, the phase of ion orbital motion is random at these conditions. 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 (36) is accomplished by applying an azimuthally spatially uniform electric field that oscillates sinusoidally with time $E(t) = E_0 \cos \omega_0 t$. All ions with the cyclotron frequency of the excitation field will be accelerated to a larger orbital radius.

$$r = \frac{E_0 t_{exc}}{2B_0}$$ (2.24)

The excitation is then turned off, and the coherent packet of ions starts 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 (7) is normally applied to activate the cyclotron motion of ions in a broad molecular range. In order to provide broadband excitation, many frequencies are applied during the excitation event. In an RF chirp, 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 induced image current that results from ions of several mass-to-charge ratios is a composite of sinusoids with 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 (37-39). In SWIFT, the desired excitation profile is first plotted in the frequency domain. It is then transformed into the mass domain by FT. The obtained digital profile is used to generate the excitation. Besides the excitation of cyclotron frequency, SWIFT is often used to isolate certain ions based on their $m/z$ value (7).

### 2.2.5 Experimental Setup

The FTICR instrument employed in this work consists of a commercial ion source (IonSpec Corp., Lake Forest/CA, USA) (Figures 2.7, 2.8) with a home-built open cylindrical cell adapted for optical spectroscopy experiments and a 4.7 T superconducting magnet (Bruker, Fällanden, Switzerland). Ions are produced by a Z-spray ESI source (Micromass, Manchester, UK).

In Z-spray, the ESI emitter is orthogonal to the first skimmer, called sample cone, which separates the atmospheric and the first vacuum stages. The second skimmer, called extractor cone, is in turn orthogonal to the sample cone. In this way, neutrals are effectively discriminated against, which allows one to avoid chemical contamination in the high vacuum chamber. Ions pass through a transport hexapole Q1 and are accumulated in a storage hexapole Q3. Accumulation time is an experimental variable. For fluorescence detection we target to maximize the number of ions, so the accumulation time was normally adjusted to maximize the ion signal. Note that so-called “space charge effects” come into play at high ion densities in the cell, which compromises the accuracy of FTICR-MS analysis (40). However being of great importance for identification purposes using exact mass measurements (40), space charge effects are of minor concern in most of our LIF experiments since the molecular sequence is usually known. In photoinduced dissociation (PID) consequence spectroscopy experiments, the accumulation time is normally decreased in order to reduce ion density and achieve higher mass accuracy.

A mechanical shutter controls ion injection from the Q3 into the ion guide hexapole and then into the ICR cell. A short gas pulse is used in order to equilibrate the ion cloud in the cell prior to
laser-induced spectroscopy or MS analysis. A detailed description of the optical setup will follow in Chapter 3.

The FTICR mass spectrometer with internal MALDI source was only used in this study to validate the functioning of the new optical setup before the ESI ion source had been delivered. The description of this MALDI-FTICR instrument can be found elsewhere (4,9).

Figure 2.7: ESI-FTICR-MS instrument.
Figure 2.8: Ion source operation during ion accumulation (top) and ion injection into FTICR cell (bottom).
2.3 Fluorescence

2.3.1 Basic theory of fluorescence

Fluorescence is the emission of a photon by a molecule or atom activated by the absorption of light. A photon of frequency $\nu$ interacting 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 the energy of one of the allowed electron transitions. Upon the absorption of light, the system is promoted to an excited electronic state, which implies a new electron configuration. This configuration is unstable, and can be deactivated in a number of ways. One of these ways is associated with the emission of photons and is called luminescence. Specifically, when the emission occurs from the singlet $S_1$ level back to $S_0$, it is referred to as fluorescence. Fluorescence is only one of the possible deactivation processes, which are usually summarized in a so-called Perrin-Jablonski diagram (Figure 2.9).

![Simplified Perrin-Jablonski diagram](image)

**Figure 2.9:** Simplified Perrin-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 (41).

In Figure 2.9, the electronic singlet states $S_0$, $S_1$, and the triplet $T_1$ along with a number of vibrational energy sublevels are shown. In the ground state, the system is in the lowest vibrational level of $S_0$. At room temperature, higher vibrational energy levels are in general not populated (less than 1% according to Boltzmann statistics). The energy of the absorbed photon determines which vibrational level of $S_1$ becomes populated. Right after the absorption, which is a very fast process ($\approx 10^{-15}$ s), the chromophore undergoes rapid vibrational relaxation to lower levels of $S_1$ within several picoseconds. In solution this process is promoted by collisions with solvent molecules, while in the gas phase it proceeds via equilibration of the excess energy in the chromophore system with other vibrational modes in the molecule. Moreover, in solution phase the system interacts with its environment by means of electronic forces (e.g., dipole-dipole), which changes the energy of the excited electronic state $S_1$ (Figure 2.10).

**Figure 2.10:** Perrin-Jablonski diagram accounting for solvent relaxation. Red and blue arrows schematically represent dipoles of a fluorophore and solvent molecules.
Normally, this interaction decreases the energy of $S_1$ due to the relaxation of solvent dipoles around the excited molecule. It is this relaxation that is responsible for large Stokes shifts – the difference between fluorescence and absorption maxima on the energy scale – observed in solution. The higher the solvent polarity the stronger shifts are induced. In the gas phase, the environmental stabilization factors are absent or largely reduced and only moderate Stokes shifts are observed as a result.

An important feature of a fluorescence spectrum is its independence of the excitation frequency (Kasha’s rule). This reflects the slow nature of fluorescence emission ($\approx 10^{-9}$ s) compared to the vibrational relaxation and solvent relaxation of $S_1$ in the excited state. Hence, fluorescence generally occurs from the lowest vibrational level of a stabilized $S_1$ state.

A number of non-radiative mechanisms can result in the return transition to the ground state besides fluorescence emission. Among those are internal conversion (IC) and intersystem crossing (ISC) followed by IC. IC is a vibrational dissipation of the excited electronic state energy. This can be both due to internal molecular vibrations and collisions with surrounding molecules. The smaller the gap between $S_1$ and $S_0$ the more probable IC is to occur. High temperatures are another factor that promotes IC. ISC is a transition from the excited singlet $S_1$ to the excited triplet $T_1$ state. The 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 sufficiently large to make it possible. 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 $T_1$ state has a long lifetime, up to $10^3$ s. The emission of a photon from this state is called phosphorescence. Its spectrum is red shifted relative to fluorescence.

In solution, an excited state of a molecule can be deactivated by external influences. The transfer of the excited state energy occurs either via collisional quenching or due to the complex formation with a quenching agent. Especially effective ubiquitous quenchers are molecular oxygen, iodide anions, and amines. The isolation of a fluorophore in the gas phase frees it from the influence of potential quenchers and therefore generally results in longer lifetimes of the excited state, although exceptions are known (42).
For a detailed consideration of fundamental aspects and applications of fluorescence one can refer to (43-46).

### 2.3.2 Labeling of biological molecules with chromophores

Only a small fraction of biological molecules exhibits intrinsic fluorescence. With few exceptions, proteins exhibit no or quite weak fluorescence due to tryptophan (Trp), tyrosine (Tyr) or phenylalanine (Phe) residues. Quantum yields of Trp, Tyr and Phe fluorescence are very moderate, which often prevents their use in real applications. In order to enable analysis by optical spectroscopy, non-fluorescent biomolecules can be tagged with extrinsic fluorophore probes, which are small molecules with specified optical properties. Nowadays, a number of highly fluorescent labels are commercially available. Labeling is usually specific to a certain reactive group of a biological molecule. For example, many labeling reagents have been developed that specifically react with primary amino (Figure 2.11) or thiol groups at the surface of proteins or with peptides.

**Figure 2.11:** Different reactions for the covalent attachment of fluorophores to ligands containing primary amino groups: A) cyanate ester, obtained by activation with cyanogen bromide, leading to an isourea derivative; B) N-hydroxysuccinimide ester yielding an amide derivative; C) p-nitro-phenyl carbonate obtained by activation with p-nitro phenylchloroformate or DSC, giving carbamate derivatives. Adapted from (47).
Labeling of amino acids, peptides and proteins with a fluorescent probe has been used for investigations described in Chapters 4 and 5.

2.4 References


Chapter 3

Exploring Fluorescence and Fragmentation of Ions Produced by Electrospray Ionization in Ultrahigh Vacuum

Adapted from
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Exploring Fluorescence and Fragmentation of Ions Produced by Electrospray Ionization in Ultrahigh Vacuum,
3.1 Overview

Fluorescence spectroscopy and mass spectrometry have been extensively used for characterization of biomaterials, but usually separately. An instrument combining fluorescence spectroscopy and Fourier-transform ion cyclotron resonance mass spectrometry has been developed to explore both fluorescence and mass spectrometric behavior of ions produced by electrospray ionization (ESI) in ultra high vacuum (< 5×10⁻⁹ mbar). Using Rhodamine 6G (R6G) as a sample, the instrument was systematically characterized. Gas-phase fluorescence and mass spectral signal of the same ion population are detected immediately after each other. Effects of gas pressure, ion density and excitation laser power on the fluorescence signal intensity and mass spectral fragmentation patterns are discussed. Characteristic times of ion photodissociation in ultra high vacuum were recorded for different irradiation powers. Photofragmentation patterns of Rhodamine 6G ions in the Penning trap of an FTICR spectrometer obtained by photoinduced dissociation (PID) with visible light and sustained off-resonance irradiation collision-induced dissociation (SORI-CID) were compared. The lowest energy dissociation fragment of Rhodamine 6G ions was identified by relating PID patterns of Rhodamine 6G and Rhodamine 19 dyes at various irradiation powers. The unique instrument provides a powerful platform for probing the intramolecular relaxation mechanisms of nonsolvated ions when interacting with light, which is of great fundamental interest for better understanding of their physical and chemical properties.

3.2 Introduction

Fluorescence spectroscopy is widely applied for sensitive probing of the structure of matter due to its high specificity to the microenvironment. It can provide several types of information on a large variety of samples, such as solid surfaces, biological membranes, living cells, etc (1). One important feature is that it allows one to obtain structural details of biomolecules. For instance, fluorescence spectroscopy allows determining binding sites of a protein, as well as its conformational transitions or intramolecular distances. Also, this technique yields information on flexibility, helix structure, torsion dynamics and carcinogenesis of nucleic acids (1).
Recently fluorescence spectroscopy has started to be used to probe ions in the gas phase (2-7). This complements solution phase data and is important for elucidating the role of the solvent in stabilizing a native structure. Also, gas-phase data are much easier to relate to theoretical calculations because in such a case it is not necessary to consider the interaction of a molecule with solvent molecules, which greatly simplifies the calculations. Non-volatile molecules are usually brought into the gas phase in ionic form by soft ionization techniques, such as electrospray ionization (ESI) (8) or matrix-assisted laser desorption / ionization (MALDI) (9). Ions produced are typically guided into a quadrupolar trap where they can be stored for a long time as a compact cloud, which enables recording of their fluorescence (3,4,6,7). In their pioneering work, Parks and coworkers monitored dissociation of double-stranded oligonucleotide anions in the gas phase by observing the change in the efficiency of fluorescence resonance energy transfer (FRET) (10) between a pair of fluorophores BODIPY-TMR (donor) and BODIPY-TR (acceptor) labeling two strands of the duplex (11). Later, the same group described the gas-phase behavior of a small Trp-cage protein such as its folding/unfolding dynamics (12). Again, they used BODIPY-TMR dye as a reporter because its fluorescence intensity depends strongly on its proximity to the intrinsic Trp residue of a protein. Quenching of the dye fluorescence was due to photoinduced electron transfer (PET), which depends exponentially on fluorophore-quencher separation (13). The same methodology was applied to explore conformational changes in unsolvated ions of polyproline peptides and a β-hairpin peptide (14).

Fourier-transform ion cyclotron resonance mass spectrometry (FTICR-MS) (15) is a powerful technique which has been successfully applied in a variety of rapidly developing research areas of modern biochemistry, such as proteomics and petroleomics. Up to now, fluorescence detection in the Penning trap of an FTICR spectrometer has only been performed in home-built instruments with internal ionization sources, such as electron impact (EI) (16) or internal MALDI (5). This substantially limits the application range of these experimental setups.

Our previous experiments were performed in an FTICR mass spectrometer with an internal MALDI ionization source described in detail elsewhere (5). Clear evidence of FRET occurring in the gas phase was demonstrated for the system Rhodamine 6G (donor) and Sulforhodamine B (acceptor) covalently bound through a phenyl ring linker (17). One key limitation of this setup
was the low molecular weight range (m/z < 5 000), because the relatively fast ions produced by the MALDI internal source could not be efficiently slowed down before entering the ICR cell. For this reason, heavier ions having higher kinetic energy could not be trapped. Also, since no ion optics were used between the MALDI target and the ICR cell, the ion cloud entering the cell was not focused in the radial direction. Therefore, quadrupolar axialization was always necessary to bring the ions onto the z-axis to provide a good overlap with the LIF excitation laser beam. The axialization in turn requires elevated buffer gas pressure (>10^{-5} mbar), since frequent collisions with buffer gas are required. No fluorescence signal was observed without axialization.

Here we present a novel experimental setup for fluorescence and dissociation spectroscopy experiments on ESI produced gas-phase ions in an ICR mass spectrometer. The setup opens new possibilities for spectroscopic probing of biomolecular ions under conventional FTICR-MS experimental conditions. The high mass accuracy of the instrument allows the identification of fragmentation mechanism such as radical or neutral loss. In particular, it offers high versatility as the fluorescence collection is essentially decoupled from the ion optics between the ESI source and the Penning trap. Therefore, our setup can be easily installed on other commercial FTICR instruments, no modifications being required except the installation of the measuring cell described in detail in the Experimental section.

### 3.3 Experimental

The experiments described here were performed on an FTICR mass spectrometer with an electrospray ionization (ESI) source (IonSpec Corporation, Lake Forest, USA) and a 4.7 Tesla superconducting magnet (Bruker, Fällanden, Switzerland). A completely new fluorescence setup, including a new ICR cell was engineered in our laboratory because the previous setup was not compatible with the z-axis ion introduction by external ESI (5).

Ions produced by ESI at atmospheric pressure were guided through a hexapole transport system with differential pumping into an open cylindrical cell. The diameter of the cell is 5 cm. All the electrodes were machined from OFHC copper (Haeuselmann Metal AG, Switzerland). Holes of 6
mm in diameter were drilled into the excitation and detection plates to allow for a laser beam to be introduced into the cell.

The latest version of our experimental setup for the LIF measurements is shown schematically in Figure 3.1 (top). The trapped ions were irradiated by a CW Ar ion laser (Innova 300, Coherent, USA) at a wavelength of 488 nm. The laser beam was introduced into the vacuum chamber through a high vacuum glass viewport (PF610010-X, Pfeiffer Vacuum, Germany). Due to space limitations caused by the magnet core size (Figure 1) the beam was introduced into the ICR cell through a Right Angle Reflector (74-90-UV, OceanOptics, USA). It consists of a mirror mounted on an anodized aluminum holder so that the incident light is reflected at a 90° angle into the cell. To efficiently absorb the laser beam after it has crossed the ion cloud, a conical beam dump (PL15, Newport, USA) was mounted on the opposite side. Light would have to reflect at least three times from black surfaces (reflectivity of less than 10^-4) to escape, but most of it undergoes many more. Note that we cannot use a “Woods horn” beam dump because its installation would require much more free space in the radial direction of the vacuum chamber than available. In order to suppress scattered light further, two home-built baffle systems were introduced as shown in Figure 3.1. Finally, the inner walls of the FTICR cell were coated with vacuum compatible, electrically conductive paint (Aquadag E, Acheson Co., USA), which dramatically reduced the scattered excitation laser light.

Alignment of the laser beam path was done when the cell was outside of the vacuum chamber. A long baffle was rigidly braced to the viewport on the flange. Two slits 2 mm in diameter were mounted inside the baffle. The distance between the slits was 50 cm. These slits were then used to guide the laser beam when the cell was inside the vacuum chamber. Figure 3.1 (bottom) shows a photograph of the ICR cell during the alignment procedure. The conical beam dump was removed in order to control the position of the beam in the cell.

The ion cloud fluorescence was collected by a system of lenses (lens 1: ACH 25X50 MGF2TS, Edmund, f = 50 mm; lens 2: 01LAG117/066, Melles Griot, f = 25 mm) and focused onto the cleaved edge of a 3 mm core diam. plastic fiber with a high NA value of 0.51 (PGR FB 3 000, SEDI, France). A long pass filter (LP02-488RU, RazorEdge®, Semrock, USA) was used after the collimating lens in order to block a part of the scattered laser light. The distance between the collimating and the focusing lens was 10 cm.
Figure 3.1: Experimental setup (top); ICR cell during the laser beam alignment procedure (bottom). The conical beam dump was removed in order to show the position of the beam inside the cell.
The fiber passes the vacuum flange through a Kel-F feedthrough (18). Light exiting the fiber was focused onto the active surface of a photomultiplier tube (R446, Hamamatsu, Japan). A long pass filter (FEL0500, Thorlabs, Germany) was used to block the scattered excitation laser light that was not blocked in the vacuum chamber. The signal from the photomultiplier tube was recorded by an 800 MHz gated photon counter/multiscaler (PMS 400, Becker & Hickl GmbH, Berlin, Germany).

Visible spectra were collected by coupling the plastic fiber to a spectrograph (Shamrock SR-163, Andor, Northern Ireland; HoloSpec f/1.8i, Kaiser Optical Instruments Inc, Ann Arbor, USA) with a 100 µm entrance slit and a CCD camera (DV420A-OE, iDus, Andor, Northern Ireland; LN/CCD-2500-PB/VISAR, Princeton Instruments, Trenton, USA), instead of a PMT used in the photon-counting mode (Figure 3.1, top).

The sequence of events in a typical experiment started from creating ions by ESI. These ions were then guided through a system of hexapole filters into the ICR cell. When an increase of the buffer gas pressure was necessary, a pulse of helium gas was introduced through a pulsed leak valve. Ions were stored in the cell as long as necessary (from 1 s to 5 min). Cyclotron motion of the trapped ions was then excited to record a mass spectrum. The Ar⁺ ion laser and the PMT were running constantly during the entire experiment. Background signal was therefore detected continuously (between 350 - 1 500 counts per second, depending on the excitation laser power). When the ions entered the cell, the signal increased due to their fluorescence (up to 10 000 counts per second for R6G ions). Excitation of the cyclotron motion broke the spatial overlap of the axialized ion cloud and the Ar⁺ laser beam, i.e. fluorescence was no longer recorded after that event. Note that the recorded mass spectrum shows all the photo-induced fragments produced during the ion cloud storage in the cell. Figure 3.2 summarizes time profile of the major events during a single experiment.

For the CID experiments, sustained off-resonance irradiation (SORI) was applied with argon as a collision gas. The peak pressure was 4×10⁻⁶ mbar, the offset frequency 800 Hz, irradiation duration 300 ms, and the amplitude ranged from 0.5 to 2.5 V.
Figure 3.2: Sequence of events in a typical experiment.

The very first version of the ESI-compatible LIF collection setup engineered in our laboratory (19) was quite different from the one described in the experimental section. It is worthy to highlight some general issues one has to pay attention to when designing a LIF detection experiment in vacuum.

In practice, it is extremely difficult to dispose of the whole laser beam after it has passed through the ion cloud in the ICR cell. The amount of laser light absorbed is limited by the capacity of the beam dump and by the beam divergence. As a result, a part of the laser beam is scattered inside the cell. Therefore, it is first of all important that no fluorescing materials are used in the cell assembly. Spurious signals originating from such materials can spectrally overlap with the ion fluorescence and thus cannot be easily filtered out later. An example of a fluorescing material is PEEK plastic, which is often used as an isolator in ICR cells. One feature of our setup which yielded an appreciable improvement was minimizing the PEEK content in the cell assembly.
Generally speaking, the earlier the scattered light is suppressed the less spurious fluorescence it can produce. In our setup it was particularly important that the major part of the scattered laser light was blocked before it reached the collection fiber because the fiber itself was found to produce non-negligible fluorescence, which substantially compromised the detection of the ion fluorescence. For this reason, an optical filter stage was introduced in the vacuum chamber. Also, when blocking the laser scattered light, interference filters were found to be better compared to absorbing colored glasses as the latter typically produce spurious fluorescence as well.

The distance between the collimating and the focusing lens was 10 cm to make sure that only the well collimated part of light passed both lenses and the filter and finally reached the fiber. Of course, a shorter distance between the lenses would allow for a bit more efficient fluorescence collection. In this case, however, the light passing through the lenses would not be so well collimated, and the edge filter performance would be much worse because it has strong angle dependence.

Figure 3.3 shows how the background spectrum (no ions in the cell) changed after all the issues described above were taken into consideration. The peak at 488 nm corresponds to the remaining laser line. The broad bands to the right of the laser line in the range of ca. 500-700 nm on the first spectrum correspond to various spurious fluorescence sources that were discussed above. Clearly, the ion fluorescence signal-to-background ratio would be greatly affected by this spectrally overlapping background signal. The second background spectrum corresponds to our latest setup. Virtually no spurious fluorescence can be observed. The laser light however is not blocked completely, thus, the second filter is used between the fiber and the PMT in the photon counting mode (Figure 3.1).

As discussed above, the laser beam path was aligned when the cell was outside of the vacuum chamber. Fine adjustment was performed after the cell had been installed to maximize the fluorescence signal.

In the very first version of our setup a fiber was used to deliver the laser light directly into the ICR cell in order to avoid the troublesome alignment procedure (19). However, the fiber was found to produce very strong fluorescence signal induced by the high laser power coupled. Thus, a laser-line filter (LL01-488-12.5, MaxLine®, Semrock, USA) was placed right after the fiber to
get rid of this spurious fluorescence signal but it resulted in perturbation of the beam shape leading to stronger laser light scattering inside the cell. This approach was finally abandoned in favor of steering the laser beam into the cell through a window followed by a series of baffles (Figure 3.1).

![Figure 3.3: Evolution of the background wavelength spectrum (from the left to the right spectrum) as a result of modifying the experimental setup (see the Results and Discussion section).](image)

### 3.4 Results and Discussion

#### 3.4.1 Ion fluorescence in ultra high vacuum

It is worth noting that the light absorption/emission of polar dyes can significantly change when going from solution to the gas phase. Thus, gas-phase R6G ions absorb the 488 nm laser line much stronger than the other major line of the Ar$^+$ laser at 514 nm (17), suggesting that the absorption maximum lies around 488 nm. Absorption of R6G in ethanol is meanwhile maximized at around 530 nm. Considerable blue shifts of approximately 40 nm in R6G emission/absorption can therefore be estimated to occur when moving from solution to the gas phase. This example nicely illustrates how sensitive the fluorescence spectroscopy is to the changes in microenvironment.
Figure 3.4 shows fluorescence recorded from R6G ions irradiated at 488 nm at 0.2 mW/mm². This low irradiance was used to make sure no photodissociation took place during the experiment. This was confirmed by the mass spectrum, which showed only molecular ions of R6G (not shown). It can be seen that it takes some time after the ions enter the cell before the signal reaches a plateau (black curve). When eight 10 ms pulses of helium gas with a 200 ms interval were introduced into the cell right after the ion injection, the peak pressure being $5 \times 10^{-5}$ mbar, the signal reached its maximum much faster (red curve). We believe that this results from more efficient cooling of the ion axial motion (along the lines of the magnet field) to the room temperature of buffer gas molecules. At elevated pressure, a much shorter time is needed to focus ions axially in the ICR cell, which makes for a faster complete overlap of the ion cloud with the laser beam (16). In case of lower pressure, a much longer time is required in order to cool the ion cloud and therefore complete overlap is achieved much later. The cooling can be indirectly observed by monitoring the intensity of the MS signal at different times. For short ion storage periods when the ion cloud in the cell is not completely focused, MS signal intensity is lower compared to that after the ions have been equilibrated. This is due to the fact that the excitation field is stronger in the center of the ICR cell, and therefore a better focused ion cloud would produce a stronger signal (20). Also, the fact that the fluorescence signal grows while the ion cloud is getting more compact further indicates that the laser beam does go through the center of the cell. Otherwise, if the interaction with the ion cloud would take place at the periphery, the signal would decay while the ions were being focused towards the center of the cell.

Another interesting observation is that the fluorescence signal decays whether or not gas was introduced, although no ion photodissociation occurred. We believe that the reason for this observation is the magnetron expansion of the ion cloud inside the ICR cell (15), which causes a gradual deterioration of the overlap between the laser beam and the ion cloud, resulting in the signal decay. Also, the MS signal intensity starts to decrease after some time as a result of the ion losses in the cell. The fluorescence signal decay was faster when helium gas was introduced (Figure 3.4, red curve), which enhances the magnetron expansion as its rate strongly depends on the background pressure (15).
Quadrupolar axialization is often performed in order to avoid magnetron expansion of the ion cloud in the ICR cell (15). This, however, requires elevated pressure in the trap and therefore was not applied in our experiments.

Figure 3.4: Two fluorescence signals recorded from Rhodamine 6G ions irradiated at 488 nm with an irradiance of 0.2 mW / mm². Increase in the signals started right after the ions had entered the cell. Black curve: no neutral gas was introduced during the ion cloud storage in the ICR cell. Red curve: eight 10 ms helium gas pulses with a 200 ms interval were introduced right after the ions had entered the cell. The signals were background subtracted.

Figure 3.5 shows a correlation of the fluorescence signal with the intensity of a molecular peak for R6G ions in the mass spectrum, in the absence of photodissociation. In ICR the latter depends linearly on the number of ions in the cell. One can see that the dependence in the figure deviates from linearity at higher MS signal intensities. This can be explained by incomplete overlap between the ion cloud and the laser beam, since the ion cloud expands at high ion densities due to space charge effects. Better overlap can be achieved using a laser beam of a bigger diameter. This would, however, result in more pronounced laser light scattering inside the cell. For this reason, no attempt was undertaken in order to improve the overlap in this fashion.

Alternatively to the single photon counting mode, fluorescence can be expanded into a wavelength spectrum using a monochromator and then detected by a CCD (Figure 3.6). When
complemented with solution-phase data, fluorescence spectra of gas-phase ions can be a source of valuable information on their properties. Fluorescence spectroscopy of gas-phase ions is used in the investigations described in Chapters 4, 6 and 7. It is worth noting that the spectral representation of fluorescence is associated with significant losses in signal intensity, which is due to the lower sensitivity of CCD detection compared to PMT.

Figure 3.5: Dependence of the fluorescence signal on the intensity of the molecular peak for Rhodamine 6G ions in the mass spectrum.

Figure 3.6: Fluorescence spectrum of Rhodamine 6G ions trapped in FTICR.
3.4.2 Effects of gas pressure and laser power on the fluorescence signal

Excitation of fluorescent molecules with visible light in high vacuum results in gradual gain of internal energy during successive photon absorption/emission cycles. Photofragmentation happens when accumulated vibrational energy is high enough to break the weakest chemical bond. This is very similar to the mechanism of infrared multiphoton dissociation (IRMPD) (24) where vibrational energy is accumulated directly through successive absorption of IR quants, without exciting the higher electronic energy level.

In earlier studies it was demonstrated that the fluorescence signal from gas phase ions depends strongly on the buffer gas pressure (4,6,7,17). Buffer gas molecules vibrationally cool excited ions, preventing them from undergoing rapid dissociation via multiple absorption/fluorescence cycles (vide infra). The higher the pressure the more efficient the collisional cooling, which allows using higher excitation laser power while preserving the ions intact. In other words, the pressure of buffer gas controls the temperature of ions during the experiment. Figure 3.7 demonstrates the dependence of the highest achievable fluorescence signal recorded from R6G ions in an ICR cell on buffer helium gas pressure (17).

The attainable signal grew as the pressure increased, due to the possibility to use higher laser powers without destroying fluorescent ions. Quadrupolar axialization was applied in order to keep the ions in the center of the cell while the fluorescence was recorded. No photodissociation

![Figure 3.7: Dependence of the attainable fluorescence signal on the pressure of helium gas inside the ICR cell. Adapted from (17).](image)
occurred at laser irradiances up to 50 - 65 mW / mm² at a background gas pressure around 10⁻⁴ – 10⁻⁵ mbar, at least on the time scale of 10 s. Also, the fluorescence yield was found to depend on what kind of buffer gas was used for the quadrupolar axialization (21). In particular, no fluorescence from R6G ions could be detected when argon gas was applied. The highest fluorescence yield was obtained by helium gas as a buffer. Temperature dependence of the fluorescence spectrum is considered in detail in Section 6.4.3.

Clearly, in ultrahigh vacuum where collisions with neutrals are very rare, vibrational heating of ions should proceed very rapidly. Figure 3.8A shows the fluorescence signal from R6G ions recorded in our new setup at various excitation laser irradiances. Five time profiles were accumulated for each laser irradiance. The background signal was subtracted and the resulting signals were then normalized. The rapid increase of the signal at around 4 s corresponds to the time when the ions enter the ICR cell. A short helium gas pulse was introduced as soon as they had been trapped in order to accelerate axial focusing of the ion cloud. The decay is due to photodissociation of parent R6G ions into nonfluorescing fragments (Figure 3.8B). It is worth noting that at least the first fragment of R6G at m/z 415, produced by loss of the ethylene, was found to fluoresce as well, which could be seen by illuminating the isolated ion species at m/z 415 (not shown). The structural and photochemical properties of the first fragment will be discussed in detail below. The decay curves thus represent the net fluorescence profile from both R6G ions and their fragments. These curves were well fit with a double exponential (y = A1exp(-t/a1) + A2exp(-t/a2) + y0, A1 = 431, a1 = 3 s, A2 = 1219, a2 = 89 s, R² = 0.97 for the decay at 10 mW/mm² in Figure 3.8A (blue curve)), which suggests that the role of the fragments in producing the net signal cannot be neglected. We interpret the second, slower time constant to be due to the fluorescence from fragments that exhibit quite a different branching ratio between the different excited state decay channels (fluorescence, nonradiative decay, photodissociation), presumably due to the structural changes. Figure 3.8B shows how the mass spectrum evolves due to photofragmentation of R6G. The spectra correspond to the blue decay curve in Figure 3.8A, providing a complete time profile of photochemical dissociation reactions that take place in the ICR cell. Indeed, rapid photodissociation limits the fluorescence detection efficiency. It can be seen from Figure 3.8A that at a laser irradiance of 15 mW / mm², the fluorescence signal decays completely within about 5 s after the ions have entered the cell. However, as mentioned earlier,
at elevated pressures no photodissociation of R6G ions in an ICR cell could be seen at a laser irradiance as high as 65 mW / mm$^2$, at least on a time scale of 10 s (17). For comparison, the probability of R6G photobleaching in solution, where vibrational cooling is much more efficient than in the gas phase due to frequent collisions with solvent molecules, is in the order of $10^{-6}$ – $10^{-7}$ for irradiances below 10 W / mm$^2$, which results in fluorescence decay times on the order of thousands of seconds (22).

Figure 3.8. Top: Fluorescence signal decay due to photodissociation of Rhodamine 6G ions at different excitation irradiances. Bottom: Evolution of the MS spectrum for Rhodamine 6G ions due to photodecomposition. Laser irradiance was 10 mW / mm$^2$ (blue curve in Figure 3.8A). All spectra are normalized to the signal intensity of its corresponding largest peak. The y-axis shows the relative intensity of each spectrum with respect to the M$^+$ signal of the spectra at 7 s.
3.4.3 Photoactivation of ions in ultra high vacuum

In order to obtain information on the structure of biomolecular ions in FTICR mass spectrometry, sustained off-resonance irradiation collision-induced dissociation (SORI-CID) is often used (23). However, it only allows for activation of ions with a certain \( m/z \) value and therefore prevents further dissociation of produced fragments. For large biological molecules it can significantly limit attainable structural information on a parent ion (24). Also, since SORI-CID is performed at an elevated pressure, the fragments produced may diffuse radially due to magnetron expansion promoted by the gas load. This can significantly compromise the accuracy of the mass measurement (25). In case of photo-induced activation, however, no gas introduction is necessary and daughter ions are still available for further fragmentation as long as they are spectroscopically active. Here we show that even for small ions a difference exists between SORI-CID and photoinduced dissociation (PID) MS spectra. Figure 3.9 demonstrates SORI-CID and PID spectra for R6G ions obtained at different activation energies.

![Figure 3.9: SORI-CID (left) and PID (right) MS spectra obtained at different activation energies (SORI-CID voltage was 1.2 V and 1.5 V, laser irradiance was 10 mW / mm² and 20 mW / mm² for the top and bottom spectra respectively). Inset: SORI-CID MS spectrum of the fragment at \( m/z \) 415. In PID, ions were excited at 488 nm with the power of 200 mW for 2 s (top) and 10 s (bottom).]
It can be seen that at low CID energy ions at m/z 415 are preferentially produced. Once produced, these ions are not available for further fragmentation in SORI-CID. Low-energy PID gives the same fragment at m/z 415. It was observed, however, that despite some structural changes this fragment also absorbs light at the same wavelength as the parent R6G ions. Therefore, it can dissociate further, resulting in a more complete fragmentation pattern as can be seen by comparing top left and top right spectra in Figure 3.9. For example, the fragments at m/z 386 and m/z 365 are absent in the SORI-CID spectra, while being easily detected by PID. The fragmentation patterns suggest that these species are much easier to produce from the fragment at m/z 415 than directly from the parent R6G ions. In order to prove this hypothesis, SORI-CID was performed on the fragment at m/z 415, revealing abundant signals at m/z 386 and 365 (inset in Figure 3.9). The difference between CID and PID patterns is not so pronounced as soon as the fragment at m/z 415 is no longer the main dissociation channel (Figure 3.9, bottom).

For molecules that absorb visible light but do not fluoresce (quenchers), excitation with visible light will, however, result in a much faster accumulation of vibrational energy due to internal conversion from the excited electronic state and therefore very rapid fragmentation. If dissociation proceeds faster than complete vibrational energy redistribution (IVR), then it should be characteristic of the chromophore’s local microenvironment, which can be used for probing the gas-phase ion structure. For instance, Bossio et al. showed decarboxylation of the polypeptide C terminus in an ICR spectrometer when a UV chromophore at the N terminus was in close proximity due to the flexibility of the peptide chain (26). Gabelica et al. studied electron photodetachment dissociation of gas-phase DNA anions activated at 260 nm (DNA excitation) (27) and DNA anions labeled with different chromophores activated at >300 nm (chromophore excitation) in a quadrupole ion trap (28). Radicals produced were shown to keep no memory of the activation method by comparing their MS³ fragmentation, suggesting that complete IVR took place (28). Therefore, in order to perform localized dissociation controlled by the position of a chromophore, it is important that the rate of de-excitation by IVR and by inelastic collisions with a bath gas is slow compared to vibrational energy accumulation by the chromophore due to internal conversion of its electronic energy. As discussed earlier, the lower the background pressure the faster the ion vibrational energy is accumulated as it is not dissipated due to collisions with neutrals. Thus, it is believed that conformation-specific fragmentation could be
easier to achieve in an ICR spectrometer compared to ion traps. Vibrational cooling could be the reason why complete IVR was always found in studies on the electron photodetachment dissociation in a quadrupole ion trap (28). Alternatively, excitation to the second excited state can be achieved for higher laser irradiances, in which case photophysics is essentially the same as for UV excitation (29).

The lowest activation energy at which fragmentation starts to occur is characteristic of R6G photostability. It is thus important to know how the first fragment at \( m/z \) 415 is created. The structure of an R6G ion is shown in Scheme 3.1 (left). The difference of 28 units is obviously due to the loss of an ethylene. It is not obvious, however, which \( \text{C}_2\text{H}_4 \) group dissociates, among the three available. We believe that most probably it is the one from the ester group. If this is the case, then the resulting fragment should look like in Scheme 3.1 (right), the structure of Rhodamine 19 (R19), which is commercially available. In order to prove our hypothesis, PID spectra of these two compounds were obtained at different excitation laser powers (Figure 3.10).

![Scheme 3.1: Structures of Rhodamine 6G (left) and Rhodamine 19 (right) ions.](image)

A remarkable agreement between the two sets of data could be seen in Figure 3.10. This finding suggests that with high probability the lowest energy fragmentation channel of R6G ions is dissociating into R19 ions due to loss of ethylene from the ester. Also, R19 was found to be fluorescent when activated at 488 nm, which is consistent with our finding that some fragments of R6G contribute into the net signal, as discussed above. The fluorescence yield of R19 was
about two times lower than that of R6G, which is probably due to the shift in absorption/emission maxima.

Figure 3.10: PID spectra of R6G (black line) and R19 (red line) ions at different excitation laser powers. The peaks corresponding to parent species are labeled.

3.5 Conclusions

In combination with fluorescence/photofragmentation spectroscopy FTICR-MS provides unique capabilities for probing ions in the gas phase due to the long ion storage times, ultra low pressure and its high mass selectivity. A unique setup was built in our lab for this purpose, allowing us to perform fluorescence spectroscopy inside an FTICR spectrometer on ions produced by
electrospray ionization. The new platform was thoroughly described and preliminary data on gaseous Rhodamine 6G ions were shown.

3.6 References


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Chapter 4

Direct Access to Isolated Biomolecules under Ambient Conditions

Adapted from
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Direct Access to Isolated Biomolecules under Ambient Conditions,
4.1 Overview

Intrinsic properties of biological molecules, which are disguised by their surroundings, can be directly accessed in the gas phase. Producing intact, isolated molecules in the gas phase is of general interest, but has traditionally been restricted to volatile compounds. Isolated nonvolatile compounds, such as proteins, nucleotides, polysaccharides and synthetic polymers, can be studied in high vacuum using a combination of soft ionization methods with mass spectrometry (MS), often enhanced by other spectroscopic methods. However, structural information that can be obtained in this case is greatly limited due to the modest number of accessible analytes as well as the sophisticated instrumentation required. Here, we report on the discovery that electrospray ionization (ESI) is capable of generating substantial amounts of isolated gas-phase species (instantaneous numbers \( \approx 10^9 - 10^{10} \)) in the ambient atmosphere. This was demonstrated by correlating fluorescence spectra recorded in the ESI plume with those of isolated ions trapped in high vacuum. Thus, naked ions are now directly accessible for spectroscopic investigation under ambient conditions, without any need for ion trapping and transport. We anticipate our discovery to re-energize the interest in studies of biomolecules in the gas phase and to enrich the experimental arsenal for probing their intrinsic properties by relatively simple and inexpensive methods.

4.2 Introduction

The study of large molecular systems in the gas phase has experienced an enormous boost by the introduction of soft ionization methods for mass spectrometry (MS), in particular electrospray ionization (ESI) (1), matrix-assisted laser desorption/ionization (MALDI) (2) and related ambient ionization techniques (3). A quite heterogeneous population of species, including abundant charged and neutral droplets/clusters of different size and composition (4,5), are created by ESI and MALDI. The eventual formation of isolated molecular ions is believed to be due to their observation by MS: the vacuum-based detection effectively completes the desolvation and declustering processes (Figure 4.1). The formation of ambient gas-phase ions
may be promoted by using vigorous desolvation conditions, but the experimental support for this is quite indirect (6,7).

**Figure 4.1:** Transfer of biological molecules into the gas phase. Thermal evaporation is extremely inefficient due to the rapid chemical degradation (left). Generally, nonvolatile species are studied in the gas phase by soft ionization methods such as ESI or MALDI in combination with MS (right). No method has been established so far to access isolated large biomolecular systems in the ambient gas phase (center).

In recent years, the attention has shifted from merely observing intact gas-phase ions by MS to studying their structure and conformation (8-11), which generally requires much more sophisticated instrumentation, including but not limited to combining MS with ultraviolet (12,13), infrared multi-photon (14-16), UV/IR double resonance (17,18) or visible-light (19,20) optical spectroscopy of ions trapped in the high vacuum of a mass spectrometer. The necessity for ion trapping, transport, and sometimes manipulation renders such instruments expensive and difficult to operate. Furthermore, direct spectroscopic interrogation is often not possible due to the low achievable ion density, dictated by the limited trapping capacity and ion guide efficiency.
of the associated MS equipment. Instead, one typically has to resort to “action spectroscopy”, i.e., the observation of fragment ions produced by optical excitation. Experimental access to isolated nonvolatiles already at ambient conditions, without the need for complex instrumentation, would thus be highly advantageous.

Here we unambiguously show that ESI is capable of generating high numbers of gaseous analytes at ambient conditions. This was demonstrated by correlating fluorescence spectra recorded in the ESI plume with those of isolated ions in the high vacuum of a mass spectrometer. Therefore, isolated biomolecular ions are now directly accessible for spectroscopic investigation at ambient conditions, without the need for ion trapping and transport.

4.3 Experimental

An experimental setup for optical spectroscopy in an ESI plume is shown schematically in Figure 4.2(a). A commercial ESI source (Micromass, Manchester, UK) was used. The inner diameter of the capillary for sample introduction was 100 µm, the electric potential was in the range of 3.5 - 5 kV. The distance between the tip of the capillary and its counter-electrode was 2 cm. A solution containing fluorescent compounds was pumped through the capillary at a flow rate of 0.2 - 2 µl min⁻¹. Fluorescence was excited by CW Ar⁺ laser (Innova 300, Coherent, USA) radiation at 488 nm and detected orthogonally by fiber optics coupled to a holographic imaging spectrograph (HoloSpec f/1.8i, Kaiser Optical Instruments Inc, Ann Arbor, USA).

A home-built ion source was used for ESSI experiments according to the scheme described in (6). The inner diameter (ID) of a capillary for liquid sample delivery was 50 µm. A coaxial capillary was used for nitrogen gas introduction of ID 530 µm. Both capillaries were made of fused silica. High voltage was applied directly to the solutions. Nebulizer nitrogen gas pressure was 25 bar. Ions produced by ESSI were guided through a copper tube cooled by liquid nitrogen. The copper tube in the cold spectroscopy experiments was 20 cm long and 5 mm in diameter. The part that was in contact with liquid nitrogen was ca. 7 cm long. Ac-Ala₋₅-Lys₋₅-R₅₇₅ peptide was synthesized by Eurogentec (Seraing, Belgium).
4.4 Results and Discussion

We found that the detected fluorescence changes dramatically when different regions of the ESI plume were excited. The spectra shown in Figure 4.2(b) were collected for Rhodamine 6G (R6G) sprayed from methanolic solution (10 µM) with an integration time of only 10 s. In the region close to the capillary tip, where the largest aerosol droplets occur, the fluorescence maximum of in-plume R6G exhibits no or only a slight shift relative to that in solution ($\lambda_{\text{max,MeOH}} \approx 555$ nm). As the laser beam was translated down the plume, a new component started to appear in the spectrum, with a maximum around 505 nm. At some point (about 10 mm far from the spray tip), only the 505 nm fluorescence peak remained visible while the one at 555 nm had disappeared (Figure 4.2(b), bottom). No changes in the spectral shape could be observed when translating the laser beam further down, except for a decrease in signal intensity caused by the plume expansion.

Figure 4.2: a) Experimental setup for optical spectroscopy in an ESI plume; b) fluorescence spectra obtained from different regions in the ESI plume when a methanol solution of R6G was electrosprayed; c) a photograph of the upper region of the ESI plume that was taken when the excitation laser beam was unfocused so that the whole plume area was illuminated. The blue color in the figure is due to laser light scattering from droplets in the plume; d) fluorescence spectra of R6G ions recorded by FTICR-MS – LIF spectroscopy (blue) and in the ESI plume in Figure 4.2(b), bottom (green).
Figure 4.2(c) shows a photograph of the ESI plume that was taken when the whole plume area was illuminated using a defocused excitation laser beam. The blue color in the figure is due to laser light scattered by the droplets constituting the plume. Indeed, the larger the droplet size \( r \), the stronger the scattering \( \sim r^6 \). Figure 4.2(b) can thus be interpreted in the sense that the spectrum follows the gradual decrease in droplet size. These data rationalize that the point beyond which no further shift of the peak maximum occurs corresponds to the location where gas-phase R6G ions are formed.

In order to prove the correlation between the spectral evolution observed and the formation of naked ions, we carried out a reference experiment in which LIF of R6G ions was recorded in high vacuum \( (10^{-9} \text{ mbar}) \), inside the ion trap of a Fourier-transform ion cyclotron resonance mass spectrometer (FTICR-MS). Complementary FTICR mass spectral analysis confirmed that the fluorescence measurements were done specifically on gas-phase R6G ions. A remarkable consistence between the LIF spectrum of R6G ions trapped in the FTICR with that obtained under ambient conditions in the lower part of the ESI plume was found, as shown in Figure 4.2(d). This observation provides direct evidence that gas-phase R6G ions are present in high abundance in the lower part of the ESI plume at ambient conditions.

By adjusting the position of the excitation laser, liquid droplets can effectively be discriminated against, such that isolated ions can be selectively probed. The size of the blue spike in Figure 4.2(c) is a characteristic of how fast desolvation is completed and needs to be minimized for easier access to gaseous ions. It was found that by decreasing the sample introduction rate (below 1 \( \mu \text{l min}^{-1} \)) together with increasing the high voltage on the ESI probe more efficient sample vaporization is generally achieved.

At the operation conditions used, no evidence for the occurrence of neutral R6G species in the experiment was found, probably because R6G is already preformed as a cation in solution. In order to discern the presence of neutral species, an alternative setup was used in which the counter electrode was placed orthogonal to the axis of the spray capillary. In this case, charged species are attracted towards the electrode, away from the spray axis, while the neutrals pass straight. No fluorescence was detected on axis starting from ca. 10 mm below the tip of the spray capillary in this case.
Unexpectedly, our data also allow direct insight into the mechanisms of the gas-phase ion production by ESI. A fluorescence profile of the ESI plume was recorded for R6G with 0.5 mm spatial resolution (Figure 4.3(a); each spectrum presented is normalized to its fluorescence maximum).

![Figure 4.3](image)

**Figure 4.3.** Evolution of the fluorescence spectrum when moving down (from red to violet color) along the ESI plume for R6G (top) and Ac-Ala₅-Lys-R575 peptide (bottom).

A two-state spectral characteristic of the transition from parent ESI droplets (red curve) to gas-phase ions (violet curve) is observed as the distance from the capillary tip is increased. Two components were deconvoluted from the experimental data, showing maxima at around 505 nm and 555 nm that corresponded to the presence of gas-phase ions and solvated species, respectively. Although the positions of the maxima were allowed to shift when fitting the spectra, a variation of $\leq 3$ nm was obtained for $\lambda_{\text{max,MeOH}}$, while $\lambda_{\text{max,gas}}$ was found to be constant.
We predict theoretically (TD-B3LYP/6-31+G*) that the addition of only one solvent molecule to
isolated R6G should shift the absorption maximum by about 2 nm. No evidence for the
formation of partially solvated analytes, with a fluorescence maximum at an intermediate
wavelength (between fully solvated and gas-phase), was found, suggesting that their
concentration was very low in the plume. It can therefore be proposed that gas-phase R6G ions
are preferentially formed directly from the liquid droplets (Ion Evaporation Model (22,23))
rather than by gradual solvent evaporation (Charged Residue Model (24,25)). In the latter case, a
gradual shift of fluorescence maximum from 555 to 505 nm should have been observed.

We also investigated the possibility to selectively access gaseous biological molecules using our
method. The peptide Ac-Ala$_5$-Lys was chosen as a model compound because Ac-Ala$_n$-Lys
peptides are the subject of numerous theoretical and experimental studies, due to the high
proposed stability of its helical structure in the gas phase (18,26,27). In the condensed phase,
where the internal stabilizing factors are dominated by interactions with solvent, these short
peptides adopt randomized conformation (28). Ac-Ala$_5$-Lys was labeled by a fluorescent tag,
Rhodamine 575 (R575), at the lysine side chain. In analogy to the R6G experiment, the
fluorescence of the peptide was found to shift as the distance to the capillary tip was increased
(Figure 4.3(b)). Again, starting from a certain point, no further change in the spectrum could be
seen, indicating that formation of gas-phase ions was complete, which was also confirmed by
FTICR-MS combined with trapped ion LIF spectroscopy. The mechanism of ion formation was
found to be similar to that for R6G as no spectral indication of partially solvated species could be
found. Interestingly, the gas-phase fluorescence of the R575-tagged Ac-Ala$_5$-Lys was found to
be significantly shifted relative to the gas-phase fluorescence of R575 ions ($\lambda_{\text{max}} \approx 507$ nm). Both
the order of magnitude ($\approx 650$ cm$^{-1}$) and the direction (towards longer wavelengths) of this shift
can be explained by the Stark effect due to a helix macrodipole (29,30).

The sensitivity achieved when directly probing ESI-produced gas-phase species in the ambient is
substantially higher than that of any setup involving trapped ions. This is because the number of
ions that can be probed by MS is limited by the trapping capacity of the MS instrumentation,
which is generally on the order of a few millions. In contrast, we estimate the total number of
gaseous ions instantaneously present in the plume to be on the order of $10^9$-$10^{10}$ ions. This
estimate was obtained by measuring the electrical current on the counter electrode (Figure
4.2(a)). These numbers underscore that our method can substantially simplify the spectroscopic characterization of gas-phase biological molecules and allow very demanding measurements where the signals are expected to be weak. As an example, we show that despite the low quantum yield of chlorophylls (~0.1) intrinsic LIF spectra can be detected within 10 s, using the in-plume strategy (Figure 4.4).

![Figure 4.4: Fluorescence spectra of gas-phase (red) and solution-phase (green) chlorophyll b ions obtained using our setup. The spectrum recorded from bulk solution (blue) is shown for reference.](image)

Chlorophylls are unequalled convertors of the solar irradiation into “green” electrical energy, and their intrinsic photophysics is therefore of great general interest (31). The intrinsic fluorescence is an important characteristic of the conversion efficiency, but is difficult to obtain experimentally. Using LIF on trapped chlorophyll ions in the FTICR-MS setup, we failed to obtain any fluorescence signals, probably due to the rapid ion decomposition in high vacuum upon absorption of light. On the other hand, when doing spectroscopy on ions created at ambient conditions, they are thermalized much more efficiently, and heat-induced dissociation can be
avoided. Also, unlike MS measurements, direct ESI plume spectroscopy deals with ion flows rather than with a trapped ion population. Therefore, the time over which each ion interacts with the irradiation laser is short (<1ms) compared to that in trapped ion spectroscopy, so that no significant heating occurs. This also allows the use of high laser powers to enhance the signals.

Electrosonic spray ionization (ESSI) (6) is a variation of ESI in which a supersonic nebulizing gas is employed in order to promote efficient desolvation of the droplets. As a result, much narrower peaks are observed in ESSI mass spectra of proteins as compared to conventional ESI. We profiled an ESSI plume by fluorescence spectroscopy, analogous to the experiments demonstrated in Figure 4.2. It was found that the desolvation of R6G was complete starting from ca. 1 cm after the sprayer tip. This observation is very important as it validates the results from several earlier studies that relied on the assumption that gas-phase ions measured by MS were produced in the ambient (6,7,32,33). Owing to the efficient nebulization, ESSI tolerates high sample introduction rates. We were able to observe gas-phase ions using flows of up to 10 µl min\(^{-1}\), which allows further enhancement of the achievable gas-phase ion density as compared to ESI. A high linear velocity of an ESSI plume allows one to guide the ion population without any ion optics. Thus, a small fraction of ionic species produced in ESSI can pass a coiled tube without being neutralized, as observed by MS (34). We were able to detect gas-phase R6G ions that passed a 30 cm long coiled tube, although the LIF signal sensitivity was greatly reduced.

Spectroscopy of vibrationally cooled biomolecular ions is of high interest as it substantially enhances structural information that can be obtained. Current experimental approaches require complex instrumentation and sometimes high-risk investments (17-19,35,36). We built up a setup that allows us to considerably decrease the temperature of unsolvated gas-phase ions, down to ca. 170 K, at ambient conditions (Figure 4.5(a)). Ions created by ESSI were guided into a copper tube. The end of the tube was in direct contact with a liquid nitrogen reservoir. The species exiting the tube were directly probed by LIF. Figure 4.5(b) shows two fluorescence spectra of R6G obtained at room temperature or upon cooling. It can be seen that the “cold” spectrum is sharper and blue-shifted by ca. 10 nm relative to the room temperature spectrum, which is in good agreement with the results obtained using an MS-based approach (35). Further cooling, e.g., by liquid helium, may even result in vibrationally resolved spectra. The 555 nm emission band of the cold spectrum in Figure 4.5(b) corresponds to solution-phase R6G species,
which we attribute to re-condensation of bare ions with solvent droplets occurring at low temperature.

![Diagram](image)

**Figure 4.5**: a) Setup for the cold spectroscopy of gas-phase ions. b) Fluorescence spectra of gas-phase R6G ions obtained at 300 K (red) and 170 K (blue). Determining the precise location of the maximum in case of the cold spectrum was not possible, due to attenuation caused by the excitation laser line filter (488 nm; green shaded area).

### 4.5 Conclusions

The discovery that high densities of unsolvated nonvolatile ions can be produced inside an ESI plume at ambient conditions substantially enhances the experimental arsenal for probing their intrinsic properties. Isolated biomolecules are accessible for characterization by optical spectroscopy, as well as for further investigation using tandem techniques such as gas-phase reactions (7), dissociation (34), heating (37) / cooling (38), soft landing (27,39), etc. using low-cost, easy-to-operate equipment.
4.6 References


Chapter 5

Rhodamines in the Gas Phase:
Cations, Neutrals, Anions, and Adducts with Metal Cations
5.1 Overview

Optical spectroscopy of biological molecules in the gas phase has recently gained considerable attention, being able to provide complementary structural information in the absence of native matrix. Biomolecules can change their properties when brought into the gas phase, and so can chromophores associated with them. Understanding the photophysics of chromophore labels is central for the correct interpretation of experimental data. In this report, the structure and the optical properties of Rhodamine 19 (R19) in the gas phase were examined by a combination of Fourier-transform ion cyclotron resonance mass spectrometry and visible-light laser spectroscopy. While R19 in solution is found either in neutral (R19\textsuperscript{0}) or protonated (R19+H\textsuperscript{+}) forms, other structures can be generated in the gas phase, such as anions (R19-H\textsuperscript{-}) and adducts with metal cations (R19+M\textsuperscript{+}). Experimental evidence for the lactone structure of gas-phase neutral R19 is presented for the first time. The different properties of gas-phase compared to solution-phase R19 are discussed in view of structural analysis of labeled gas-phase biological molecules by optical spectroscopy.

5.2 Introduction

Optical properties of dye vapors started to be explored in 1970s, mainly due to the interest in gas-phase lasers (1-5). This research has been re-energized (6-11) by the recent advances in optical spectroscopy of gas-phase ions that allows the use of chromophore tags to address structural properties of unsolvated biomolecules (10-17). It has been noticed that the photophysics of a chromophore can significantly change when transferred to the gas phase (10,11,15,18). This is due to the lack of specific solvent-chromophore (e.g., dipole-dipole) interactions but also because internal molecular forces are amplified in the absence of solvent shielding. For example, electrostatic interactions, which are about two orders of magnitude stronger in the gas phase than in solution, can be partially responsible for the change in optical properties of a chromophore upon desolvation (10,11).
Rhodamines and their derivatives are extensively used in chemistry and biology, e.g., as fluorescent probes (19), laser dyes (20) and chemosensors (21). In solution they can adopt protonated, zwitterionic and lactone forms (Scheme 5.1).

![Diagram of Rhodamine 19 forms](image)

**Scheme 5.1:** Protonated, zwitterionic and lactone form of Rhodamine 19 in solution.

While the first two forms are quite similar with regard to their optical properties, the lactone is not optically active in the visible. Its formation is promoted by aprotic solvents such as acetone, dichloromethane or benzene (20). The photophysics of gas-phase protonated rhodamine cations has recently been addressed by a number of research groups (6-9,11). Rhodamine cations were produced by electrospray ionization (ESI) (22) and could be spectroscopically probed inside an ion trap of a mass spectrometer (7,9,11) or directly inside the ESI plume (15). Strong visible light absorption and fluorescence has been detected for protonated Rhodamine 6G (R6G+H
\(^+\)) (9,15), Rhodamine 19 (R19+H
\(^+\)) (7,8) and Rhodamine 101 (R101+H
\(^+\)) (11) cations. The experimental access to the properties of neutral rhodamine molecules in the gas phase is more challenging. This is mainly due to the low volatility of rhodamine dyes. Normally, one has to resort to evaporation at elevated temperature in order to build up measurable vapor concentration in the analyzer volume (3-5). Visible light absorption was reported for neutral forms of R19 (5), R110 (5) and Rhodamine B (RB) (2). However, the necessity for elevated temperature in these experiments and therefore possible sample degradation render the interpretation ambiguous. A recent theoretical study by Cavallo et al. suggests that neutral gas-phase rhodamines tend to form lactones (23). Therefore, a controversy currently exists between the experimental evidence
speaking for the optical activity of rhodamine vapors in the visible range (2,4,5) and the formation of the lactone, which should be transparent to visible light, as predicted by theory (23).

In this work, a combination of Fourier-transform ion cyclotron resonance mass spectrometry (FTICR-MS) (24) with visible-light laser spectroscopy was used to study the optical properties of gas-phase Rhodamine 19 (R19) at room temperature. Ionic forms of R19 were generated by ESI: protonated (R19+H+) and cationized (R19+M+, M = Li, Na, K, Rb, Cs) in positive ion mode, and deprotonated (R19-H-) in negative ion mode. Besides that, R19 was covalently tagged to glycine (Gly) and arginine (Arg) via their amino groups, and to the peptide FmocGluGly$_3$LysGly$_3$Glu at the lysine (Lys) side chain (Scheme 5.2).

![Chemical structure for GlyR19 (a), FmocGluGly$_3$Lys(G19)Gly$_3$Glu (b) and ArgR19 (c).](image)

**Scheme 5.2:** Chemical structure for GlyR19 (a), FmocGluGly$_3$Lys(R19)Gly$_3$Glu (b) and ArgR19 (c).

The R19 moiety of the GlyR19 conjugate stays neutral in negative ion mode, which allows spectroscopic interrogation of R19$^-$ in the gas phase of a mass spectrometer. Due to its small size, the Gly residue is unlikely to interact with the chromophore. The optical properties of the xanthene chromophore are only affected by electrostatic interaction with the negatively charged COO- group of the lysine. This effect is however expected to be small due to the considerable
spatial separation between the chromophore and the negative charge (Scheme 5.2a). A similar strategy of attaching a remote charge carrier has recently been applied in order to study the optical properties of a neutral Green Fluorescent Protein (GFP) chromophore in the gas phase in an electrostatic ion storage ring (25). In the work reported herein, gas-phase R19+H⁺, R19-H⁻ and R19+M⁺ showed optical absorption, while R19ⁿ was found to be transparent in the visible range. It can therefore be concluded that the neutral form of R19 forms a lactone in the gas phase. This result is confirmed by the earlier theoretical calculations (23) and those done in our group (vide infra). The intrinsic properties of R19 can change when bound to large biological molecules as a result of possible intramolecular interactions. Thus, we found that R19ⁿ stays transparent when conjugated with FmocGluGly₃Lys(R19)Gly₃Glu, while starts to absorb visible light when tagged with Arg, suggesting that the flexible side chain of Arg interacts with the R19ⁿ moiety to destabilize the lactone structure.

5.3 Methods

5.3.1 Experimental

The experimental setup for optical spectroscopy of gas-phase ions has recently been described (9). Ions are generated by ESI and detected by Fourier-transform ion cyclotron resonance mass spectrometry (FTICR-MS) (24). While trapped inside the analyzer cell, the ions are exposed to continuous wave visible laser light at the 457, 476, 488, 496, 502 or 514 nm lines of an Ar⁺ laser (Innova 300, Coherent, USA). The power (5 mW – 1.5 W) and the duration of laser irradiation (0.5 s – 1 min) are experimental variables. The ions of interest are pre-selected in the FTICR cell prior to the laser light exposure based on their mass-to-charge ratio. All the analytes were electrosprayed from a 1 µM water/methanol mixture (1:1). Gas-phase complexes of R19 with alkali metal cations were generated by the addition of metal salts (LiCl, NaCl, KCl, RbCl, CsI, 100 µM) into the working ESI solutions.

Rhodamine 19 was obtained commercially (05750, Exciton, USA). FmocEG₃KG₃E was custom-synthesized (Peptide 2.0, USA) with 98% purity. Labeling of Gly, Arg and FmocEG₃KG₃E by a
succinimidyl activated R19 (C6157, Invitrogen, USA) was done in-house following standard protocols for cross-linking chemistry.

5.3.2 Theoretical Calculations

Structure optimization (quadratic approximation algorithm) for gas-phase R19+H+, R19-H -, R19+M+ and R19n was done using Gaussian 03 software (26,27). C1 symmetry was applied for R19n and R19-H structures; Cs symmetry was applied for R19+H+ and R19+M+. Different sites of metal binding were tested to find the lowest energy structure: salt-bridge (SB) or charge solvated (CS). The tight optimization criterion (maximum gradient below 2.5×10⁻⁵; root mean square gradient below 8.3×10⁻⁶ Hartree Bohr⁻¹) was applied for all structures. The Hessian was used to check whether the presented geometry is a stationary point (no imaginary frequencies) on the potential energy surface (PES).

The second-order Möller–Plesset (MP2) theory and density functional theory (DFT) with B3LYP, BLYP, BMK, and PBE1PBE density functionals were applied for geometry optimization. Double-ζ 6-31G(d) and 6-31+G(d) Pople-type basis sets were used. R19n/R19+H+ structures were used to evaluate the difference between DFT with different functionals and MP2 predictions: no considerable difference was found. B3LYP density functional was used as a default theory level for other calculations (e.g., for R19-H -).

Single-point energy calculations with the 6-311+G(2d,p) basis set were conducted. A tight self-consistent field (SCF) convergence criterion (ΔE < 10⁻⁸ Eh) was applied.

A basis set superposition error (BSSE) correction (28,29) was not performed since the amplitude is not expected to be greater than 2 kcal/mol. The latter can be neglected in view of the method (e.g., B3LYP/6-31+G*) accuracy of about 4 kcal/mol (30).
5.4 Results and Discussion

Besides gas-phase R19+H⁺ (m/z 415) detected by ESI-FTICR-MS in positive ion mode, we were able to observe R19-H⁻ (m/z 413) upon switching the polarity of the instrument. Although the ion yield of R19-H⁻ was substantially lower than that of R19+H⁺, a detectable MS signal was recorded upon extended ion accumulation. Interestingly, R19-H⁻ can neither be generated in aqueous nor in ethanolic solution, even at very basic conditions, which is due to the high pKa value of the R19 imino group (31). Deprotonation of R19 in our experiments is promoted by the ESI process and the anion is stabilized in the gas phase due to the absence of surrounding solvent. Both R19+H⁺ and R19-H⁻ were established to absorb visible light, as followed from the photoinduced fragmentation observed upon irradiation at 488 nm (5 s, 35 mW for R19+H⁺; 5 s, 5 mW for R19-H⁻).

Both protonated and deprotonated ions of GlyR19 could be detected by ESI-FTICR-MS. The R19 moiety is protonated in GlyR19+H⁺ (m/z 516), while it stays neutral in GlyR19-H⁻ (m/z 514). The negative charge of gas-phase GlyR19-H⁻ is created by deprotonation of the –COOH group of Gly (Scheme 5.2a). Photoinduced fragmentation of GlyR19+H⁺ was observed upon irradiation at 488 nm (5 s, 20 mW), in agreement with the established visible-light absorption of gas-phase R19+H⁺. In contrast, the neutral R19ⁿ moiety of GlyR19-H⁻ revealed no fragmentation, even upon extended exposure to visible light at high intensity (2 min: 457-514 nm, 200 mW-1.5 W). It is known that R19ⁿ can assume two isomeric states in solution: zwitterionic (R19±) in protic solvents, and lactone (R19¹) in aprotic solvents (20). While the visible light absorption of R19± is similar to that of the cationic form R19+H⁺ (31), lactones are transparent to visible light due to the loss of the planarity of the chromophore (Scheme 5.1). We thus conclude that R19ⁿ in the gas phase - an ideally nonpolar environment - adopts the lactone conformation.

The structural assignments for gas-phase R19+H⁺, R19ⁿ and R19-H⁻ derived from the experimental observations are supported by the quantum chemical calculations (Figure 5.1). Both R19+H⁺ and R19-H⁻ preserve the planar structure of the xanthene chromophore responsible for the absorption of visible light. R19ⁿ forms a lactone, as predicted earlier by Cavallo et al. (23).
Figure 5.1: Theoretical predictions (B3LYP) for gas-phase structures of R19: protonated cations $R19^+H^+$ and deprotonated anions $R19-H^-$ have planar xanthene chromophore responsible for the absorption of visible light, while neutral $R19^0$ forms a lactone.

Rhodamines are widely used as fluorescent tags (19). In aqueous solutions, an equilibrium exists between the zwitterionic and protonated forms, which are similar although not identical with respect to their optical properties (31). Therefore, over a wide range of experimental conditions, the lasing properties of rhodamines stay nearly constant. The situation is more complex in the gas phase. Thus, as shown above, the R19 moiety can be completely transparent to visible light in the gas phase. We carried out a number of experiments in which gas-phase biomolecular ions tagged with R19 were exposed to visible light. The peptide FmocGluGly$_3$Lys(R19)Gly$_3$Glu has only one site for protonation, which is on the R19 moiety (Scheme 5.2b). Therefore, it comes as no surprise that gas-phase FmocGluGly$_3$Lys(R19+$H^+$)Gly$_3$Glu ions ($m/z$ 1410) showed visible light absorption, while FmocGluGly$_3$Lys(R19)Gly$_3$Glu-$H^-$ ($m/z$ 1408), in which R19 is neutral, did not. We observed the same phenomenon for gaseous single-stranded DNA tagged with R19, which revealed no visible-light absorption in negative ion mode (data not shown).

Biomolecular ions produced by ESI are usually multiply charged. One can imagine a biomolecule that contains a number of basic groups, e.g. guanidine side chains of arginines. When tagged with a rhodamine label and observed in positive ion mode, such a molecule would fluoresce in the gas phase as a function of charge state: only when a charge state is reached where the rhodamine tag accommodates a proton would the compound become fluorescent.
Furthermore, intramolecular interactions are amplified in the gas phase due to the absence of solvent shielding, greatly affecting the lasing properties of a chromophore. We examined the absorbing capacity of an Arg-R19 conjugate (Scheme 5.2c) in the gas phase. It was quite surprising to find that ArgR19+H⁺ (m/z 615) showed optical activity despite the extremely high basicity. We expected that Arg would carry the proton, while R19ⁿ would form a lactone, analogous to GlyR19-H. The clear visible light absorption of ArgR19+H⁺ can be explained by intramolecular interaction between the carboxyphenyl moiety of R19 and the flexible side chain of Arg in the gas phase, which apparently destabilizes the lactone.

The propensity of rhodamines to easily switch their fluorescence on and off in the gas phase depending on their microenvironment renders their use as chromophoric labels inconvenient. One may need to design protection groups for basic moieties and rigid linkers when attaching the chromophore in order to make sure that the rhodamine tag is fluorescent in the gas phase. Furthermore, two or more chromophores are used in some techniques, e.g., in Förster resonance energy transfer (FRET) (32). Close proximity of the second chromophore (acceptor) to the first one (donor) in FRET leads to a decrease in the fluorescence of the donor chromophore. The quenching efficiency is distance-dependent and therefore provides valuable information on intramolecular distances (32). However, a decrease of the rhodamine emission in the gas phase can also result from deprotonation caused by the addition of the second tag with higher proton affinity, which would render the interpretation of such FRET experiments ambiguous.

Chemical modification of the carboxylic group of rhodamines, e.g., esterification such as in Rhodamine 6G (R6G) avoids lactone conformation (33). R6G is a cationic dye and its lasing properties are not dependent on pH (33). The use of R6G is advantageous both in solution, due to the absence of zwitterions with slightly different properties than the cationic form, as for R19 (31), and in the gas phase, due to the inability of forming the lactone. Ironically, the name of the fluorescent tag called “carboxyrhodamine 6G” which is commercially available from a leading manufacturer of fluorescent labels (C6157, Invitrogen, USA) is not precise. This rhodamine tag does not have an esterified carboxylic group and should rather be named carboxyrhodamine 19. This confusion in nomenclature, while of minor consequence for most of solution-phase measurements, can be fatal when performing gas-phase spectroscopy experiments.
As mentioned earlier, deprotonated anions of R19 can only be stabilized in the gas phase, in the absence of solvent. We were also able to observe complexes of R19 with alkali metal ions (R19+M^+: M^+ = Li^+, Na^+, K^+, Rb^+, Cs^+) in the gas phase. All the complexes absorbed visible light, which directly followed from the photofragmentation observed. This suggests that the metal cations bind to the COO^- moiety of R19, thereby stabilizing the zwitterionic form and preventing the lactone formation. Similarly, metal cations have recently been found to stabilize the zwitterionic form of some amino acids (34,35). The fragmentation yield of the parent complex ions was monitored as a function of the excitation laser wavelength by MS. Action spectra thus obtained are roughly representative of the visible light absorption (Figure 5.2a). The absorption of R19+M^+ shifted to shorter wavelengths as the size of the cation increased. This is due to the increasing dipole moment of the Ph-COO^-M^+ moiety, which has a strong influence on the optical properties of the xanthene chromophore (8,31,36). Theoretical predictions made for gas-phase R19+Li^+, R19+Na^+ and R19+K^+ complexes support a salt-bridge (SB) structure of R19+M^+ complexes (Figure 5.2b). Alternatively, one could expect the metal cations to be chelated by the xanthene π electron system and one of the oxygen atoms in the carboxylic group, which would promote the lactone formation (Figure 5.3, Table 5.1).

No shifts in the visible light absorption of R19 were observed in water/methanol solutions when different metal salts were introduced, even at high concentrations. It can therefore be suggested that the gaseous metal complexes of R19 are created as the binding partners are brought to the gas phase, rather than pre-formed in the solution, where metal cations are fully solvated. Cation–ligand association is expected to take place at the latest stages of the ESI process when the solvent molecules shielding R19 ions are essentially evaporated out of the charged droplets. This finding emphasizes that the intrinsic interactions between R19 and metal cations cannot be addressed in the solution phase.
Figure 5.2: a) Normalized dissociation action spectra for gas-phase complexes of R19 with proton and alkali metal cations; b) theoretically predicted structure for R19+Na⁺.
Figure 5.3: Two possible coordination sites of alkali metal cation to bind R19: 1) the COO\(^-\) group to form salt bridge structure (SB); 2) the xanthene chromophore and one of the oxygen atoms in the carboxylic group to form charge solvated structure (CS). The formation of salt bridge in the gas phase is preferential, as follows from the energy difference between SB and CS (Table 5.1).

Table 5.1: Energy difference (in kcal mol\(^{-1}\)) between the salt bridge (SB) and charge solvated (CS) forms of gas-phase R19+M\(^+\) for M = H and M = Na.

<table>
<thead>
<tr>
<th></th>
<th>Energy difference (kcal mol(^{-1}))</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>MP2</td>
</tr>
<tr>
<td>R19+H(^+)</td>
<td>67.7</td>
</tr>
<tr>
<td>R19+Na(^+)</td>
<td>3.0</td>
</tr>
</tbody>
</table>

Single point energy calculation with 6-311+G(2d,p) basis set at 6-31G(d) optimized geometries.
5.5 Conclusions

Although mass spectrometry is commonly thought of as a tool to probe molecular ions, it can also be applied to study the properties of neutral species when tagged to a remote charged carrier. When modified this way, the neutral species can be delivered into the analyzer region, where they can be probed by more structure-sensitive methods. This strategy allowed us to experimentally establish the lactone conformation of neutral Rhodamine 19 in the gas phase by a combination of ESI-FTICR-MS with laser spectroscopy. This result was confirmed by quantum chemical (DFT/MP2) calculations. The Rhodamine 19 lactone can unfold in the gas phase upon protonation, cationization or due to intramolecular noncovalent interactions with a substrate molecule. The interaction between a biological molecule and its tag is generally much stronger in the gas phase than in solution due to the lack of a solvent shield. A variety of forms that rhodamines can assume in the gas phase can be associated with considerable optical shifts that are not observed in analogous solution-phase spectroscopy measurements. “Recalibrating” the behavior of fluorophores from solution to vacuum is crucial to correctly interpret the observations in gas-phase spectroscopy experiments.

5.6 References


Chapter 6

Optical Properties of Protonated Rhodamine 19 Isomers in Solution and in the Gas Phase
6.1 Overview

Visible light absorption and fluorescence of three positional isomers of protonated Rhodamine 19 (\(o\), \(m\) and \(p\)-R19H\(^+\)) were studied in solution and in the gas phase. In solution, strong solvatochromic effects lead to spectral shifts between rhodamine isomers. In contrast, in the gas phase, these species were found to exhibit very similar fluorescence, while pronounced differences were observed in the absorption spectra. The \(o\)-R19H\(^+\) was found to have the largest Stokes shift in the gas phase (around 10 nm), suggesting that an intramolecular relaxation operates in the excited electronic state for this isomer. Several mechanisms for this relaxation are proposed, such as the change of the dihedral angle between the carboxyphenyl group and the xanthene chromophore or that between the carboxylic group and the phenyl ring.

6.2 Introduction

The special fluorescence properties of rhodamines and their derivatives, such as their extremely high quantum yields, photostability and matrix sensitivity (1,2) are responsible for their wide application in many disciplines as fluorescent probes (3), laser dyes (1,2), thermo- (4,5) and chemosensors (6-9), molecular switches (10,11), or fluorescent standards (12-14). Understanding the processes that determine the optical behavior of rhodamines is important for establishing a connection between a phenomenological description of their fluorescence behavior and a rigorous theoretical treatment. The presence of numerous, simultaneously acting mechanisms considerably complicates the examination of these photophysical properties. One needs to account for a complex interplay of intrinsic factors, determined by molecular structure, and external factors, for example environment polarity, viscosity, pH, temperature, concentration, impurities etc. Altogether, these variables often render unambiguous data interpretation difficult (1,2,15-25).

Here we report on investigation of the optical properties of isomeric protonated Rhodamine 19 (\(o\), \(m\) and \(p\)-R19H\(^+\), Scheme 6.1), both in solution and in the gas phase. The number of factors that contribute to the observed optical behavior is dramatically reduced in the gas phase, which facilitates interpretation of experimental observations.
Scheme 6.1. Gas-phase cations of rhodamine dyes produced by ESI: schema and theoretically predicted structures in the ground electronic state (B3LYP/6-31G*). A dihedral angle between the carboxyphenyl and the xanthene rings (φ) in the unsolvated ground state of \( o \)-R19H⁺ is 90°, while it is ca. 45° for \( m \)-R19H⁺ and \( p \)-R19H⁺ isomers.

6.3 Methods

Gas-phase R19H⁺ cations were produced by electrospray ionization (ESI) (26). Their visible light absorption was studied by photodissociation action spectroscopy in the high vacuum (<10⁻⁸ mbar) of a Fourier-transform ion cyclotron resonance mass spectrometer (FTICR-MS) (27). Fluorescence measurements were conducted both on ions trapped in the FTICR (27) and directly inside the ESI plume (28). In order to avoid possible carry-over effects during the ESI experiments from the sample accumulation in transport tubing and capillaries of the ion source, disposable ESI units were employed (Figure 6.1): a 50 μm ID fused silica capillary was passed through a hole at the bottom of a 0.5 mL Eppendorf tube. The tube was then filled with a 1 mM
solution of one of the rhodamine isomers in methanol. ESI was initiated by applying a voltage of 6.5 kV directly to the solution via a stainless steel needle.

**Figure 6.1.** Schematic of a home-built unit used in the ESI experiments. ID = inner diameter, SS = stainless steel.

### 6.3.1 Dissociation spectroscopy

Ions trapped inside the ICR cell were subjected to continuous-wave (CW) Ar⁺ ion laser irradiation (*Innova 300*, Coherent, USA) at six discrete wavelengths (457, 476, 488, 496, 502, 514 nm) at equal fluence. Dissociation action spectra were obtained based on the relative depletion of the parent signal intensity in the mass spectrum, resulting from the photoinduced ion fragmentation inside the measuring cell \( A = \log(I_0/I) \). The laser fluence was adjusted in order to maximize the dynamic range of the action spectrum for each isomer.

### 6.3.2 Fluorescence spectroscopy of gas-phase ions

Fluorescence spectroscopy of gas-phase ions was performed both in high vacuum of the ICR cell and at ambient conditions in the ESI plume, as described elsewhere (27,28). In both cases, the fluorescence emission was analyzed by a holographic imaging spectrograph (*HoloSpec f/1.8i*, Kaiser Optical Instruments Inc, Ann Arbor, USA) with CCD detection (*LN/CCD-2500-PB/VISAR*, Princeton Instruments, Trenton, USA). The spectral response of the CCD camera was
calibrated using a halogen lamp (HLX 64342, OSRAM, Germany). Fluorescence signals recorded from ion populations trapped in the ICR are substantially weaker than those from gas-phase ions in ESI plume. This is due to both the lower instantaneous analyte concentration in the ICR analyzer volume and the limited optical detection efficiency in the ICR (28). In order to achieve detectable signals in ICR experiments, fluorescence of gas-phase R19H\(^+\) isomers was excited at 488 nm, which is near their absorption maximum (vide infra). Conversely, in the ESI experiments, we were able to detect fluorescence from gas-phase R19H\(^+\) ions excited with 488 nm as well as with 476 nm. Activation at 476 nm allowed us to record emission spectra that were less affected by the excitation laser line.

Laser fluences in the range of 8 - 20 mJ/mm\(^2\) were used in the ICR experiments. A net fluorescence spectrum was averaged from ca. 200 acquisitions. Applying higher fluences resulted in notable dissociation due to vibrational heating of the trapped ions (29). Inside the ESI plume, each ion is exposed to laser light for < 1 ms (28) (<0.2 mJ/mm\(^2\)) and therefore absorbs considerably fewer photons than in ICR experiments. The overall acquisition times in the experiments done in the ESI plume were in the range of 1-60 s. All spectra collected in the ICR and the ESI plume were background corrected.

6.3.3 Solution measurements

Absorption spectra of R19H\(^+\) isomers in solution were recorded on a spectrophotometer (Lambda 6, Perkin Elmer, Waltham, USA). Fluorescence measurements were done using the same detection platform as for the gas-phase experiments. The concentration of R19H\(^+\) isomers was 0.5 \(\mu\)M (in water, methanol or isopropanol). Protonation of the isomeric rhodamines was achieved by acidifying the working aqueous, methanolic and isopropanolic solutions to pH \(\approx\) 2 by the addition of trifluoroacetic acid (15). Since both R19 and TFA are strong electrolytes, and expected to be fully dissociated in all solvents used, the R19H\(^+\) cation should be effectively shielded from the CF\(_3\)CO\(_2\)^- anion, and the influence of CF\(_3\)CO\(_2\)^- on the optical properties of R19H\(^+\) should be minimal.
6.3.4 Materials

The ortho R19 isomer (o-R19) was commercially available (05750, Exciton Inc, Dayton, USA). The other isomers (m-R19 and p-R19) were custom synthesized by the same supplier. All compounds were used in their free-base form. For all experiments described here, spectroscopy-grade methanol (Acros-Organics, Basel, Switzerland), isopropanol (Sigma-Aldrich, St. Louis, USA) or ultra-high purity water (ETH facilities) were used as solvents.

6.3.5 Theoretical calculations

Structure optimization (quadratic approximation algorithm) for the gas-phase R19H⁺ isomers (note that here we found it more convenient to use the notation R19H⁺ instead of R19+H⁺ employed in Chapter 5) was done using Gaussian 03 software (30,31). C₁ symmetry was applied with the tight optimization criterion (maximum gradient: 2.5×10⁻⁵; root mean square gradient: 8.3×10⁻⁶ Hartree Bohr⁻¹) for all structures. The Hessian was used to check whether the presented geometry is a minimum on the potential energy surface (PES).

A second-order Møller–Plesset (MP2) level of theory and B3LYP, BLYP, BMK, PBE1PBE (PBEO) density functionals were applied for geometry optimization. Structure predictions of R19H⁺ produced the same results by all the methods. For all the other isomers, B3LYP was applied by default. A 6-31+G(d) Pople-type basis set was used. Single-point energy calculations (tight SCF convergence criterion) with 6-311+G(2d,p) basis set were conducted.

A basis set superposition error (BSSE) correction (32,33) was not performed since the amplitude is not expected to be greater than 2 kcal/mol. The latter can be neglected in view of the method accuracy (±4 kcal/mol) (34).
6.4 Results and Discussion

6.4.1 Dissociation spectroscopy

When continuously irradiated with visible light, protonated fluorescent o-R19H⁺, m-R19H⁺ and p-R19H⁺ cations undergo many photon absorption/emission cycles. In high vacuum, in the absence of efficient collisional cooling, ions gradually accumulate vibrational energy according to the “Frank-Condon walking” mechanism (29). Ion fragmentation occurs as soon as the build-up of internal energy exceeds the unimolecular dissociation threshold, on the millisecond to second time scale. This mechanism is analogous to that observed for infrared multi-photon dissociation (IRMPD), blackbody infrared radiative dissociation (BIRD) or sustained off-resonance irradiation collision-induced dissociation (SORI-CID), in which slow ion activation is achieved by multiple absorption of infrared photons or collisions with buffer gas molecules, respectively (35).

In action spectroscopy the dependence of the fragmentation yield on the wavelength of the excitation light can be thought of, in rough terms, as the absorption spectrum of the analyte. Nevertheless, any quantitative analysis of the visible light absorbance needs to be applied very carefully, since the internal energy of the system can significantly increase on the time scale of the dissociation process. This situation is analogous to that in IRMPD spectroscopy measurements, in which the spectral lines are broadened due to vibrational heating (36).

In our experiments R19H⁺ cations trapped inside the ICR cell were subjected to CW laser irradiation at six discrete wavelengths (457, 476, 488, 496, 502, 514 nm) with equal fluence. Dissociation action spectra were obtained based on the relative decrease of the parent signal intensity in the mass spectrum that resulted from the photoinduced ion fragmentation inside the measuring cell (Figure 6.2, dark blue color). The spectral bands of o-R19H⁺ were found to be considerably blue-shifted (by ≈ 5-10 nm) relative to m-R19H⁺ and p-R19H⁺, which showed close similarity. Our observations reflect the intrinsic photophysics of the S₀–S₁ transition since all the external factors associated with the condensed phase are not operative in the gas phase (e.g., hydrogen bonding, ion-dipole, dipole-dipole, dispersion interactions, etc.). According to our theoretical calculations (see structures in Scheme 6.1), the mutual arrangement of the carboxyphenyl and the xanthene rings in the unsolvated ground state of o-R19H⁺ is expected to
be orthogonal, while a dihedral angle of ca. 45° should be found for \( m\)-R19H\(^+\) and \( p\)-R19H\(^+\) isomers (angle \( \phi \) in Scheme 6.1). The latter orientation is close to that found in biphenyls (37). \( o\)-R19H\(^+\) conformers that are formed by the 180° turn of the carboxylic group around the phenyl ring (angle \( \theta \) in Scheme 6.1) were found to have almost the same energy (difference < 0.1 kcal mol\(^{-1}\)). We therefore estimate both of these conformers to be equally represented in the gas phase.

![Normalized optical spectra of R19H\(^+\) isomers](image)

**Figure 6.2.** Normalized optical spectra of R19H\(^+\) isomers in the gas phase and in solution. **Dark blue:** photodissociation action spectra in the gas phase. The spectra are discrete due to the limited number of excitation lines provided by the Ar\(^+\) ion laser. **Blue:** fluorescence recorded in the gas phase of the ESI plume (smoothed). **Dark red:** absorption spectra in methanol. **Red:** fluorescence spectra in methanol.

The processes responsible for the blue-shifted absorption of \( o\)-R19H\(^+\) relative to the other isomers are at first glance obscure. One reason for this shift could be in the spatial proximity between the carboxylic group and the xanthene chromophore of \( o\)-R19H\(^+\). The Ph-CO\(_2\)H dipole (38) can directly perturb the xanthene \( \pi \) electron system via electrostatic interactions, as was proposed by earlier solution-phase studies (15,16). This effect is much weaker in \( m\)-R19H\(^+\) and
$p$-R19H$^+$ isomers due to the remote location of the Ph-CO$_2$H dipole relative to the chromophore core. An alternative way to account for the observed optical shift in R19H$^+$ isomers is to consider the mutual orientation of the xanthene and aryl rings, characterized by dihedral angle $\phi$. The quadrupole moment (39) of the aryl moiety induces perturbations in the $\pi$-electron system of the xanthene moiety, which is manifested in optical spectra. The different spectrum of $o$-R19H$^+$ compared to $m$-R19H$^+$ and $p$-R19H$^+$ can then be explained by the orthogonal configuration of the interacting rings ($\phi = 90^\circ$) (Scheme 6.1).

6.4.2 Absorption in solution

We performed a set of complementary solution-phase measurements in which visible light absorption of the R19H$^+$ isomers was recorded in water, methanol and isopropanol (Figure 6.3).

![Figure 6.3. Visible light absorption spectra of R19H$^+$ isomers in water, methanol, and isopropanol.](image)
The results obtained corroborate our inference from the gas-phase experiments, since visible light absorption of $o$-R19H$^+$ was also found to be blue-shifted relative to $m$-R19H$^+$ and $p$-R19H$^+$. The magnitude of the shift decreases in the order isopropanol $>$ methanol $>$ water, likely due to a more efficient solvation of the carboxyl group. The increased shielding weakens the effect of the Ph-CO$_2$H group on the xanthene chromophore, as proposed in earlier solution-phase studies of rhodamines (15,16).

For all isomers, the absorption of R19H$^+$ cations in the gas phase is considerably blue-shifted relative to the solution phase (by ca. 30 nm: Figure 6.2). Both the ground and the excited states of the chromophore are stabilized by solvation (40). The observed position of the absorption bands for R19H$^+$ cations in solution indicate that the energy of the excited state is affected by external factors to a larger extent than that of the ground state, due to a higher magnitude of its dipole moment (40) (Scheme 6.2). The blue shift in the absorption of rhodamine ions upon desolvation has also been observed in earlier studies (27,29,41).

Scheme 6.2. Schematic representation of the processes responsible for fluorescence behavior of rhodamines in the gas phase and in solution.
6.4.3 Temperature of R19H\(^+\) in the gas phase upon exposure to laser irradiation

The gas-phase R19H\(^+\) ions produced inside the ESI plume rapidly equilibrate to the ambient temperature. The internal energy of ions can change during their transfer to high vacuum. Still, the ion population that is ultimately trapped in the FTICR relaxes back to room temperature due to collisions with buffer gas molecules in the hexapole ion guides. Here we consider how the temperature of initially cold ions (ca. 300 K) changes upon exposure to CW laser irradiation, both in the ESI plume and in the FTICR instrument.

In the ESI experiments, each ion is exposed to a laser fluence of less than 0.2 mJ/mm\(^2\) (see Section 6.3.2). During the exposure, ions are vibrationally heated by multiple absorptions of light while, at the same time, cooled down due to the frequent collisions with ambient gas molecules. Fluorescence spectra of R19H\(^+\) recorded at various laser fluences did not reveal any notable difference in spectral shape and position of the maximum. Therefore, we conclude that the internal energy of gas-phase R19H\(^+\) in ESI is not measurably altered by the laser irradiation, which is both due to the low laser fluence and the high efficiency of collisional cooling by the ambient air.

Conversely, ions trapped in the FTICR instrument are gradually heated because of the absence of a dissipation channel for excess energy in high vacuum. Also, these ions are exposed to higher laser fluence. Figure 6.4 shows fluorescence spectra of trapped o-R19H\(^+\) ions obtained at different fluences.

A slight shift of the spectrum to longer wavelengths can be seen as the fluence is increased, reflecting the increasing temperature of o-R19H\(^+\) ions during the measurement. This observation is consistent with earlier reports on blue shifts of the fluorescence of rhodamine ions upon cooling (28,42). The issue of vibrational heating in FTICR introduces some uncertainty regarding the temperature of the trapped ions. As can be seen from Figure 6.4, laser irradiation needs to be minimized in order to maintain rhodamine ions near room temperature during the measurement. Figure 6.5 shows fluorescence spectra of o-R19H\(^+\) ions obtained in the ESI plume and in ICR at a laser fluence of 8 mJ/mm\(^2\).
Figure 6.4. Fluorescence spectra of $o$-R19H$^+$ ions trapped in FTICR obtained upon exposure to different laser fluences. Smoothed spectral lines are shown.

Figure 6.5. Fluorescence spectra of gas-phase $o$-R19H$^+$ ions trapped in the FTICR (gray) and in the ESI plume at ambient conditions (blue). Laser fluence was 8 mJ/mm$^2$ in the FTICR experiments and < 0.1 mJ/mm$^2$ when probing the ESI plume. The spikes in the spectra are due to cosmic rays recorded by the CCD camera. The spikes are much more abundant in the ICR spectrum, reflecting longer acquisition time needed to accumulate the spectrum (10 s in ESI vs. 10 min in ICR). The negative intensity values are due to background subtraction.
The good agreement between the spectra suggests that there is no substantial heating under these experimental conditions, highlighting the possibility to record relatively cold spectra. However, using low fluences in ICR experiments compromises the ability to acquire spectra with high signal-to-noise ratio (Figure 6.5). For this reason, fluorescence spectra of R19H+ isomers obtained in the ESI plume are used for further discussion.

As opposed to the experiments, which are carried out at room temperature (≈ 300K), the quantum chemical calculations are performed at 0 K. The internal temperature is known to not have any effect on the time-averaged structures obtained using standard methods (e.g., DFT-B3LYP), provided the dissociation threshold has not been reached (43). However, the standard deviations of atomic positions do increase upon heating due to vibrational motions of the molecule, which would only influence the accuracy of spectral (not structural) predictions at 300 K (44).

6.4.4 Fluorescence in solution and in the gas phase

While there is a good qualitative correlation between the optical absorption of the R19H+ isomers in solution and in the gas phase, no such agreement was found for the measured fluorescence profiles (Figures 6.2, 6.6). Most surprisingly, the fluorescence emission of all three R19H+ isomers was very similar in the gas phase, which contrasts with the results from the absorption measurements. The maximum in the fluorescence spectra was found to be unchanged when excited at either 476 or 488 nm, for all the R19H+ isomers in the gas phase. This observation reflects that the vibrational energy of the chromophore is redistributed across other vibrational modes of R19H+ (e.g., phenyl ring) prior to the emission of light. The Stokes shift – the difference between the fluorescence and absorption maxima – was found to be very small for gas-phase m-R19H+ and p-R19H+ isomers (Figure 6.2). This is expected given the absence of external influences that would affect the energy of the excited state in the gas phase (Scheme 6.2). A non-zero Stokes shift is, however, apparent for gas-phase o-R19H+, indicating that some intramolecular relaxation takes place prior to the emission of light. Since the fluorescence of all isomers in the gas phase peaks at the same wavelength, it can be suggested that this relaxation is associated with the formation of an excited-state conformation in which the position of the
carboxylic substituent is not significant for the photophysical behavior (Scheme 6.2). Below we discuss two possible conformational changes.

As proposed above, direct electrostatic interaction between the carboxyphenyl dipole and the π electron system of the xanthene chromophore may be responsible for the different absorption of \(\alpha\)-R19H\(^+\). A 180° rotation of the CO\(_2\)H group (\(\theta\)) will dramatically change the orientation of the overall Ph-CO\(_2\)H dipole moment (38). In the ground state, the net electrostatic effect of the Ph-CO\(_2\)H moiety on the xanthene core is a superposition of the local dipole moments of the rotamer populations. Experimental gas-phase fluorescence spectra of the positional R19H\(^+\) isomers are nearly identical. This suggests that the influence of Ph-CO\(_2\)H becomes weaker in the excited state, possibly due to the reorientation of its dipole moment. Thus, the conformation in which the OH group is closer to the chromophore has less influence on the xanthene core and can become the preferential orientation for \(\alpha\)-R19H\(^+\) in the excited state. In other words, the larger Stokes shift for \(\alpha\)-R19H\(^+\) may be due to a population redistribution of the carboxylic group rotamers upon excitation.

**Figure 6.6.** Fluorescence spectra of R19H\(^+\) isomers in water, methanol and isopropanol.
The shifts in absorption for R19H\(^+\) isomers can also be related to a different dihedral angle \(\phi\) as discussed above. The absence of the corresponding shifts in the gas-phase fluorescence suggests that the ring orientation in the excited state becomes essentially identical for all three isomers. The conformation of the \(m\)- and \(p\)-R19H\(^+\) cations should not significantly change upon excitation, which follows from their vanishing Stokes shifts. It can thereby be proposed that rotation around the central C–C bond in the \(o\)-R19H\(^+\) cation can be another factor responsible for the observed photophysical behavior. No conformational change in the excited electronic state was indicated by recent theoretical studies for rhodamine 6G (R6G) (45). However, some rotamers, in particular those formed by rotation around \(\theta\), were not considered in that work. Also, it is not clear to what extent the predicted structure of R6G that has a bulky CO\(_2\)Et substituent is representative of R19H\(^+\), where the steric constrains on the internal rotation (\(\phi\)) are smaller. Besides that, the reliability of current theoretical methods for structural predictions of many-atom rhodamine cations in the excited state is disputable (46). In this study we did not attempt structure predictions for R19H\(^+\) in the excited state. Our findings emphasize the importance to carefully consider sparsely populated rotamers in the ground electronic state. The excited-state repopulation will manifest itself in a different dipole moment and therefore influence the optical properties.

The solution-phase fluorescence emission of R19H\(^+\) isomers is affected by the interaction of the excited-state chromophore with solvent. Absorption of light promotes a change in the orientation and magnitude of the inherent dipole moment, as shown in Scheme 6.2. The interaction with solvent stabilizes the excited state via an orientational relaxation of molecular dipoles prior to the emission of light (~ \(10^{-10}\) s) (40). This relaxation mechanism is the major contributor to the net Stokes shift in solution and is responsible for the solvatochromism of rhodamine dyes (19). In a simplified picture, \(p\)-R19H\(^+\) is the isomer that offers the least obstacles for the solvent molecules to interact with the xanthene chromophore, while in case of \(o\)-R19H\(^+\), the carboxylic group partially blocks the xanthene chromophore core. One may propose that this leads to a situation in which the energy of the excited state for \(p\)-R19H\(^+\) is reduced the most, and hence \(p\)-R19H\(^+\) should exhibit the largest Stokes shift in solution. The opposite is true for \(o\)-R19H\(^+\): poorer stabilization of the excited state and therefore a smaller Stokes shift due to the less efficient solvation. This is exactly what is observed (Figures 6.3, 6.6). Furthermore, a trend can be seen
that the Stokes shifts get smaller in the order water > methanol > isopropanol. For example, the Stokes shift for \( o-R19H^+ \) in water is \( \approx 7 \) nm greater than in isopropanol. This reflects a higher capacity of water to stabilize the excited state of R19H\(^+\), which is due to the high polarity, capacity for H-bond formation and mobility of water molecules.

Due to the complex interplay of intrinsic and external factors in the condensed phase, it is not evident whether the excited-state conformational relaxation proposed for gas-phase \( o-R19H^+ \) also occurs in solution. In solution, intramolecular relaxation competes with the orientational relaxation by solvent molecules. If the latter mechanism is fast, system equilibration via the internal orientational relaxation may no longer be operative.

### 6.5 Conclusions

A combination of trapped ion mass spectrometry with laser spectroscopy allows the optical properties of nonvolatile species to be probed in the gas phase, without any solvent or matrix effects. Gas-phase spectra add new information that contribute to the understanding of the intrinsic molecular photophysics of these compounds, and complements that available from conventional condensed-phase measurements, which is dominated by interactions with the solvent. In this study, the presence of an intrinsic relaxation for the excited electronic state of protonated gas-phase \( o-R19H^+ \) cations was discovered using laser-induced fluorescence and dissociation spectroscopy. We attribute the observed relaxation of \( o-R19H^+ \) in the gas phase to a conformational change upon excitation. To what extent this intrinsic mechanism is relevant to the solution-phase behavior is an open question that awaits further investigation.

### 6.6 References


Chapter 7

Absorption of the Green Fluorescent Protein Chromophore Anion in the Gas Phase Studied by Laser-Induced Photodissociation Spectroscopy
7.1 Overview

The optical absorption of the green fluorescent protein chromophore anion (HBDI⁻) in the gas phase has been addressed by a number of experimental and theoretical studies; however, there is no consensus yet. In this report, the intrinsic absorption of HBDI⁻ was probed by photo-induced dissociation (“action”) spectroscopy using discrete lines of a continuous-wave (CW) laser source. The observed spectral profile of dissociation efficiency revealed a pronounced dependence on the laser irradiance. At very low irradiance (< 0.4 mW cm⁻²) dissociation of gas-phase HBDI⁻ is mostly promoted by single-photon transitions and suggests an absorption maximum of gas-phase HBDI⁻ < 458 nm. This estimate is consistent with the extrapolation of absorption spectra in solution and points to a considerable bathochromic effect of the protein on the absorption of free HBDI⁻. Our data raise doubts on the interpretation of earlier results obtained in the multi-photon regime with pulsed lasers.

7.2 Introduction

The green fluorescent protein (GFP) is an intrinsically fluorescent protein, which is extensively used as a genetic marker in cellular biology (1). It has a barrel structure that consists of β-sheets with a chromophore-containing α-helix passing through the center (2,3). In wild-type GFP, the chromophore 4’-hydroxybenzylidene-2,3-dimethyl-imidazolinone (HBDI) is normally present in either neutral or deprotonated (HBDI⁻, Scheme 7.1) form, leading to the absorption bands at around 395 and 480 nm, respectively (4).

![Scheme 7.1: Anionic form of the model GFP chromophore: HBDI⁻.](image)
The native protein conformation is directly responsible for the bright fluorescence of HBDI ($\lambda_{\text{max}} \approx 510$ nm, quantum yield $\approx 0.8$, lifetime $\approx 3$ ns) (5), which is weakened by at least three orders of magnitude upon protein unfolding (6). The underlying mechanisms of the protein-chromophore interaction which enables fluorescence are not entirely understood (7). It is thought that the protein modulates the chromophore photophysics via both a steric and an electronic influence (8). The tight packing of the chromophore provided by the native GFP conformation restricts its rotational freedom. The extent of this restriction affects the rate of quenching isomerization reactions in the excited state and has been shown to directly influence the fluorescence quantum yield (9). Besides the steric influence that the protein interior imposes on the chromophore rotational freedom, a considerable effect on the energy of electronic transitions is expected.

Visible light absorption of HBDI in solution is generally significantly blue-shifted compared with the absorption of HBDI in wt-GFP, consistent with a strong protein-chromophore interaction (10,11). A multivariant Kamlet-Taft fit (12) of the HBDI absorption maxima in different solvents allowed Dong et al. to separate the contributions from selective (H-bonding) as well as from nonselective (dipole-dipole interaction) solvation to the electronic transition (10). Extrapolation of this fit to both zero hydrogen bond donor/acceptor properties and zero polar solvation parameters yields an estimate for the intrinsic absorption maximum of HBDI in the absence of solvent around 437 nm, which is ca. 45 nm blue-shifted relative to the absorption of HBDI inside the protein. A very similar estimate was obtained (440±5 nm) upon direct extrapolation of absorption maxima measured in non-polar solvents to the vacuum dielectric constant, $\varepsilon = 1$ (13). Unfortunately, there is no single solvent parameter which adequately describes the solvatochromism of HBDI (7). Therefore, the bathochromic shift in the absorption of HBDI inside the protein cannot be entirely assigned to a specific interaction (e.g., H-bonding) but rather reflects a unique microenvironment provided by the protein interior.

The effect of the protein on the electronic transition energies in HBDI deduced from solution-phase experiments has been questioned by the results of pioneering photoabsorption action spectroscopy experiments carried out on HBDI in the gas phase by the groups of Andersen (14,15) and Jockusch (16). In these experiments, gas-phase HBDI ions trapped inside an electrostatic storage ring (14) or a quadrupolar ion trap (16) were exposed to irradiation produced by a tunable laser source. Both ionic (16) and neutral (14) fragment yields as well as parent ion.
depletion (16) were monitored as a function of the excitation wavelength and revealed a maximum at around 480 nm, which is very close to the absorption maximum of HBDI– in the protein. It was therefore proposed by Andersen and coworkers that the GFP scaffold offers a near-vacuum environment for the chromophore, although with restrictions on its rotational freedom (14). In our opinion, this conclusion is quite speculative, as the lack of a shift in an electronic transition does not automatically imply that “the actual environment of the chromophore inside the protein cavity is much closer to vacuum than to bulk solution” (14). In fact, the absorption of HBDI– in DMSO and DMF was also found to be very similar to that in the protein (10,17). Indeed, the very fact that the absorption of HBDI– in the gas phase deduced from action spectroscopy is very different from that extrapolated from solution-phase studies is quite unsettling. The key difference between the solution-phase measurements and those in the gas phase is that multi-photon processes become important in the latter (16). For a more accurate determination of the absorption at $\varepsilon =1$ (vacuum), the contribution of these multi-photon processes needs to be minimized, by employing lower activation power, which is difficult or impossible to implement with pulsed laser sources (vide infra).

Herein, we present the results of photoabsorption action spectroscopy experiments performed on HBDI– ions trapped inside the Penning trap of a Fourier-transform ion cyclotron resonance mass spectrometer (FTICR-MS) using low power, continuous-wave (CW) laser irradiation.

7.3 Experimental

The experimental setup for laser spectroscopy of gas-phase ions has recently been described in detail (18). HBDI– ions ($m/z$ 215) were produced by electrospray and then trapped inside the ICR cell ($5 \times 10^{-9}$ mbar). Trapped ions were excited with discrete lines of an Ar$^+$ ion laser (458, 476, 488, 496 and 502 nm). Depletion of the parent HBDI– ion signal in the mass spectrum ($I(m/z$ 215)/$I_0(m/z$ 215)) was monitored as a function of the excitation wavelength (16).
7.4 Results and Discussion

The limited number of available wavelengths in our experiments does not allow us to record dissociation spectra with high resolution; however, the position of the maximum can still be assessed. Two major fragmentation channels were found, neutral methyl loss (fragment ion at \( m/z \) 200) and photoelectron detachment (\( ePD \)), in agreement with the findings of Forbes and Jockusch (16). To detect the detached electrons we used SF\(_6\) buffer gas, which efficiently traps low-energy electrons to form SF\(_6^–\) anions (\( m/z \) 146) (19). It is worth noting that the operation of \( ePD \) mechanism in earlier experiments could not be rigorously proven, since neither the detached electron nor the neutral product can be directly detected in ion trap mass spectrometers (16).

In order to investigate the effect of the laser irradiance on the measured absorption maximum of HBDI\(^–\), a series of measurements were performed in which laser irradiance (\( I \)) and exposure time (\( t \)) were simultaneously varied such that a constant laser fluence (\( F \)) was maintained (\( F = I \cdot t = 43 \) mJ cm\(^{-2}\)). Under these experimental conditions, the final parent ion depletion is expected to be independent of irradiance for a single photon process, while it should grow with laser irradiance for a multi-photon absorption process. Figure 7.1a shows the dependence of the fragmentation yield (\( \Phi_{215} = 1 - I(m/z \ 215)/I_0(m/z \ 215) \)) for HBDI\(^–\) on laser irradiance for five different excitation wavelengths. The fragmentation yields of HBDI\(^–\) were separately plotted as a function of excitation wavelength to obtain a spectral representation (Figure 7.1b).

The fragmentation yield in Figure 7.1a clearly grows as a function of laser irradiance at each wavelength until it saturates near \( \Phi_{215} = 1 \) (all the parent HBDI\(^–\) ions are destroyed), indicating that ion fragmentation occurs mainly via a multi-photon absorption process. Note that data points obtained in the saturation regime (\( I > 2 \) mW cm\(^{-2}\) for 458, 476, and 488 nm in Figure 7.1a) are indeed no longer indicative of the actual dissociation rate. As the laser irradiance is decreased, single-photon dissociation becomes more pronounced. This can be deduced from the decreasing slope of the fragment yield with respect to laser irradiance (Figure 7.1a, dashed line). For example, for the curve that corresponds to excitation at 458 nm, the single-photon regime is reached at ca. 0.4 mW cm\(^{-2}\). Even at the lowest irradiance used in our experiments (0.2 mW cm\(^{-2}\)), multi-photon processes are still notable at other wavelengths. We attribute the onset of the single-photon dissociation regime for 458 nm to be due to the higher photon energy, which
allows easier access to the single-photon dissociation channel. A non-zero fragment yield of HBDI\textsuperscript{−} is predicted even for vanishingly low laser irradiance (but infinitely long exposure time) at 476 and 488 nm (Figure 7.1a), suggesting that the single-photon dissociation channel is available at these excitation wavelengths. Extrapolation of the fragment yield dependence at 496 and 502 nm to zero irradiance yields near-zero fragmentation, indicating that the energy of a single photon is below the threshold energy of both dissociation channels (ePD and methyl loss), in a good agreement with the theoretical estimate (B3LYP/6-311+G**) of 2.5 eV (16).

Figure 7.1. (a) Dependence of the fragmentation yield (Φ) on CW laser irradiance (I) for gas-phase HBDI\textsuperscript{−} anions at different excitation wavelengths (violet – 458 nm, dark blue – 476 nm, cyan – 488 nm, light green – 496 nm, dark green – 502 nm). All the data points we obtained at a constant laser fluence (\(F = 43 \text{ mJ cm}^{-2}\)), while the irradiance was varied. In order to keep the laser fluence constant, the increase in irradiance was in each case compensated by a corresponding decrease in the exposure time (t) (e.g., 0.215 mW cm\(^{-2}\) ∙ 200 s = 43 mJ cm\(^{-2}\)). (b) Wavelength dependence of photodissociation for gas-phase HBDI\textsuperscript{−} obtained upon exposure of trapped ions to CW laser irradiance of 0.215 (open circles) and 1.194 mW cm\(^{-2}\) (closed circles) power over 200 and 36 s correspondingly.
The spectral trend obtained upon excitation with 1.2 mW cm\(^{-2}\) (strong multi-photon character) implies a dissociation maximum between 476 and 488 nm (Figure 7.1b, solid circles), which is in very good agreement with the results of earlier photoabsorption action spectroscopy experiments (14,16). Both the sharp edge of the spectrum at \( \sim 496 \) nm and the tailing to the blue agrees well with the observations by Forbes and Jockusch (16). In their experiments, trapped HBDI\(^-\) ions were excited by a train of low-energy sub-picosecond pulses (\( \tau = 130 \) fs; \( E = 50 \) pJ/pulse; \( P_{\text{total}} = 4 \) mW; \( \tau_{\text{total}} = 250 \) ms). These authors also concluded that dissociation of HBDI\(^-\) mostly proceeded via multi-photon absorption around 480 nm, while single-photon dissociation was operating in the deep-blue region of the spectrum (16). The close agreement of the spectral representation obtained upon activation with CW laser irradiance of 1.2 mW cm\(^{-2}\) with the results of pulsed-excitation experiments reflects that multi-photon absorption governs the photofragmentation process under these conditions. In other work, Andersen and coworkers induced dissociation of gas-phase HBDI\(^-\) ions trapped in a storage ring by a single laser pulse of high energy (\( \tau = 3 \) ns, \( E = 1-2 \) mJ). In this work, it was reported that HBDI\(^-\) photodissociates as a result of single-photon absorption at 495 nm (15), which is quite surprising given the high peak power of the excitation (\( \sim 0.5 \) MW). The reason that their observations were interpreted as single-photon dissociation could be due to the fact that the products of ePD were not observed to an appreciable extent in the storage ring (16). This was also proposed (16) to account for the absence of the blue tailing in the spectrum recorded by Andersen and coworkers (14,15). Besides that, it was noted by the authors that it was exceedingly difficult to establish the correct power dependence of the neutral yield in pulsed experiments (15). In an earlier work from the same group on the absorption of gas-phase HBDI\(^-\), two-photon dissociation was reported (14).

While the spectral profile recorded at 1.2 mW cm\(^{-2}\) excitation laser irradiance shows a maximum at ca. 480 nm, the dependence recorded at 0.2 mW cm\(^{-2}\) reveals a gradual decrease from 458 to 488 nm (Figure 7.1b, open circles). We interpret this remarkable difference between the two profiles to be due to the large contribution from single-photon dissociation upon excitation with 0.2 mW cm\(^{-2}\). Although multi-photon dissociation is still partially operative under these conditions (Figure 1a), we will further refer to this spectral dependence as “single-photon”. This spectral profile suggests an absorption maximum of gas-phase HBDA anions below 458 nm. The position of the maximum (< 458 nm) is at least 40 nm away from the red edge of the spectrum (>
500 nm). This considerable shift suggests that the absorption maximum of HBDI\(^{-}\) in the gas phase does not correspond to the shortest adiabatic transition. The same phenomenon was found for HBDI\(^{-}\) both in solution and inside wt-GFP, based on the results of fluorescence spectroscopy experiments carried out at different temperatures (17,20). The fact that the shortest adiabatic transition is not the most intense in the spectrum is very important, as it suggests a significant structural change between the ground and excited states (17), which is central in theoretical models of ultrafast radiationless decay in HBDI (21).

Multi-photon dissociation of HBDI\(^{-}\) can proceed via a number of pathways. For example, absorption of the last photon, which induces dissociation, may take place from a twisted intermediate state (22). Alternatively, absorption of the last photon can occur from a vibrationally hot ground state populated via internal conversion from the excited electronic state of HBDI\(^{-}\). The latter mechanism is supported by the observed pressure dependence of multi-photon dissociation kinetics for gas-phase HBDI\(^{-}\) (23). Whatever mechanism is operating, the dissociative transition does not occur from the zero vibrational level of the ground state. On the contrary, the intrinsic absorption is the one originating from the zero vibrational level of the ground state via a single-photon process. The dissociation action spectrum of gas-phase HBDI\(^{-}\) recorded in the multi-photon regime is therefore not representative of its actual optical absorption. The photoabsorption action spectrum of gas-phase HBDI\(^{-}\) obtained by Forbes and Jockusch using pulsed excitation consists of two bands with maxima at ~480 nm and ~450 nm (16). The authors assigned these bands to the \(S_0\rightarrow S_1\) adiabatic transition at 480 nm and a highly active vibrational mode of the excited state (+1450 cm\(^{-1}\)) (16). The peak at 480 nm was identified to result from multi-photon absorption, while a significant contribution of single-photon dissociation to the second band was expected (16). The resonance around 480 nm present in the earlier action spectroscopy experiments can therefore alternatively be interpreted to be due to the transition from the twisted intermediate state or hot vibrational level of the ground state, as proposed above. If this is true, then the “semi-resolved” shape of the spectra obtained in a multi-photon absorption regime can be explained by a superposition of bands corresponding to the \(S_0\rightarrow S_1\) as well as to the aforementioned transitions and is therefore not representative of vibronic structure, as was proposed earlier (16). In the condensed phase, the vibronic structure of the electronic spectra for HBDI\(^{-}\) can become quite distinct if the \textit{cis-trans} isomerization channels in
the excited state are suppressed, e.g. inside the protein at low temperatures (20) and in some glasses (17). The suppression of isomerization in the excited state of HBDI\(^-\) imposed by the restriction of rotational freedom is also thought to be a key contributor to the high fluorescence yield (20). The fact that no fluorescence has been observed for gas-phase HBDI\(^-\) ions (16) suggests that the isomerization channel does operate in the excited state, which should result in a solution-like, unresolved spectrum at room temperature.

Unfortunately, HBDI\(^-\) represents a very difficult case for ab initio methods. The results are extremely method-dependent. For example, CASPT2 calculations with the same basis set (6-31G\(^*\)) and size of the CAS yielded an estimate for the vertical \(S_0 \rightarrow S_1\) transition energy of HBI\(^-\) (HBDI\(^-\) sans 2\(\times\)CH\(_3\)) of 2.63 (471 nm) or \(\sim 2.9\) eV (428 nm) (13), depending on the zero-order Hamiltonian employed. It is also noteworthy that the precision of CASSCF calculations used to simulate the absorption spectrum of HBI\(^-\) (22) is greatly limited for a number of reasons, e.g., for the principal inefficiency to account for dynamic correlation effects (24). Most recent state-of-the-art theoretical calculations utilizing different computational algorithms (TDDFT, CASPT2, QMC) consistently predict a vertical excitation energy of HBDI\(^-\) around 2.9-3.0 eV (415-428 nm) (13). This estimate agrees reasonably well with the extrapolation from soluton-phase measurements (437 nm) (10) and the results of our action spectroscopy in the single-photon regime (<458 nm). To what extent available theoretical predictions take into account the dissociative nature of the excited state is not yet clear (25).

Figure 7.2a demonstrates the yield of the fragment formed by the neutral methyl loss (\(\Phi_{200} = I(m/z\ 200)/I_o(m/z\ 215)\)) as a function of laser irradiance at 488 nm. It can be seen that the signal in the mass spectrum corresponding to the fragment \(m/z\ 200\) grows at low laser irradiance while it starts to decay at higher irradiance. This observation is explained by the fact that the fragment \(m/z\ 200\) also absorbs visible light and can also undergo \(ePD\). When exposed to laser irradiation, the number of parent HBDI\(^-\) ions in the cell continuously decreases (Figure 7.1a, cyan color), which results in slower formation of the fragment (\(dI(m/z\ 200)/dt \sim I(m/z\ 215)\)). At some point, the fragmentation rate of \(m/z\ 215\) ions becomes lower than the rate of \(ePD\) from \(m/z\ 200\) ions, and the number of \(m/z\ 200\) ions starts to decrease. The dissociative nature of the fragment \(m/z\ 200\) renders it difficult to precisely account for the contribution of the \(ePD\) and neutral methyl loss dissociation channels in HBDI\(^-\) in the multi-photon absorption regime. Thus, we suggest that
the intensity of the band at 480 nm in the ePD spectrum reported by Forbes and Jockusch (16) can be greatly overestimated due to ePD from m/z 200 ions. It is worth noting that even at low powers, when the parent HBDI' ions undergo single-photon dissociation, daughter ions can still fragment on the time scale of the experiment due to faster dissociation kinetics. Figure 7.2b shows the yield of m/z 200 ions as a function of excitation wavelength in the single-photon dissociation mode (0.2 mW cm\(^{-2}\)). The yield peaks around 480 nm, which is in agreement with the observation by Forbes and Jockusch (16).

![Figure 7.2](image-url)

**Figure 7.2.** (a) Yield of the HBDI' fragment formed by the neutral methyl loss (\(\Phi_{200} = I_m/z\ 200)/I_o(m/z\ 215)\)) as a function of laser irradiance at 488 nm. (b) Yield of m/z 200 ions as a function of excitation wavelength in the single-photon dissociation mode (0.2 mW cm\(^{-2}\)).

Interestingly, no heat-induced fragmentation was observed for HBDI' anions upon exposure to light: in reference experiments, gas-phase HBDI' was subjected to sustained off-resonance
irradiation collision-induced dissociation (SORI-CID), which is known to be a slow heating method (26). Neutral loss of CH₂ was found to be the lowest energy dissociation channel. This fragmentation channel was inactive in photodissociation experiments, suggesting that trapped HBDI⁻ ions are not significantly heated upon irradiation. Given that collisional cooling is extremely inefficient in high vacuum, gas-phase molecules can gain internal temperature and ultimately dissociate via successive photon absorption / fluorescence emission cycles (18). The absence of notable ion heating during our experiments is consistent with a negligible fluorescence yield and a very low rate of internal conversion for HBDI⁻ ions in high vacuum. This conclusion is in line with earlier unsuccessful attempts to directly observe fluorescence of HBDI⁻ in the gas phase (16). Note that as soon as the energy of SORI-CID is sufficient to induce the neutral CH₃ loss, this becomes the major dissociation channel, superseding the CH₂ cleavage. The CH₃ loss was also observed by Andersen and coworkers in their CID experiments preformed on HBDI⁻ trapped in a storage ring (15).

7.5 Conclusions

The results of our photoabsorption action spectroscopy experiments performed for gas-phase HBDI⁻ anions cast doubt on the interpretation of earlier results obtained with pulsed lasers (14-16). Our data suggest that the photodissociation spectrum of HBDI⁻ via absorption of multiple photons is not indicative of the actual ground-state absorption spectrum. The wavelength dependence of photodissociation collected in the single-photon regime predicts an absorption maximum of gas-phase HBDI⁻ below 458 nm, which is in fairly good agreement with a maximum at ≈ 437 nm derived from solution-phase data (13).

7.6 References


This chapter summarizes the results from preceding chapters and presents the projects that are currently underway in our lab.
8.1 Summary

This work began with the construction of a new setup for laser spectroscopy of trapped biomolecular ions. A unique platform was built that allows laser-induced fluorescence and dissociation spectroscopy measurements inside a Fourier-transform ion cyclotron resonance (FTICR) mass spectrometer on ions produced by electrospray ionization (ESI). The new instrument was thoroughly characterized using Rhodamine 6G (R6G) as a test compound (Chapter 3). The current setup is advantageous over previous installations for optical spectroscopy of gas-phase ions employed in our laboratory for a number of reasons. First, a combination of an external ESI source with an FTICR allows for the generation and trapping of intact macromolecular ions, such as proteins, oligonucleotides, polysaccharides, polypeptides etc. An earlier-generation optical setup in our lab employed internal MALDI source, which greatly limited the accessible molecular range of analyte ions. Second, externally generated ions arrive in the ICR region thermalized, which allows one to entirely take advantage of the benefits of FTICR mass analysis, such as supreme mass accuracy and resolution. This is particularly helpful for identification purposes in dissociation spectroscopy. Most importantly, gradual improvement of the optical detection system resulted in the capability of recording fluorescence with spectral resolution rather than in counting mode, as in previous works in our lab. It was the possibility to record fluorescence spectra of gas-phase ions trapped in an FTICR that resulted in the observation of naked gas-phase ions inside the ESI plume at ambient conditions (Chapter 4). An ESI plume of R6G solution was profiled by fluorescence spectroscopy. It was found that in certain parts of the plume recorded fluorescence is equal to that from gas-phase R6G ions trapped in FTICR. This discovery allowed us to move a large part of fluorescence experiments out of the high vacuum into ambient conditions, where the analysis is substantially easier and much more sensitive. It was shown that the instantaneous number of analyte ions inside ESI could reach $10^9-10^{10}$, which is several orders of magnitude higher compared with the capacity of trapping devices. Complementary to LIF, laser-induced dissociation spectroscopy was applied to probe absorption of gas-phase ions. A combination of fluorescence and dissociation spectroscopy was used to describe optical properties of gas-phase Rhodamine 19 (R19) neutrals (Chapter 5) and cations (Chapter 6). Experimental evidence was provided that neutral R19 assumes a lactone structure in the gas phase for the first time. The cationic form of gaseous R19 was found to
fluoresce with a notable Stokes shift. Reference experiments on R19 isomers led to the hypothesis that the cationic form of gas-phase R19 undergoes conformational change in the excited state. Finally, the photophysics of gaseous green fluorescent protein chromophore anion (HBDI\textsuperscript{-}) was addressed by laser-induced dissociation spectroscopy (Chapter 7). Fragmentation yield was found to strongly depend on whether experiments were run in a “single-photon dissociation” or “multi-photon dissociation” regime. Armed with this knowledge, we critically revisited the interpretations of earlier studies done in the multi-photon mode. We proposed that the results obtained in the single-photon regime could in fact better reflect the $S_0 \rightarrow S_1$ transition in HBDI\textsuperscript{-} and explain the pronounced inconsistency between earlier dissociation spectroscopy experiments and inference from solution-phase data.

### 8.2 Outlook

Chapters 3-7 report on the projects that have by now been successfully completed. A number of experiments are however still continuing or being designed. Some of ongoing projects are overviewed below. Future research is being developed in two directions: 1) probing structure of macromolecular ions, such as proteins and polypeptides, by fluorescence/dissociation spectroscopy; 2) exploring the area of applications for the discovery that ESI/ESSI is capable of generating high amounts of gas-phase ions at ambient conditions.

#### 8.2.2 Fluorescence of a green fluorescent protein chromophore analog with restricted dihedral freedom in the gas phase

Fluorescence of a model GFP chromophore, the HBDI anion (Scheme 8.1), is quenched in the absence of the protein via cis-trans or other isomerization reactions in the excited state (1). The high quantum yield of HBDI\textsuperscript{-} in the protein ($\sim 0.8$) is attributed to partial restriction on the dihedral freedom of the chromophore imposed by the protein interior, which disables isomerization in the excited state (2,3). A synthetic analog of the HBDI chromophore, PyHBDI (4), offers a chelation site between the ring systems of the chromophore (Scheme 8.1).

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Upon chelation, the chromophore is “locked”, and isomerization is disabled. Chelation of PyHBDI with Zn\(^{2+}\) and Cd\(^{2+}\) was found to dramatically increase the fluorescence quantum yield of PyHBDI in solution (Figure 8.1a). However, chelation with other transition metal cations did not result in any enhancement of fluorescence. The presence of the chelated species was confirmed by MS analysis for all metals tested (Figure 8.1b). Therefore, locking the chromophore dihedral freedom is not the only factor responsible for the fluorescence enhancement.

We performed a number of laser spectroscopy experiments of gas-phase complex trapped inside the FTICR cell adapted for optical studies (5). Considerable absorption of gas-phase PyHBDI\(^{-}\)···MCl\(_2\) in the blue-green region of the visible spectrum is predicted based on the results of photoinduced dissociation spectroscopy. However, no fluorescence of the complex was observed for all metals tested, including Zn and Cd. A possible explanation may be the efficient
dissociation of PyHBDI···MCl$_2$ in the excited state via neutral loss of HCl (Figure 8.1b). As a next step, we are planning to test whether other salts of Zn can form more stable complexes with PyHBDI in the gas-phase. The work is ongoing.

![Figure 8.1](image)

**Figure 8.1:** a) Increase in fluorescence yield of PyHBDI solution upon the addition of ZnCl$_2$ salt. b) Mass spectrum of PyHBDI···ZnCl$_2$ in negative ion detection mode. Upon collision or laser-induced activation, trapped PyHBDI···ZnCl$_2$ ions undergo a rapid neutral loss of HCl.

### 8.2.2 Evolution of the green fluorescent protein conformation during transfer from solution to the gas phase probed by fluorescence spectroscopy

What will happen to a protein if the solvent shell is removed: will it maintain a near-native conformation, unfold, or assume a new folded structure? Once these questions have been answered, the role of the solvent in stabilizing the native structure can be directly assessed. The green fluorescent protein (GFP) is highly fluorescent in its native conditions, while it does not fluoresce upon unfolding (1). Fluorescence of GFP can therefore serve a very direct measure of its conformation.
We performed a series of laser spectroscopy experiments in order to probe the conformation of gas-phase protein ions produced by ESI/ESSI directly inside the plume and when trapped in the high vacuum of an FTICR mass spectrometer. In enhanced green fluorescent protein (EGFP), the equilibrium between the neutral and deprotonated form of the chromophore is shifted towards the anion by a single-point mutation (1). In our experiments, EGFP tagged to a carrier protein moiety used for extraction was studied. The mass of the whole assembly was ca. 36 kDa. For simplicity, we will refer to this ligate as EGFP in the following discussion. In aqueous solution, the protein was found to have an absorption maximum around 488 nm and fluorescence around 514 nm. An aqueous solution of EGFP was electrosprayed in positive ion mode. The solution-phase fluorescence spectral shape was found unchanged along the plume, both in ESI and ESSI. The signal intensity decreased due to the divergence of the spray (Figure 8.2).

![Figure 8.2](image_url): Evolution of EGFP fluorescence along the ESSI plume. No distinct change in the spectral change can be observed.

It has been suggested earlier that ESSI is capable of generating free gas-phase protein ions at ambient conditions (6). The fact that no change in the spectral shape was observed for EGFP, even at vigorous desolvation conditions (nebulizer gas pressure 40 bar, distance from the sprayer tip 30 cm), suggests that within the limited time scale of the ESSI process (on the order of milliseconds) bare EGFP ions preserve a compact solution-phase-like conformation.
The most important question is how the conformation of gas-phase protein ions will evolve on a longer time scale, when the ions have time to assume a new, stable gas-phase conformation. Inside the ICR cell, ions can be trapped for several minutes. MS analysis of EGFP revealed quite a low charge-state distribution of ions (Figure 8.3a) reflecting ionization from a near-native environment. The ion population was subjected to CW laser irradiation (457, 476, 488, 496, 502, 514 nm) of various power (up to 1.5 W) and exposure time (up to 2 min).

![Figure 8.3](image)

**Figure 8.3:** Mass spectra of EGFP obtained from native (a) and denaturing (b) solutions. Inset in panel b shows a SORI-CID-MS spectrum of the 31⁺ charge state.

Interestingly, neither fluorescence nor dissociation was observed for trapped EGFP ions. The zero fluorescence signal could be directly attributed to protein unfolding in vacuum. On the other hand, the fluorescence signal can simply be too low and therefore undetectable due to the low EGFP ion density inside the trap. This low density results from the fact that EGFP ions are multiply charged, which greatly affects trapping efficiency in ICR. Work is currently underway to modify the cell geometry from an open trap electrode configuration to a closed shape, which
will allow for both higher ion capacity and improved fluorescence collection efficiency. More surprisingly, no laser-induced dissociation could be observed, even at high laser power (1.5 W) and extended exposure time (2 min). In contrast, myoglobin ions absorbing light in the same optical range in solution were found to fully dissociate within 40 s upon irradiation with 0.5 W. Again, one could directly conclude that gas-phase EGFP ions do not absorb visible light in the range 457-514 nm. There is indirect evidence, however, that prevents us from rushing into this conclusion now. First, GFP is an extremely stable protein, which maintains its folded conformation in solution upon heating up to 90°C. We failed to generate fragmentation of EGFP in collision-induced dissociation experiments using SORI-CID. Such dissociation was, however, easily obtained for myoglobin ions. Furthermore, no laser-induced fragmentation occurred for EGFP ions labeled with Rhodamine R19 (R19) or BODIPY FL. The presence of the labeled species was confirmed by MS analysis. Therefore, even if the protein is labeled with another fluorescent moiety, it cannot be destroyed by laser irradiation. This actually suggests that EGFP ions have a very stable, compact conformation in the gas phase. In order to induce dissociation, the protein ion first needs to unfold. One can speculate that this may require laser powers that are inaccessible in our experiments. This hypothesis is supported by the results of collision-induced dissociation experiments for EGFP ions electrosprayed from denaturing conditions. As can be seen in Figure 8.3b, unfolded proteins contain many more charges than folded ions due to the higher number of basic groups available for protonation. Importantly, we were able to induce fragmentation of these unfolded species (Figure 8.3b, inset), which points at a much weaker stability of gas-phase EGFP ions obtained from denaturing solution than by native electrospray.

In conclusion, despite the fact that neither fluorescence nor photofragmentation was observed from trapped EGFP ions, a number of observations suggest that the protein is still folded in the gas phase. Therefore, we cautiously believe that LIF from trapped EGFP ions should be observable with the improved optical setup.

8.2.3 FRET spectroscopy of polyalanine helices in the gas phase

The propensity of a polypeptide to form a helix in vacuum differs from that found in solution. The charge plays a critical role in stabilizing α-helices and destabilizing β-sheets in vacuum.
Alanine (Ala) has a high helix forming propensity in solution (7). It was proposed that locating a charged lysine (Lys) residue at the C-terminus of a neutral polyalanine chain should stabilize its helical conformation in the gas phase (8). The structure of gas-phase Ac-Alaₙ-LysH⁺ ions was explored by ion mobility spectroscopy (IMS) (9). The experimentally measured average ion cross-section per residue was found to be independent of the number of Ala monomers, n, suggesting that these peptides have helical conformations. On the other hand, the cross sections per residue for the AlaₙH⁺ and Ac-LysH⁺-Alaₙ peptides clearly decreased with increasing size, indicating that these peptides have conformations that are more compact than helices. The helical motif of Ac-Alaₙ-LysH⁺ ions is stabilized through favorable interactions of the charged lysine side chain with the helix macrodipole (10,11) and through capping interactions with dangling carbonyl groups at the C-terminus (12,13).

We propose to use FRET to characterize the conformation of Ac-Alaₙ-LysH⁺ ions in the gas phase. We are now working on labeling the C and N termini of Ac-Alaₙ-LysH⁺ with a FRET donor (CR6G) and acceptor (BODIPY) moieties, respectively (Scheme 8.2).

\[ \text{Scheme 8.2: } \text{BODIPY-Ala}_n\text{-Lys-CR6G}^+ \]
FRET efficiency between the C and N termini of the polypeptide should provide much more direct information on the end-to-end distance in the polymer than the ion cross-section measured in IMS. Once the rigid gas-phase structure of Ac-(Ala)$_n$-(Lys+H)$^+$ ions has been rigorously proven, it can be employed as a molecular ruler for measuring intramolecular distances by FRET spectroscopy.

### 8.2.4 Raman spectroscopy of gas-phase biomolecular ions

The discovery that high amounts of gas-phase ions ($\sim 10^9$-$10^{10}$) can be instantaneously generated inside the ESI plume was described in detail in Chapter 4. It was shown that probing gas-phase ions directly inside the ESI/ESSI plume provides an enormous gain in the sensitivity of the analysis compared to the measurements performed on trapped ions in vacuum. Fluorescence spectroscopy of gas-phase ions has commonly been considered as a very challenging task from the technical aspect, but in view of our discovery it may soon become quite routine. This fact invites attempting experimental methods for structural analysis of gas-phase biomolecular ions where the signals produced are weaker than those in fluorescence spectroscopy.

Like LIF, Raman spectroscopy employs CW laser activation and CCD signal detection. Although the experimental equipment in our lab is best suited for fluorescence measurements, a step towards Raman spectroscopy looks very natural. For proof-of-principle experiments, a resonant Raman system needed to be found, i.e. one that absorbs visible light in the gas phase. The resonance character of a process provides an enormous gain in Raman signal. The major factor that hinders the observation of Raman signal in the optical spectrum is fluorescence. Therefore, two major requirements for a good test system for Raman spectroscopy are a strong absorption in the blue-green region of the visible spectrum provided by our Ar$^+$ laser and a quenched fluorescence channel. As follows from the discussion in Chapter 7, HBDI$^-$ anions meet these requirements perfectly: the absorption is maximized in the blue region of the spectrum and fluorescence is quenched (14), presumably via isomerization reactions in the excited state (1). Scheme 8.3 shows two more chromophore analogs of HBDI that also meet these requirements, although when in the form of cations. Rough estimations based on solution-phase measurements predict Raman signals from these systems to be $\sim 10^2$ times weaker than fluorescence signals.
from rhodamine at the same experiment conditions. Unfortunately, no Raman signals have so far been detected from the compounds shown in Scheme 8.3, even with a 40-min signal accumulation. It is worth noting that the current setup was designed for mapping fluorescence along the plume. In order to enhance the signal collection efficiency, a fiber bundle as well as matching focusing optics will be used instead of single-fiber detection in further experiments.

![Scheme 8.3: Compounds proposed for trial Raman spectroscopy experiments in the gas phase.](image)

**8.2.5 Ion soft landing under ambient conditions**

The coupling of biological molecules and nanomaterials is of great importance for the development of new devices for forthcoming biological, medical and electronic applications (15). Ion soft landing is a mass spectrometric technique based on the deposition of specific biomolecular ions on a solid surface at low kinetic energies, thus preserving their molecular structure. Gas-phase ion chemistry combined with soft landing provides a unique opportunity for preparing novel synthetic materials (16). We propose to use gas-phase ions formed inside an ESI/ESSI plume for direct soft landing at ambient conditions. The advantage of this approach is the ease of implementation: no mass spectrometer is required! Furthermore, a much more rapid sample deposition is expected compared to soft landing by MS, where the deposition of a
measurable amount of sample may take up to several days. Of course, by doing experiment at ambient conditions one loses some of the selectivity of soft landing: ions cannot be preselected in the mass spectrometer. For complex samples a purification step is therefore necessary prior to soft landing, e.g., liquid chromatography separation.

As a trial experiment, we propose to repeat a recent experiment by Mazzei et al. on covalent immobilization of microperoxidase-11 protein on a carboxyl-functionalized multi-walled carbon nanotube (MWCNT) electrode surface by means of ion soft landing at ambient conditions. Mazzei et al. reported that the immobilized protein maintained its biochemical properties, displaying an excellent electrochemical behavior on the electrode surface (17). The key prerequisite for successful immobilization is the absence of solvent during the reaction, which can be achieved by optimizing ESI for the “gas-phase-only mode”. As described in Chapter 4, one can effectively discriminate gas-phase ions against charged and neutral droplets by adjusting experimental parameters of ESI such as sample introduction rate, capillary voltage, electrode configuration etc.

8.3 References

4 Baldridge, A. et al., Inhibition of Twisting of a Green Fluorescent Protein-like Chromophore by Metal Complexation. (submitted to Chem. Comm.).


Curriculum Vitae

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2006 - 2010  doctoral research in the Department of Chemistry and Applied Biosciences (Prof. Dr. R. Zenobi), ETH Zurich, Switzerland

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List of Publications


**Conferences**


