Developing Combined Optical Spectroscopy and Mass Spectrometry Tools to Probe the Conformation of Double-Stranded DNA in the Gas Phase

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

Department of Chemistry
University of Toronto

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Abstract

Mass spectrometry (MS) has long been a popular analytical technique for the characterization of gas-phase molecules. Beginning in the late 20th century, the coupling of MS with soft ionization techniques such as electrospray ionization (ESI) and the development of various gas-phase methodologies facilitated the structural analysis of intact biomolecules. To that end, this dissertation describes the coupling of MS with optical spectroscopy for the characterization of isolated DNA-binding dyes and non-covalent double-stranded DNA (dsDNA) complexes in the gas phase. MS and optical spectroscopy analysis is performed using a quadrupole ion trap (QIT) that has been modified to perform photodissociation (PD) and laser-induced fluorescence (LIF) on mass-selected ions. First, fluorescence measurements on trapped rhodamine 640 ions are performed to visualize the cloud of trapped ions inside the QIT. Second, the intrinsic photophysical properties of the classic dsDNA intercalator, ethidium bromide, is presented via photodissociation action spectroscopy, dispersed emission spectra, and time-resolved measurements. Finally, latter chapters describe the use of non-covalent DNA-binders that exhibit “turn-on” fluorescence responses in solution and Förster Resonance Energy Transfer (FRET) methodologies to probe the conformation of dsDNA ions in the gas phase. The work described in this dissertation serves to develop methodologies that use MS and optical spectroscopy techniques to investigate the intrinsic intra- and intermolecular interactions present in biomolecules, without competing solvent effects.
Statement of Authorship and Status of Manuscripts

This work forms the basis of Chapter 2. I performed the experiments and data analysis for Figures 1-3 in the publication, in addition to many of the figures appearing in Appendix A. I also made the figures for these experiments with revisions from RAJ. I wrote an initial draft of the introduction and experimental section. Final revisions of the entire article were performed with FOT and RAJ.

This work forms the basis of Chapter 3. All experiments and data analysis were performed by me. I also wrote the initial manuscript, with subsequent revisions done jointly with RAJ.

(3) S.V. Sciuto, R.A. Jockusch, “Developing optical spectroscopy and mass spectrometry tools for the characterization of double-stranded DNA in the gas phase”.
This work forms the basis of Chapter 4. All experiments and data analysis were performed by me. The first draft of the manuscript was prepared by me, and was later revised jointly with RAJ. This chapter is in the late stages of a manuscript (to be submitted).

(4) S.V. Sciuto, R.A. Jockusch, “Gas-phase Forster Resonance Energy Transfer (FRET) measurements probe the conformation of double-stranded DNA in the gas phase”.
This work forms the basis of Chapter 5. All experiments and data analysis were performed by me. The first draft of the manuscript was prepared by me, and was later revised jointly with RAJ. This chapter is in the early stages of a manuscript (to be submitted).

I performed action spectroscopy experiments on the complex of NMM with a DNA G-quadruplex over several days. I also advised SMJW on the preparation of the DNA G-quadruplex for subsequent power dependence measurements. The rest of the experiments and data analysis were performed by SMJW. The first draft of the manuscript was prepared by SMJW, and was later revised with RAJ and me. This is in the early stages of a manuscript.
Acknowledgements

This is by far the most difficult section to write since it is hard to put into words the academic and personal support that I received from friends, family, and colleagues during my doctoral degree.

First, I would like to thank Colin Douglas and Mike Lacasse from the Zamble lab for allowing me to use their thermocycler, which allowed me to prepare double-stranded DNA in solution with remarkable precision.

To the members of the Chemistry Machine Shop: John Ford, Johnny Lo, Ahmed Bobat, and David Heath, I am always inspired by your creativity and unbelievable accuracy in machining complicated laboratory equipment. There are too many instances to count where your ingenuity and attention to detail helped solve some stressful moments in the lab.

To Bill, Heather, and Rohan: each of you will remember my awkward social skills during high school, despite an obvious passion for Chemistry and the sciences. I want to thank each of you for being my best friends and encouraging me along the way. A special shout out to Bill for being my roommate during our time together at Western. I know it must have been difficult to deal with someone who had never lived by themselves before, but I think our friendship has benefitted since then from that experience. P.S. To all three of you, thanks for always listening, even if it was about laundry.

To Joey and Patrick, my stubborn siblings: all I can say is that there was never a dull moment between us! The two of you have always been there to provide some much-needed distractions from my academic life, at times when I sorely needed it. I can’t put into words how much it meant to me that the two of you were my Best Men when I married Julia. I’m looking forward to more parties, finance discussions, and certainly more laughs.

To Dana: we all know that out of the four of us, you are definitely the most stubborn sibling. That being said, I am always inspired by your passion for school and life, your work ethic, and the uncompromising empathy that you show on a daily basis. I am so proud to call you my sister because you’ve always been there for me and were never afraid to give me helpful advice, especially as I pursued new things.

To Mom and Dad: Thank you from the bottom of my heart for being there for me and encouraging me to work hard at school from the very beginning. If it weren’t for your emotional and financial support, completing my undergraduate degree and making the decision to pursue graduate school would have been that much more difficult. Thank you also for playing such an integral role in our wedding; your efforts made the day all the more enjoyable and will be something that I will never forget.

To all current (Jocky, Sydney, Rita, Matt, JoAnn, Neena, Iden, and Huihui) and past (Martin, Andrea, and Sandee) members of the Jockusch group: thank you for your fruitful scientific discussions and for the many practice presentations that you gave me comments for. I was able to learn so much from each and every one of you. Thank you also to Matt and Jocky for the extra effort the two of you gave in fixing the QIT. I know at times it was frustrating, but by sharing our
ideas I felt we were able to make the situation more manageable while reducing future instrument downtime.

To the members of my doctoral committee, Profs. Kagan Kerman and Ulli Krull: Thank you for your consistently helpful suggestions and comments. I always left our annual supervisory meetings with a renewed passion for the characterization of DNA with mass spectrometry and optical spectroscopy. Thank you also for pushing the boundaries of what I thought I knew during my comprehensive exam; that single event has made me realize that there is still lots to learn and understand.

To Francis: Thank you for instilling in me a healthy skepticism of anything that I claim, and for making me realize that value of time in the scientific field. There are too many times to count where I have left from a discussion between the two of us with more knowledge than I had before it, and this is all thanks to the expert knowledge that you bring to mass spectrometry and optical spectroscopy. Thank you also for helping me to develop a standard operating procedure for fluorescence alignment of the QIT. Future generations of the Jockusch lab will benefit from this, mostly due to your ingenuity and problem solving skills.

To my doctoral supervisor, Rebecca Jockusch: There are so many skills that I learned during my doctoral degree that can be directly attributed to your attention to detail and expert knowledge. When we both agreed to pursue the characterization of dsDNA in my 1st year, I can confidently say that I thought identifying an appropriate fluorescence “turn-on” fluorophore would be more straightforward. Nevertheless, your comments and suggestions have helped develop my skills as an analytical chemist that is ready to pursue work in the industrial field. I wish you the best with your future research endeavors and I am confident that under your supervision, future generations of scientists will have more than the necessary skills to succeed as chemists.

To my beautiful and loving wife, Julia: We have been together since my 1st year of graduate school, and I can safely say that you are the most important person in my life. Your loyalty, love, and sheer tenacity have helped support me during the highs (and especially the lows) of graduate school. I cannot explain how much more difficult graduate school would have been without you cheering me on. Our wedding last September was the happiest moments of my life thus far, and I can’t wait to see the adventures that we take together. I know that making the decision to pursue graduate school hasn’t always been easy for the two of us, but I am confident that it is going to be the first step toward a wonderful life together. I love you and thank you for always being there.
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Scheme 3.1. The structure of ethidium.

Scheme 4.1. Structures of the DNA-binding dyes studied in this work. (a) Hoechst 33342, (b) Hoechst 34580, (c) Hoechst 33258, (d) DAPI, (e) ethidium, (f) acridine orange, (g) SYBR Green I. The fluorescence “turn-on” response relative to the unbound dye ($\Phi_{Fl}$↑) is indicated next to the dye.

Scheme 4.2. dsDNA, made from complementary oligonucleotides ss1 and ss2

Scheme 5.1. (a) Structures of the donor, BODIPY Fl (blue) and the acceptor, BODIPY 576/589 (green). The attachment to the 5’ ends of ss1 and ss2 are indicated. (b) The 20-mer dsDNA complex made from complimentary oligonucleotides ss1 and ss2.
### List of Terms

#### Chapter 1

3’  
5’  
A  
Å  
$A_D$  
$A_{DA}$  
A-DNA  
BBO  
B-DNA  
BIRD  
C  
CCS  
cf.  
CH$_3$OD  
CD$_3$OD  
CID  
CPMD  
DABCYL  
DAPI  
DET  
DNA  
DsDNA  
EB  
ECD  
EDANS  
EDD  
EM-CCD  
EPD  
ESI  
ETD  
eV  
FRET  
fs  
FTICR  
G  
H33258  
H33342  
HDX  
$h_{VF}$  
$h_{VP}$  
ICC  
$I_D$  
$I_{DA}$  
IM-MS

3’ hydroxyl  
5’ phosphate  
Adenine  
Angstrom  
Donor-corrected absorption  
FRET complex corrected absorption  
A conformation of dsDNA  
Beta Barium Borate crystal  
B conformation of dsDNA  
Blackbody infrared dissociation  
Cytosine  
Collisional cross section  
Compare  
Singly deuterated methanol  
Completely deuterated methanol  
Collision-induced dissociation  
Carr–Parrinello molecular dynamics  
dimethylaminoazobenzenesulfonic acid  
4,6-diamidino-2-phenylindole  
Dexter energy transfer  
Deoxyribonucleic acid  
Double-stranded DNA  
Ethidium bromide  
Electron capture dissociation  
5-(2-Aminoethylamino)-1-naphthalenesulfonic acid  
Electron detachment dissociation  
Electron-multiplying charge coupled device  
Electron photodetachment dissociation  
Electrospray ionization  
Electron transfer dissociation  
Electron volt  
Förster Resonance Energy Transfer  
Femtosecond  
Fourier transform ion cyclotron resonance  
Guanine  
Hoechst 33258  
Hoechst 33342  
Hydrogen-deuterium exchange  
Fluorescence photon  
Phosphorescence photon  
Ion charge control  
Donor-control fluorescence intensity  
Donor fluorescence intensity in FRET complex  
Ion mobility mass spectrometry
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>IRMPD</td>
<td>Infrared multiple photon dissociation</td>
</tr>
<tr>
<td>K</td>
<td>Kelvin</td>
</tr>
<tr>
<td>$k_{\text{diss}}$</td>
<td>Dissociation rate constant</td>
</tr>
<tr>
<td>$k_{\text{ic}}$</td>
<td>Internal conversion rate constant</td>
</tr>
<tr>
<td>$k_{\text{isc}}$</td>
<td>Intersystem crossing rate constant</td>
</tr>
<tr>
<td>$k_{\text{nr}}$</td>
<td>Non-radiative rate constant</td>
</tr>
<tr>
<td>$k_{\text{q}}$</td>
<td>Quenching rate constant</td>
</tr>
<tr>
<td>$k_{\text{r}}$</td>
<td>Radiative rate constant</td>
</tr>
<tr>
<td>kV</td>
<td>Kilovolt</td>
</tr>
<tr>
<td>$k_{\text{vr}}$</td>
<td>Vibrational redistribution rate constant</td>
</tr>
<tr>
<td>LMCO</td>
<td>Low-mass cutoff</td>
</tr>
<tr>
<td>M</td>
<td>Molecular ion mass</td>
</tr>
<tr>
<td>$m/z$</td>
<td>Mass-to-charge ratio</td>
</tr>
<tr>
<td>MALDI</td>
<td>Matrix-assisted laser desorption ionization</td>
</tr>
<tr>
<td>mbar</td>
<td>millibar pressure</td>
</tr>
<tr>
<td>MHz</td>
<td>Megahertz</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>MS/MS</td>
<td>Tandem mass spectrometry</td>
</tr>
<tr>
<td>nanoESI</td>
<td>Nanoelectrospray ionization</td>
</tr>
<tr>
<td>ND$_3$</td>
<td>Deuterated ammonia</td>
</tr>
<tr>
<td>NH$_4$OAc</td>
<td>Ammonium acetate</td>
</tr>
<tr>
<td>ng</td>
<td>nanogram</td>
</tr>
<tr>
<td>NH$_2$</td>
<td>Amine</td>
</tr>
<tr>
<td>niECD</td>
<td>Negative ion electron capture dissociation</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometer</td>
</tr>
<tr>
<td>ns</td>
<td>nanosecond</td>
</tr>
<tr>
<td>OPO</td>
<td>optical parametric oscillator</td>
</tr>
<tr>
<td>PET</td>
<td>Photoinduced electron transfer</td>
</tr>
<tr>
<td>pg</td>
<td>Picogram</td>
</tr>
<tr>
<td>pH</td>
<td>Potential of hydrogen</td>
</tr>
<tr>
<td>ps</td>
<td>Picosecond</td>
</tr>
<tr>
<td>PTM</td>
<td>Post-translational modification</td>
</tr>
<tr>
<td>QIT</td>
<td>Quadrupole ion trap</td>
</tr>
<tr>
<td>$q_z$</td>
<td>QIT trapping parameter</td>
</tr>
<tr>
<td>r</td>
<td>Donor-acceptor distance</td>
</tr>
<tr>
<td>$R_0$</td>
<td>Förster distance</td>
</tr>
<tr>
<td>RhoB</td>
<td>Rhodamine B</td>
</tr>
<tr>
<td>S$_0$</td>
<td>Ground electronic state</td>
</tr>
<tr>
<td>S$_1$</td>
<td>1$^{\text{st}}$ electronic state</td>
</tr>
<tr>
<td>SPAD</td>
<td>Single-photon avalanche photodiode</td>
</tr>
<tr>
<td>ssDNA</td>
<td>Single-stranded DNA</td>
</tr>
<tr>
<td>T</td>
<td>Thymine</td>
</tr>
<tr>
<td>TCSPC</td>
<td>Time-correlated single photon counting</td>
</tr>
<tr>
<td>td</td>
<td>Drift time</td>
</tr>
<tr>
<td>Ti:Sapphire</td>
<td>Titanium Sapphire laser</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>UVPD</td>
<td>Ultraviolet photodissociation</td>
</tr>
</tbody>
</table>
Vis  Visible
x-mer DNA molecule with “x” bases
z  Number of protons
z+ Charge state
Z-DNA Z conformation of dsDNA
μm Micron
T Fluorescence lifetime
τD Donor-control lifetime
τDA Donor lifetime in FRET complex
Φ Fluorescence quantum yield
E FRET efficiency
ε Molar extinction coefficient
ηA Acceptor quantum yield and wavelength detection correction factor
ηD Donor quantum yield and wavelength detection correction factor

Chapter 2
AC Alternating current
CAD Collisionally activated dissociation
f Focusing distance
FWHM Full-width at half maximum
ITSIM Electrostatic axially harmonic optical trapping
kHz kilohertz
LIF Laser induced fluorescence
mW Milliwatt
pHe Helium pressure
r Radial
r0 Radial distance in trapping region of QIT
rf Radiofrequency
Rh640 Rhodamine 640
SIMION Ion optics simulation program
SRS Stanford research systems
t_irr Irradiation time
VRF RF potential applied to ring electrode
z Axial
z0 Axial distance in trapping region of QIT
λex Excitation wavelength

Chapter 3
\( \frac{k_r}{n^2} \) Ratio of radiative rate to square of index of refraction
\( I_{gas\ phase}^{Rh575H^+} \) Protonated rhodamine 575 fluorescence intensity
\( I_{gas\ phase\ ethidium} \) Ethidium gas-phase fluorescence intensity
\( I_{precursor\ laser\ off} \) Precursor ion intensity during laser off period
\( I_{precursor\ laser\ on} \) Precursor ion intensity during laser on period
Protonated rhodamine 575 gas-phase molar absorptivity coefficient

Ethidium gas-phase molar absorptivity coefficient

Excitation wavelength

1st component fluorescence lifetime

2nd component fluorescence lifetime

Protonated rhodamine 575 gas-phase quantum yield

Ethidium gas-phase quantum yield

Wavenumbers

Cubic centimeters

Charge-transfer

Deuterated water

Dimethyl sulfoxide

Water

Megahertz

Dimethyl amino

Neodymium-doped yttrium orthovanadate

Nuclear magnetic resonance spectroscopy

Laser power

Photodissociation

Rhodamine 575

Protonated Rh575

Signal-to-noise ratio

Sustained off-resonance irradiation collision-induced dissociation

Volume percentage

Photodissociation yield

1st order photodissociation power dependence fitting parameter

2nd order photodissociation power dependence fitting parameter

3rd order photodissociation power dependence fitting parameter

FWHM of gaseous emission spectrum

FWHM of solution-phase emission spectrum

Gaseous emission maximum

Solution-phase emission maximum

Energy difference between gas- and solution-phase emission maxima
cucurbit[7]uril-acridine orange complex in the 1+ charge state
dsDNA complex with rhodamine green-X in the 5+ charge state
dsDNA complex with rhodamine green-X in the 6+ charge state
dsDNA complex with 4 SYBR Green I’s in the 6- charge state
Charge-reduced dsDNA radical ion in the 4- charge state
dsDNA ion in the 5- charge state

Chapter 4

FWHM$^{gas}_{em}$

FWHM$^{soln}_{em}$

$\lambda^{gas}_{em,max}$

$\lambda^{soln}_{em,max}$

$\tilde{\nu}^{gas}_{em} - \tilde{\nu}^{soln}_{em}$

[CB7+AOH$^+$]$^{1+}$

[ds-RhoGX+4H]$^{5+}$

[ds-RhoGX+5H]$^{6+}$

[ds-10H+4SG$^+$]$^{16-}$

[ds-5H]$^{4-}$

[ds-5H]$^{5-}$
[ds-6H+AOH]+\textsuperscript{3-} dsDNA complex with acridine orange in the 5\textsuperscript{th} charge state
[ds-7H+DAPI]+\textsuperscript{6-} dsDNA complex with DAPI in the 6\textsuperscript{th} charge state
[ds-9H+3Et]+\textsuperscript{5-} dsDNA complex with 3 ethidiums in the 6\textsuperscript{th} charge state

[ss1-RhoGX+3H]+\textsuperscript{4+} ss1 complex with rhodamine green-X in the 4\textsuperscript{th} charge state
[ss1-5H+Et]+\textsuperscript{4-} ss1 complex with 1 ethidium in the 4\textsuperscript{th} charge state

AOH\textsuperscript{+} Protonated acridine orange
d(G\textsubscript{4}T\textsubscript{4}G\textsubscript{4})\textsubscript{2} Bimolecular DNA G-quadruplex
ds Complementary dsDNA made from ss1 and ss2
ePD Electron photodetachment
H33258 Hoechst 33258
H33258+H\textsuperscript{+} Hoechst 33258 in the 1\textsuperscript{st} charge state
H33342+H\textsuperscript{+} Hoechst 33342 in the 1\textsuperscript{st} charge state
H34580+H\textsuperscript{+} Hoechst 34580 in the 1\textsuperscript{st} charge state

[DAPI+H]+\textsuperscript{1+} DAPI in the 1\textsuperscript{st} charge state

Φ\textsubscript{FL}↑ Increase in fluorescence quantum yield
H33342 Hoechst 33342
H34580 Hoechst 34580
Oct 1 DC Octopole 1 DC voltage
Oct 2 DC Octopole 2 DC voltage
Rh110 Rhodamine 110
Rh123 Rhodamine 123
RhoGX Rhodamine Green-X
SG SYBR Green I
SILIF Selected-ion laser-induced fluorescence
ss1 GCGGGAATTGGGCG
ss2 CGCCCAATTCCCGC
ss3 GGGGTTTTGGGG
ss4 TAAGCGGCCGGAAT

Chapter 5

[A-dsDNA] Acceptor-only dsDNA control
[DA-dsDNA]\textsuperscript{6-} Charge-reduced radical FRET complex in the 6\textsuperscript{th} charge state
[DA-dsDNA] FRET-labeled dsDNA complex
[D-dsDNA] Donor-only dsDNA control
BODIPY 576/589 Acceptor fluorophore
BODIPY Fl Donor fluorophore
d(TG\textsubscript{4}T\textsubscript{4}) Rigid 4-stranded TGGGGT quadruplex
ss1 BODIPY Fl-5’-ATTCCGGCCGCTTAGGCAGT-3’
ss2 BODIPY 576/589-5’-ACTGCCATAGCCGCGCGGAAT-3’
Chapter 1

Introduction

There is a growing need in the biological community to understand the relationship between a biological molecule’s structure and function. However, correlating function with a particular structure is difficult as there exists a large energetic landscape dictating biomolecular structure that depends on a delicate balance between inter- and intramolecular interactions. For example, it has long been proposed that solvent interactions such as those between water and a biomolecule play an important role in dictating biomolecular structure. Water molecules can form extensive hydrogen-bonding networks while also affecting electrostatic and intramolecular interactions that give rise to a biomolecule’s tertiary structure. That being said, to understand the intrinsic factors that drive biomolecular structure it is critical to remove solvent interactions and develop techniques that probe structure in the absence of solvent. Therefore, I report on the coupling of mass spectrometry (MS) and fluorescence spectroscopy in this thesis. MS and fluorescence are two popular analytical techniques that when used in tandem, offer unparalleled capabilities in probing biomolecular conformation. The sections that follow provide a detailed overview of MS and fluorescence and the role these two methodologies can play in probing the conformation of biomolecules in the gas phase.

1.1 Mass Spectrometry: The Basics and Techniques Available

Mass spectrometry (MS) is an analytical technique that separates gas-phase molecules based on their mass-to-charge \( m/z \) ratio, and has gained considerable popularity due to its speed of analysis, high sensitivity, and small amount of sample (ng – pg) required. When it was first introduced, MS served as a diagnostic tool for the characterization of small molecules. The
popularity of MS continued to grow for small-molecule characterization, as a readout of a molecules’ m/z could be obtained in a few seconds. MS can be used to isolate specific m/z values, enabling characterization of a specific molecule with a known number of charges and potentially different structures. Furthermore, tandem mass spectrometry (MS/MS) experiments can perform structural analysis of isolated precursor ions using standard dissociation techniques, offering a “fingerprint” for structural analysis of unknown molecules. However, one major limitation of early MS characterization was that transferring molecules from solution into the gas phase was sufficiently energetic that molecules frequently dissociated prior to MS analysis. This made the characterization of biomolecules particularly difficult as intra- and intermolecular interactions would frequently be lost during the ionization process. It was not until the development of “soft” ionization techniques in the late 20th century that MS could be used for structural characterization of biomolecular ions.

The development of “soft” ionization techniques such as electrospray ionization (ESI)\(^5\) and matrix-assisted laser desorption ionization (MALDI)\(^6\) has fueled rapid growth in the analysis of intact biomolecules and non-covalent complexes in the gas phase. A number of reports have extended our understanding of the ESI process,\(^7\)-\(^11\) so only a brief description is given here. In the ESI process, analyte molecules are formed in solution and pass through a capillary with a small opening (~100 μm) where an electric potential is applied (3-4 kV). Highly charged solvent droplets are formed, and due to solvent evaporation these charged droplets experience increasingly higher charge densities with time. Therefore, to minimize electrostatic interactions the charged droplet will break apart (typically referred to as droplet fission) to generate highly charged molecular ions in the form [M+zH]\(^{z+}\). Here, M represents the mass of the molecular ion, \(z\) is the number of bound protons, and \(z+\) reflects the charge state. Negatively charged molecular ions in the form [M-zH]\(^{-}\) can also be formed in ESI when the electric potential at the capillary is
reversed. There are also efforts to miniaturize the ESI-MS platform using capillaries with smaller openings (5-15 μm) and lower electric potentials (0.6 – 1.4 kV). This ESI complement, termed nanoelectrospray ionization (nanoESI) generates smaller charged droplets that undergo fewer droplet fission events and is proposed to be gentler than traditional ESI-MS techniques. Ultimately, this highlights that ESI-MS can be used to transfer intact biomolecular ions from solution into the gas phase. However, as will be expanded upon in this thesis, the observation of an intact biomolecular ion in a mass spectrum provides little detail regarding that molecule’s gas-phase properties (conformation(s), energetics, ligand binding sites, etc.). Thus, the coupling of gas-phase techniques with ESI-MS has been used to characterize these properties for gas-phase molecules.

1.1.1 Ion Mobility Mass Spectrometry

One of the properties that is particularly relevant in understanding gas-phase conformation is molecular size. A typical mass spectrum for a biomolecular ion formed via ESI will show a distribution of charge states and thus $m/z$ values. It is not unreasonable to conclude that different charge states have different conformations due to electrostatics. However, a single $m/z$ ratio can also give rise to multiple conformations, further adding to the ambiguity of a biomolecule’s structure in the gas phase. Thus, mass spectrometrists have developed techniques to characterize the conformation(s) adopted by biomolecular ions in the gas phase. One of these techniques is ion mobility mass spectrometry (IM-MS), which is a gas-phase technique that separates biomolecules based on their size, or collisional cross section (CCS). Under the influence of an electric field, biomolecular ions are subjected to collisions with a buffer gas such as helium or argon. Ions with larger sizes undergo more collisions with the buffer gas than smaller ions, thereby allowing smaller ions to reach the ion mobility detector first. Furthermore,
the time it takes for an ion to travel through the ion mobility cell, called the drift time ($t_d$), can be related to the CCS of that biomolecule either through calibration using known standards,$^{14}$ or inputting the drift time into established equations relating the mobility of charged ions in the presence of electric fields.$^{15}$ For example, IM-MS of ubiquitin ions transferred into the gas phase via ESI-MS show CCS values that are consistent with ubiquitin’s native state for charge states ranging from 6+ to 8+. $^{16, 17}$ However, high charge states of ubiquitin (10+ and above) yield CCS values that are consistent with ubiquitin’s A-state, a less-compact, predominantly helical form. Thus, the development of IM-MS has expanded MS’s applicability in determining a biomolecules’ conformation in the gas phase.

1.1.2 Fragmentation Techniques in Mass Spectrometry

Ion dissociation techniques have been extensively developed for numerous MS applications, such as biomolecule sequencing, the identification of ligand binding sites, and quantitative proteomics. The most common dissociation technique that is standard on almost all commercial mass spectrometers is collision-induced dissociation (CID). However, evidence for hydrogen scrambling$^{18}$ and loss of post-translation modifications (PTMs)$^{19}$ in CID analysis of biomolecules have led to the development of alternative dissociation techniques. One example is electron-capture dissociation (ECD), where low-energy (<2 eV) electrons are captured by $\left[ M+zH \right]^{z+}$ ions, leading to the formation of a charge-reduced $\left[ M+zH \right]^{z-}$ radical species that often undergo dissociation useful for sequencing applications.$^{20}$ Reports using ECD have shown its applicability in determining sites of ligand binding, even for ligands that are bound non-covalently to the biomolecule.$^{21}$ Furthermore, for systems where the site of ligand binding in solution is well-characterized, ECD has shown that the ligand binding site frequently remain unchanged after the ECD process$^{22}$ (though counterexamples do exist$^{23}$). This highlights the
applicability of ECD-MS to characterize solution-phase binding. Unfortunately, ECD experiments require the use of high-resolution Fourier transform ion cyclotron resonance (FTICR) mass spectrometers due to the low-mass cutoff in RF devices that makes electron trapping impossible. The substantially higher cost of the FTICR relative to other commercial mass spectrometers has led researchers to develop complementary techniques that can be used on most trapping mass spectrometers. One example is electron-transfer dissociation (ETD) which forms a similar charge-reduced species as in ECD, but involves a gas-phase reaction between a trapped molecular ion and a molecule of opposite charge. The use of optical spectroscopy methods such as ultraviolet photodissociation (UVPD) have also shown their applicability for sequencing applications, as high-energy (>3 eV) ultraviolet photons can be absorbed by gaseous molecular ions yielding rich mass spectral data.

The development of IM-MS and various fragmentation techniques have provided a means of characterizing the properties of a biomolecular ion after being transferred from solution into the gas phase. This has enabled researchers to characterize biomolecules in the absence of solvent, thereby providing new insights into the intrinsic intra- and intermolecular interactions that drive structure. MS has also allowed researchers to make highly selective environmental changes. For example, once solvent molecules are removed, selected pieces of a local environment (known number of water molecules, non-covalently bound ligands) can be added back and their effects studied separately. This highlights the versatility of MS in probing biomolecular structure.

As a model system I chose to investigate the structure of deoxyribonucleic acid (DNA), and in particular, the complex that forms when complementary single-stranded DNA molecules combine to form double-stranded DNA (dsDNA). This dissertation describes the coupling of MS and optical spectroscopy to develop tools for the characterization of dsDNA. This includes an
examination of the fluorescence properties for several DNA-binding fluorophores (e.g. ethidium) upon complexation to dsDNA in the gas phase. In the sections that follow I outline the structural properties of DNA and literature detailing what is known about the correlation between the solution-phase structures of this important biological molecule to its gaseous structure.

1.2 The structure of deoxyribonucleic acid (DNA)

Deoxyribonucleic acid is one of the most important biological molecules. DNA is the carrier of genetic information in the cell that provides a blueprint for life, encodes for proteins that perform key biological functions, and through the process of evolution protects living creatures from disease. Structurally, DNA is constructed through successive addition of building blocks called nucleotides. Nucleotides are made up of three pieces: a phosphate group, a sugar group, and four types of nitrogenous bases, adenine (A), thymine (T), guanine (G), and cytosine (C) (Figure 1.1a). Nucleotides add to one another through the phosphate backbone to form the structure of DNA. The sequence of DNA is dependent on the ordering of the nitrogenous bases. However, the DNA in the cell is not found as a single chain of polynucleotides, but rather as two chains that combine to form double-stranded DNA (dsDNA). DsDNA arises from hydrogen-bonding between G–C and A–T bases. Other non-covalent DNA structures, such as G-quadruplexes, are guanine-rich DNA sequences that can form four-stranded helices via guanine hydrogen bonds. G-quadruplex structures are known to exist in telomeric DNA, which are implicated in certain types of cancers. A structural depiction of the hypothetical DNA sequence 5’-ACTG-3’ is shown in Figure 1.1a. Below it in Figure 1.1b is a 20-mer dsDNA molecule (B-DNA conformation; see below) highlighting key structural features such as the major/minor grooves and the rise per base pair (3.3 Å). The formation of the DNA double helix depends to an extent on hydrogen-bonding between base pairs. However, additional factors such as
Figure 1.1. (a) The hypothetical DNA sequence d(ACTG) (left) hydrogen-bonding with its complementary single-stranded sequence (right) oriented in an anti-parallel (3’→5’) position to illustrate the formation of dsDNA. Hydrogen bonds are indicated by dashed red lines. (b) Structural depiction of a 20-mer B-DNA conformation illustrating major and minor grooves.
intramolecular interactions between adjacent bases and electrostatic interactions between the negatively charged phosphate groups (screened in solution by solvent and nearby counterions) also play an important role in dsDNA structure. Shown in Figure 1.1b are also the major and minor grooves that arise due to the antiparallel orientation of complementary sequences. Major and minor grooves are important biologically for the recognition of DNA-binding proteins, as well as the non-covalent binding of specific drug molecules. For example, due to its unique structure, DAPI (4,6-diamidino-2-phenylindole) typically binds to the minor-groove in dsDNA and has a particular preference for A-T regions. DAPI is known to exhibit a well-characterized fluorescence “turn-on” response upon binding dsDNA. In Chapter 4, I examine the fluorescence properties of the dsDNA-DAPI complex and other dsDNA-dye complexes, to determine whether the fluorescence “turn-on” response for several DNA-binding dyes is preserved in the gas phase.

The conformation of dsDNA is highly dependent on solvent conditions and the presence of counterions. Under physiological conditions (pH 7 in water, ~150 mM Na\(^+\) and 50 mM K\(^+\)), dsDNA adopts the classic B-DNA conformation that was proposed by Watson and Crick. However, changing the solution conditions from a polar to a non-polar solvent results in the formation of the A-DNA conformation. The A-DNA conformation is a right-handed helix that has 11 base-pairs per turn (cf. to 10 base-pairs/turn in B-DNA) and has a wider helix diameter than the B-DNA conformation (2.6 nm cf. 2.4 nm for B-DNA). The wider diameter of the A-DNA helix is due to decreased solvent screening of the negatively charged phosphate groups that repel one another due to electrostatics. Under high salt concentrations and particularly for DNA sequences with alternating GC-tracts (ie., d(CG)\(_n\)), the Z-DNA conformation can be formed. Z-DNA is a left-handed helix that has a shorter helix diameter than both A-/B-DNA at 1.8 nm, solely due to a higher concentration of counterions in solution that screen the negatively charged
phosphate groups more effectively. Thus, the conformation of dsDNA present in solution is dependent on several interactions, such as hydrogen-bonding, base stacking, and electrostatics. Furthermore, the stabilization/destabilization associated with these interactions clearly depends on the presence of competing solvent and counterion interactions. This prompted me to characterize the stability and structure of dsDNA in the absence of solvent – particularly in the gas phase of a mass spectrometer. In the following section I outline what is already known regarding MS of DNA, and what techniques have been used to probe the structure of dsDNA in the gas phase.

1.3 Characterization of DNA using Mass Spectrometry

The first mass spectrum illustrating the appearance of oligonucleotide anions using ESI-MS was reported by Covey et al., who detected a 14-mer single-stranded oligonucleotide ion with charge states ranging from 6- to 11-. The early detection of [M-2H]⁺ oligonucleotides using ESI-MS led a few researchers to work towards gas-phase sequencing of DNA molecules. The general principle of DNA sequencing using ESI-MS is to form gaseous ions, isolate a specific m/z, and then subject that ion to gas-phase fragmentation using an appropriate technique (this is typically called “top-down” fragmentation). Fragment ions can then be pieced together to elucidate the DNA sequence. Unfortunately, mainstream electron-capture dissociation techniques (ECD, ETD) cannot be used on negatively charged DNA ions due to electrostatics. This has motivated researchers to develop complementary dissociation techniques that can be used for negatively charged ions. For example, negative ion ECD (niECD) developed by Håkansson and coworkers has enabled the fragmentation of peptide anions. Electron-detachment dissociation (EDD) is another technique that has been explored by the Håkansson group to characterize the
fragmentation pathways for DNA anions. Therefore, electron-based dissociation techniques have been developed to characterize negatively charged biomolecular ions.

More frequently, techniques such as collision-induced dissociation (CID) and infrared multiple photon dissociation (IRMPD) are used for characterization of DNA anions. IRMPD is an optical fragmentation technique that involves the absorption of IR photons by gaseous ions. Sequential absorption of IR photons raises the internal energy of the molecular ion until an appropriate dissociation threshold is reached, resulting in the formation of fragment ions. Upon CID of deprotonated DNA anions, loss of neutral or charged bases is followed by subsequent 3’ C-O cleavage, forming complementary fragment ions that provide sequence information. However, CID of DNA anions also form internal fragment ions that complicate sequencing interpretations. To that end, IRMPD of DNA anions have been developed as IRMPD spectra show similar fragment ions to CID, but minimize the formation of uninformative base loss ions while producing fragmentions important for sequencing applications. Furthermore, IRMPD of DNA anions show richer MS/MS information in the low m/z region, an issue that plagues conventional CID methods in radiofrequency devices (e.g. QITs) due to low-mass cutoff (LMCO) restrictions. IRMPD has also been used in tandem with other dissociation techniques to sequence DNA anions. For example, McLafferty and coworkers used in-source dissociation, CID, and IRMPD in tandem to completely sequence an unknown DNA anion with 50 nucleotides. The researchers also partially sequenced DNA sequences as large as 100- and 108-mers, a noteworthy achievement for the MS community. However, McLafferty and coworkers employed a high-resolution FTICR mass spectrometer in order to detect low-abundance ions, an important criteria for DNA sequencing that is not as easily achieved on other mass spectrometers. Furthermore, there are already well-developed competing technologies that are capable of sequencing DNA, such as the Sanger method and other new methodologies.
these reasons sequencing applications for biologically-relevant DNA sequences using MS have not garnered significant traction. However, MS can be used for other DNA-based applications, such as identifying the binding site for DNA/ligand complexes and reporting on the structure and stability of higher-order DNA complexes such as dsDNA. As the focus of my thesis is analyzing dsDNA the following section will outline what is known regarding the detection of dsDNA by mass spectrometry.

1.3.1 Mass Spectrometry of Double-Stranded DNA

DsDNA ions in the form \([\text{ds-}z\text{H}]^{z-}\) have long been detected in the gas phase of a mass spectrometer despite the lack of solvent and electrostatic screening of negatively charged phosphate groups. Light-Wahl et al. were able to detect an intact 20-mer dsDNA ion with charge states distributed from \([\text{ds-}2\text{H}]^{2-}\) to \([\text{ds-}8\text{H}]^{8-}\). Ganem et al. investigated the relative abundance of dsDNA ions in the mass spectrum with different extents of GC-content. The authors found that for 8-mer dsDNA ions with 100% GC content, dsDNA intensities were higher than for AT-rich sequences. This suggests that the number of hydrogen bonds present in the DNA duplex in solution plays a role in the gas-phase stability of the dsDNA ion. Doktycz et al. showed that 10- and 20-mer dsDNA anions can be stored for hundreds of milliseconds in a quadrupole ion trap (QIT) mass spectrometer, even in the presence of multiple ion-molecule collisions. Schnier et al. measured base pair binding energies using blackbody infrared dissociation (BIRD) and showed that binding energies were higher for GC versus AT sequences. Finally, Pan et al. investigated a larger subset of dsDNA anions with varying GC content. Sequences with higher GC content (indicating more hydrogen bonds in solution) required higher dissociation thresholds in the gas phase, suggesting that dsDNA ions retain a memory of their hydrogen bonding network in the absence of solvent. Thus, there is significant evidence that (1) dsDNA anions be
detected in the gas phase and that (2) gaseous dsDNA stability may be dependent on the degree of stabilization present in solution.

1.3.2 Mass Spectrometry of dsDNA-ligand complexes

ESI-MS can detect complexes formed between dsDNA and non-covalent binders. Small-molecule DNA-binders can be broken up into two types of binding: intercalation and minor-groove. Shown in Figure 1.2 is an illustration of these two types of binding using ethidium bromide (Fig. 1.2a) and DAPI (Fig. 1.2b). Intercalative binders are typically positively charged, planar molecules that sandwich themselves between adjacent base pairs to participate in base stacking interactions. Examples of intercalators include anthracyclines such as daunomycin and doxorubicin, metallointercalators with ruthenium centers and bipyridine ligands, and the classic fluorescence stains ethidium bromide and acridine orange. Minor-groove binders, as the name suggests, lie across the minor-groove present in the DNA double helix and participate in hydrogen-bonding, especially with AT-rich sequences. Examples of minor-groove binders include netropsin, benenil, and distamycin. There are also minor-groove binders that exhibit classic “turn-on” fluorescence responses (i.e., their fluorescence becomes brighter) upon binding dsDNA, such as DAPI and Hoechst dyes. The “turn-on” fluorescence response for complexes of DAPI and Hoechst dyes with dsDNA is attributed to two factors: (1) imposed rigidity of the minor-groove binder when complexed to dsDNA and/or (2) sequestration of the chromophore from solvent upon dsDNA binding.30

An important investigation by Rosu et al. investigated the binding and fragmentation behaviour of several dsDNA-dye complexes in positive and negative ion mode ESI-MS.47 The authors studied complexes of dsDNA with varying GC percentages with minor-groove binders (Hoechst 33258/33342, DAPI, and netropsin) and intercalators (daunomycin, doxorubicin,
Figure 1.2. The illustration of (a) intercalation by ethidium (green) and (b) minor-groove binding by DAPI (purple) when non-covalently bound to the B-form of a 14-mer dsDNA sequence. The structures of ethidium bromide and DAPI are shown next to the complexes.
ethidium, and several others) in order to determine relative binding constants in the gas phase. Apparent association constants were determined by comparing the relative intensities of the dsDNA-drug complex relative to the dsDNA complex (assuming equal ionization efficiencies for these complexes). The authors found good correlation between reported association constants in solution to those determined in the gas phase for the dsDNA-dye complexes. Furthermore, minor-groove binders’ preferential AT-stretch binding appeared to be preserved in the gas phase, as association constants for all minor-groove binders decreased as the GC content of the DNA duplex increased. Fragmentation behaviour of dsDNA-drug complexes also supported stabilization of the DNA duplex upon minor-groove binding. For example, examination of breakdown curves (which plot intact complex percentage versus center-of-mass collision energy) illustrated that to deplete intact complex percentages by 50%, higher collision energies were required for dsDNA-dye complexes relative to the bare dsDNA ions. Other researchers have also observed this behaviour for intercalating and metallointercalating molecules. These findings demonstrate that known solution-phase binding properties correlate well with MS results for several dsDNA-dye complexes, encouraging the use of MS to probe solution-phase properties of these important class of complexes.

1.4 The conformations of dsDNA in the gas phase

A considerable amount of evidence supports the preservation of helical-like properties for gaseous dsDNA. First, the detection of dsDNA ions in the mass spectrum suggests some degree of hydrogen-bonding retention in the gas phase; dissociation thresholds for dsDNA ions increase with GC percentage, consistent with the idea that duplexes with more hydrogen-bonds in solution are more stable in the gas phase. Furthermore, minor-groove binders maintain their preferential AT-stretch binding for dsDNA ions in the gas phase, consistent with solution-phase
reports. Finally, fragmentation data for dsDNA-dye complexes support stabilization of dsDNA upon non-covalent binding. While these observations point to preservation of structural properties present in the dsDNA, a more detailed description of the conformation of dsDNA in the gas phase would be advantageous. In the following sections, I outline the literature that has provided more detail regarding the conformation of dsDNA in the gas phase and the extent to which it does or does not resemble its solution-phase structure.

1.4.1 Gas-phase Hydrogen-Deuterium exchange (HDX) of dsDNA ions

Gas-phase hydrogen-deuterium exchange (HDX) has provided some structural characterization regarding the conformation of dsDNA in the gas phase. HDX probes the extent of exchange between solvent accessible protons on an analyte and a deuterated solvent such as ND$_3$ or CD$_3$OD. Increased levels of exchange between protonated groups and the deuterated solvent reflect more solvent accessibility, suggesting a more “unfolded” conformation for a biomolecule. While HDX is predominantly a solution-phase technique, several researchers have integrated HDX with commercial mass spectrometry to probe gaseous conformation of dsDNA. In particular, Hofstadler et al. probed the gaseous HDX behaviour of a 13-mer duplex in the 5-charge state in comparison to a 12-mer single-stranded DNA (ssDNA) ion in the 3-charge state.$^{49}$ The authors showed that after normalizing for the maximum number of exchangeable sites, the duplex ion exchanged fewer protons than the ssDNA ion. This indicates that hydrogens are more protected in the dsDNA ion, potentially due to extensive internal Watson-Crick hydrogen bonding or a compact dsDNA structure. In a subsequent study, Gabelica et al. monitored the HDX behaviour for a 12-mer duplex ion in the 5-charge state.$^{50}$ The authors noted that after 50s storage at a CD$_3$OD pressure of $\sim 8.0 \times 10^{-9}$ mbar, 5 protons (out of $\sim 50$ potentially labile protons) had been exchanged with deuteriums for the dsDNA ion, indicating a high-degree
of protection. In addition, the distribution of masses at 50s storage was significantly broader than that observed at 2s, suggesting the presence of multiple conformers with different HDX rates. Thus, researchers using HDX were able to illustrate that dsDNA ions have a high degree of protection in the gas phase, potentially due to the presence of internal hydrogen-bonds or a compact dsDNA structure.

1.4.2 Molecular dynamics simulations for dsDNA ions

Molecular dynamics simulations have provided additional insight in our understanding of gaseous dsDNA conformation. Early work by Hobza and coworkers investigated the hydrogen bonding and stacking of DNA bases using ab initio calculations. The authors reported that amino groups on DNA bases possess non-planar geometries due to a partial sp³-hybridization of the amino group when participating in hydrogen bonding. The non-planarity of the amino groups is important as it establishes attractive intramolecular interactions with hydrogens oriented out of the DNA base planes. Later, Orozco and coworkers investigated the structure adopted by 12- and 16-mer DNA duplexes in the 6- and 8- charge states, respectively, in the sub-microsecond time scale using AMBER-99 force field with TIP3P. The researchers found that upon transfer from solution into the gas phase, a notable number of hydrogen bonds remained intact for gaseous dsDNA (30-50% at 448K and 60-90% at 298K) over the course of 250 ns. G–C pairs retained most hydrogen bonds and stacking interactions for gaseous dsDNA, with these interactions lost in A–T regions. The researchers observed a significantly elongated double helix for 12- and 16-mer sequences which retains most hydrogen bonds and some helical structure, but lacks stacking interactions. However, recent calculations by the Orozco group using Carr-Parrinello molecular dynamics (CPMD) methods in the ms timescale have investigated the role that charge state plays when comparing the computed structure of 7-mer dsDNA ions with the aqueous structure. The
authors reported that for 7-mer dsDNA with -2, -3, or -4 charges, computed CCS values were similar (-3 charge), smaller (-2 charge), and larger (-4 charge) than the CCS of the solution-phase conformation. Furthermore, calculated structures for the -4 charge state showed loss of intermolecular base hydrogen bonds and the presence of intramolecular hydrogen bonds between phosphate (anion) and phosphate (neutral) groups. A similar result was observed for the 7-mer dsDNA with an overall -3 charge: sampled structures appear severely distorted relative to its solution-phase conformation, though phosphate-phosphate interactions remain the prevalent hydrogen bonding contact. Thus, while MD simulations have provided detailed information regarding the conformation of dsDNA in the gas phase, clearly there are numerous factors that dictate its gaseous conformation.

1.4.3 Ion mobility mass spectrometry of dsDNA ions

Several researchers have used ion mobility to probe the conformation of dsDNA sequences shorter than 20 base-pairs. Bowers and coworkers first investigated the collisional cross sections (CCS) of poly(CG)$_n$ sequences with charge densities ranging from 50% to 64% of sequence length, i.e. 7- to 9- for 14-mer dsDNA.\textsuperscript{54} Measured CCS values for the gaseous dsDNA ions were consistent with the A-DNA conformation, suggesting a transition from the well-known B-DNA form in solution to the A-DNA form in the gas phase. More recent work by Gabelica and coworkers have suggested that the structure of dsDNA in the gas phase is dependent on several factors, such as GC content, charge state, and type of instrument.\textsuperscript{55} In contrast to Bowers’ findings, the authors noted that CCS distributions for 12-mer dsDNA ions were significantly broader than the rigid G-quadruplex, d(TG$_4$T)$_4$, which is a four-stranded DNA complex that arises from hydrogen-bonding between guanine bases. d(TG$_4$T)$_4$ serves as a useful reference for gas-phase conformation as its CCS is similar in solution and the gas phase,\textsuperscript{56} suggesting similar
conformations. Burmistrova et al. also investigated the ion mobility properties of numerous 18-mer dsDNA sequences with various GC percentages in the 7-charge state. In numerous cases, CCS distributions resolved at least two conformational ensembles, strongly suggesting the presence of multiple conformations for dsDNA ions in the gas phase. Finally, Porrini et al. demonstrated that for several 12-mer dsDNA ions in the 5-charge state with varying GC content, CCS distributions support the formation of a compact dsDNA structure whose CCS is significantly smaller than A- and B-DNA forms. With the help of calculations, the authors hypothesized that this compact dsDNA conformation arises from intramolecular phosphate-phosphate hydrogen bonding along the DNA backbone.

1.4.4 The need for complementary techniques to probe dsDNA structure

The conformation of dsDNA in the gas phase appears to be sensitive to several parameters, such as: sequence composition and length, charge state, and potentially experimental conditions present in the mass spectrometer (due to timescale, harshness of source, etc.). Furthermore, while HDX, molecular dynamics, and ion mobility measurements have provided significant insight regarding the conformation of dsDNA, little consensus exists among these techniques. Therefore, the development of new complementary techniques promises to provide useful insight into the conformation of dsDNA and other biomolecular ions. It is critical to obtain a better understanding of gaseous biomolecular conformation since it is unclear the degree to which intra- and intermolecular interactions present in solution are preserved in the gas phase. Due to the unique selectivity of MS and sensitivity of optical spectroscopy, when used in tandem these techniques provide the tools to address key interactions in complex systems. In the following section, methodologies that employ MS and optical spectroscopy to probe the structure of gaseous dsDNA are outlined.
1.5 Combining MS and optical spectroscopy to probe dsDNA structure

The combination of MS and optical spectroscopy serves to elucidate key interactions for dsDNA in the gas phase. Furthermore, the implementation of spectroscopic tools into MS systems allows the researcher to probe photophysical properties in a charge-state resolved or sequence-specific manner. This enables comparisons to be made between identical sequences of dsDNA with different charge states or different sequences in the same charge states, thereby elucidating inter- and intramolecular interactions present in dsDNA. An important early investigation by Gabelica et al. used a quadrupole ion trap mass spectrometer coupled with an optical parametric oscillator (OPO) laser to irradiate intact 12-mer dsDNA ions with 250-285 nm light.60 Three dsDNA ions in the 5- charge state were investigated with different GC percentages (33%, 66%, 100%). When irradiated with 260 nm light, [ds-5H]5- ions primarily underwent electron photodetachment, resulting in the formation of a charge-reduced radical, ie., [ds-5H]4+. Furthermore, the electron photodetachment efficiency was largest for GC-rich sequences, attributed to the low ionization potential of guanine bases. The researchers also combined UV-irradiation with traditional collision-induced dissociation (CID), coined electron photodetachment dissociation (EPD), to yield rich MS/MS spectra for dsDNA ions that lacked internal fragments and had excellent sequence coverage with informative base losses. EPD of dsDNA ions primarily resulted in base loss instead of interstrand separation, suggesting that this technique could be used to probe intermolecular interactions in DNA duplexes. In a subsequent study, Gabelica et al. investigated whether EPD could be extended to dsDNA ions with noncovalently bound chromophores via excitation at chromophore-specific wavelengths.61 A subset of the small-molecule DNA binders investigated were the minor-groove binders DAPI
and Hoechst 33258 (H33258), in addition to the intercalator ethidium. Complexes of [ds-5H]5- ions with DAPI, H33258, and ethidium (Et) that were excited with wavelengths specific to chromophore excitation (>300 nm) resulted in the formation of charge-reduced radical ions, similar to results obtained previously for bare dsDNA ions excited with light of wavelength <300 nm suitable for base excitation. Furthermore, [ds-6H+H33258]4+ and [ds-6H+Et]4+ complexes formed after chromophore excitation and subjected to CID had identical fragments compared to CID spectra obtained at excitation wavelengths specific to DNA base absorption. This indicates that EPD methodologies can be extended to dsDNA-dye complexes, merely by tuning the wavelength such that the non-covalent chromophore is preferentially excited. More recently, Rosu et al. probed the UV spectra of intact 12-mer dsDNA ions in the 5- charge state in comparison to the complementary single-strands.55 Sequences arising from different [ss-3H]3- ions had similar UV excitation maxima and shape. However, UV spectra of the [ds-5H]5- ions were dramatically broader than their respective [ss-3H]3- ions, indicating base environments that differed from the ssDNA ions in the gas phase. Furthermore, comparison of UV spectra between dsDNA ions with different GC percentages illustrated differences in vibronic signatures, likely reflecting different extents of hydrogen-bonding for these ions in the gas phase. The researchers compared the CCS distributions obtained from ion mobility for these two dsDNA sequences and illustrated that the dsDNA sequence with 100% GC content was more compact, potentially due to enhanced hydrogen bonding. While innovative, the use of UV spectroscopy by itself lacked some of the detailed information that has made calculations and ion mobility especially informative for characterization of dsDNA in the gas phase.

Fluorescence spectroscopy is capable of providing a sensitive probe of biomolecular conformation, as the fluorescence properties of many chromophores are highly sensitive to environment. This theme is highlighted several times in this thesis as the fluorescence properties
of a fluorophore can change dramatically upon transfer from solution into the gas phase. I explore this idea in Chapter 3 where the intrinsic photophysics of gaseous ethidium ions are examined and ultimately compared to the spectral properties of ethidium in solution. In the following section, an outline of MS and fluorescence-based methodologies that I used to probe dsDNA structure in the gas phase is presented.

1.5.1 Coupling MS with Fluorescence-based methodologies

The selectivity of MS and sensitivity of fluorescence-based methodologies can probe several types of interactions in the gas phase. These include the role of solvent on biomolecular structure, the effect of added metal ions, and the effect on covalently and non-covalently bound ligands upon biomolecular attachment. To this end, several research groups have coupled commercial mass spectrometers with fluorescence-based detectors to investigate the structure of biomolecular ions.62-66 However, the literature is fairly sparse in the use of MS and fluorescence to probe the conformation and dynamics of dsDNA ions in the gas phase. An important early contribution came from Danell and Parks, who used Förster Resonance Energy Transfer (FRET) in combination with trapping mass spectrometry to probe the conformational dynamics of dsDNA ions upon heating.67 FRET is a fluorescence-based tool that depends on the non-radiative energy transfer between a donor and acceptor fluorophore that are typically covalently attached to a biomolecule. FRET rates are extremely distance dependent and as such, can report on the conformation of a biomolecule (a more detailed description is given in the following section). In their work, Danell and Parks appended BODIPY fluorophores to the 5’ and 3’ ends of complementary 14-mer single-stranded DNA sequences, annealed the sequences in solution to form dsDNA, electrosprayed the dsDNA FRET complex and probed the FRET efficiency over a range of temperatures in a quadrupole ion trap mass spectrometer. As the temperature increased
the measured FRET efficiency was consistent with an “unravelling” in the preliminary AT-stretch of the DNA duplex, potentially due to the breaking of hydrogen bonds in that region. This highlights the utility of fluorescence- and MS-based techniques in probing the dynamics and conformation of biomolecular ions. Next, a more comprehensive review of the fluorescence process, in addition to several key fluorescence-based applications are presented because fluorescence is a major tool used in the work presented in this thesis.

1.6 Analytical Tools and Techniques Used

1.6.1 The fluorescence process

Fluorescence spectroscopy is a popular choice for the structural characterization of biomolecules in solution due to the sensitivity of a fluorophores’ fluorescence properties on its environment. However, fluorescence is only one (of many) processes that can occur upon fluorophore excitation. To illustrate this a typical Jablonski diagram is shown in Figure 1.3, with the possible radiative (rate constant $k_r$) and non-radiative processes (rate constant $k_{nr}$) that may occur. Absorption of a UV/Vis photon will promote a ground-state ($S_0$) fluorophore (timescale $\sim 10^{-15}$ s) to an upper electronic state ($S_1$) where it can undergo numerous additional processes. For fluorescence, an excited-state fluorophore will usually redistribute excess energy into its vibrational modes ($k_{vr}$) before returning to the ground-state via emission of a photon. Competing with fluorescence is the rapid depopulation of an excited-state fluorophore from the excited state to the ground state via internal conversion ($k_{ic}$). Furthermore, an excited-state fluorophore can undergo intersystem crossing ($k_{isc}$), populating an excited triplet state before returning to the ground-state through phosphorescence. Moreover, the fluorescence process is constantly in competition with dissociation pathways ($k_{diss}$), particularly for gas-phase fluorophores. An
Figure 1.3. Jablonski diagram illustrating the multiple pathways that a fluorophore can undergo upon electronic excitation. A list of terms and timescales relevant to processes that occur upon fluorophore excitation are listed below the energy level diagram. This image has been adapted from the work of Forbes and Jockusch.69
excited-state fluorophore can absorb multiple photons (sometimes even one photon is sufficient) causing it to reach a dissociation threshold and fragment. Depending on the timescale of dissociation in comparison to fluorescence, the fluorescence pathway can be quenched by surpassing the dissociation threshold. This highlights that although fluorescence is a popular tool for structural characterization, numerous competing processes can disrupt its fluorescence pathway, which can alter fluorophore brightness, timescale, and/or emission maximum.

The depopulation of an excited-state fluorophore via fluorescence also competes with quenching processes – mechanisms where an excited-state fluorophore transfers energy to an acceptor molecule. Förster resonance energy transfer (FRET; presented above) can occur when the electronic energy spacing of an excited-state fluorophore is resonant with acceptor absorption.\textsuperscript{70} FRET rates depend strongly on the degree of dipole-dipole coupling between the donor and acceptor fluorophore, having an inverse $r^6$ dependence, where $r$ is the donor-acceptor distance. The inverse $r^6$ dependence on FRET rates makes FRET most useful for biological characterization as it allows analysis of systems on the order of 1 – 10 nm. Dexter energy transfer (DET) occurs via electron transfer between both donor and acceptor fluorophores and has an $r$ dependence,\textsuperscript{71} making DET useful for smaller length scales (0.1 – 1 nm). Photoinduced electron transfer (PET) occurs via electron transfer from an excited donor molecule to an acceptor, resulting in the formation of $D^{+}\cdot$ and $A^{-}\cdot$ species. PET is widely used in semiconductors and photosynthesis applications.\textsuperscript{72} Finally, an excited-state fluorophore can also be quenched through other processes such as solvent-mediated proton transfer reactions or through collisions with $O_2$ molecules.

The fluorescence parameter that defines the ratio of photons emitted by a fluorophore relative to photons absorbed is called the fluorescence quantum yield ($\Phi$). Shown in equation 1, $\Phi$ measures a fluorophores’ ability to de-excite via radiative processes (with rate constant $k_r$)
through fluorescence or phosphorescence in comparison to non-radiative (with rate constant $k_{nr}$) processes.

$$\Phi = \frac{k_r}{k_r + k_{nr}}$$

Higher $\Phi$ values indicate that the radiative rate is larger than the non-radiative rate, ie. $k_r > k_{nr}$. A related concept is a fluorophores’ brightness, which is related to the product of a fluorophores’ molar extinction coefficient (which reflects the “overlap” of vibrational levels upon electronic excitation) and $\Phi$, shown in equation 2.

$$Brightness = \varepsilon\Phi$$

The quantum yield is an important measurable parameter as it is sometimes sensitive to a fluorophores local environment. For example, our group investigated the fluorescence properties of gaseous rhodamine B (RhoB), estimating that RhoB’s non-radiative rate is 4-6 times smaller in the gas phase versus in solution. The authors inferred that RhoB’s fluorescence quantum yield is higher in the gas phase due to the absence of non-radiative pathways that are dependent on coupling between solvent and RhoB. This highlights that the fluorescence quantum yield may be highly dependent on a fluorophore’s local environment. An additional parameter that relates to the timescale of the fluorescence process, the fluorescence lifetime, is also sensitive to changes in a fluorophore’s local environment. I outline below information that can be extracted from the fluorescence lifetime and the techniques available to measure it.

1.6.2 Time-resolved fluorescence measurements

The fluorescence lifetime measures the timescale of the fluorescence process and is broadly defined as the time required for $1/e$ (~37%) of an excited-state population to return to the ground state. $\tau$ can be expressed more explicitly using Equation 3:
\[ \tau = \frac{1}{k_r + k_{nr}} \]  

The dependence of \( \tau \) on the non-radiative rate makes the fluorescence lifetime of a fluorophore potentially sensitive to its local environment. For example, the fluorescence lifetime of the DNA intercalator ethidium bromide (the subject of Chapter 3) increases from \( \sim 1.8 \) ns in water to \( \sim 22.5 \) ns when bound to dsDNA, reflecting a substantial decrease in \( k_{nr} \) upon dsDNA binding.

Several techniques exist that can measure fluorescence lifetimes. Here, I focus on time-correlated single photon counting (TCSPC) because it is used to measure fluorescence lifetimes in this thesis.\(^7^4\) In TCSPC, a sample is excited with a short (~ps or fs) pulse of light that is detected by a fast photodiode. This preliminary trigger from laser excitation serves as the “start” pulse. Over numerous laser excitation cycles, the arrival time of fluorescent photons relative to the excitation pulse is detected repeatedly by a sensitive detector such as a single photon avalanche photodiode (SPAD). However, as the repetition rate of laser excitation is several orders of magnitude larger than the fluorescence detection rate, there are many laser excitation cycles where fluorescence from a single photon is not detected. Furthermore, the probability of two fluorescence photons being detected within the same excitation cycle is low, a requirement for TCSPC techniques. A schematic depiction of the TCSPC technique is shown in Figure 1.4a. The signal from laser excitation (start pulse) and the detection of a fluorescence photon by the SPAD (stop pulse) are both sent to a TSCPC PC card in order to measure the start-stop times. A histogram of start-stop times is then recorded to produce the fluorescence decay (Figure 1.4b). Note that the fluorescence setup described in this thesis (subsection 1.7.3) utilizes a reverse start-stop configuration where the emission pulse provides the start trigger, which is conceptually similar to the TCSPC process described above. Overall, TCSPC techniques have gained considerable popularity for the characterization of protein folding and conformational dynamics (particularly when combined with FRET) at the single-molecule level.\(^7^5\)
Figure 1.4. (a) Schematic depiction of the time-correlated single photon counting (TCSPC) process. A laser excitation pulse begins a start trigger and the arrival time of a fluorescence photon is measured relative to this start pulse. (b) A histogram of fluorescence photon arrival times is summed to generate the time-resolved fluorescence curve.
1.6.3 Förster Resonance Energy Transfer (FRET)

FRET is a sensitive fluorescence-based tool that is capable of probing biomolecular structure in the $1 \text{–} 10 \text{ nm}$ distance scale. FRET efficiencies report on the extent of energy transfer between donor and acceptor fluorophores that are typically covalently attached to a biomolecule of interest. Upon laser excitation of the donor fluorophore, if fluorescence emission is resonant with acceptor absorption, quenching of donor fluorescence emission may occur concurrently with acceptor excitation. The FRET efficiency ($E$) between donor and acceptor fluorophores can be characterized using equation 4.

$$E = \frac{R_0^6}{R_0^6 + r^6}$$  \hspace{1cm} (4)

In equation 4, $R_0$ is the Förster distance, which is defined as the distance at which 50% energy transfer occurs between donor and acceptor. The FRET efficiency is inversely related to the donor-acceptor distance; thus, as the donor-acceptor distance increases the extent of energy transfer decreases, resulting in less quenching of donor fluorescence. This makes FRET a versatile tool for studying conformational dynamics of biomolecular ions. Practically speaking, the Förster distance for a given donor-acceptor pair is dependent on several parameters. For example, $R_0$ is dependent on the solvent refractive index, spectral overlap of donor emission with acceptor absorption, fluorescence quantum yield of the donor, absorption cross section of the acceptor, and the relative orientation of donor and acceptor dipoles. Under well-defined experimental conditions $R_0$ can be measured. Thus, FRET efficiencies may be used to determine $r$ via equation 4. FRET efficiencies can be measured experimentally using steady-state fluorescence spectra or time-resolved measurements (equation 5).

$$E = \frac{1}{1 + \frac{\eta_A}{\eta_D} \left( \frac{I_D}{I_A} \right)} = 1 - \frac{A_D}{A_{DA}} \frac{I_{DA}}{I_D} = 1 - \frac{\tau_{DA}}{\tau_D}$$  \hspace{1cm} (5)
For steady-state fluorescence measurements the FRET efficiency can be measured in two ways. The first method is called the ratiometric method and compares the integrated fluorescence intensities of the donor \((I_D)\) and acceptor \((I_A)\) bands in the FRET complex, normalized to \(\frac{\eta_A}{\eta_D}\), which accounts for differences in detection efficiencies and quantum yields of the donor and acceptor. An alternative method is through comparison of the donor band intensity in the presence of the acceptor \((I_{DA})\) to a donor-control species \((I_D)\), normalized to their respective absorbances, \(A_{DA}\) and \(A_D\). Ultimately, energy-transfer pathways in the FRET complex introduce an alternative deactivation pathway for the donor fluorophore in the presence of the acceptor, resulting in a reduction of donor fluorescence intensity. For an appropriate donor-only species, energy-transfer processes due to FRET is absent, resulting in higher donor fluorescence intensities relative to the FRET complex. Thus, steady-state fluorescence spectra offer a relatively simple method of determining FRET efficiencies for conformational ensembles of biomolecules. However, one disadvantage of steady-state FRET spectra is that the measured FRET efficiency reflects a weighted average of the conformational ensembles present for a given biomolecular ion. To resolve conformations with different FRET efficiencies (and thus different donor-acceptor distances), time-resolved measurements are used. In time-resolved measurements, the fluorescence lifetime of the donor in the presence of the acceptor \((\tau_{DA})\) is compared to the fluorescence lifetime of a donor control \((\tau_D)\). Due to the presence of an additional non-radiative deactivation process in the FRET complex, the donor fluorophore will show a decrease in its fluorescence lifetime relative to a donor-control that lacks energy transfer to an acceptor fluorophore. Furthermore, as fluorescence lifetimes can resolve individual conformations arising from different donor lifetimes, time-resolved measurements of donor emission can report on the presence of multiple conformations for a particular biomolecular ion. Thus, due to its high-dependency on the distance between donor and acceptor fluorophores and
the multiple channels from which the FRET efficiency can be extracted, FRET represents a powerful structural tool for the characterization of biomolecular ions.

FRET has been utilized for the structural characterization of dsDNA in solution in several applications, such as molecular beacons and in instances where non-covalent binding increases the fluorescence intensity of an acceptor molecule. For example, Tyagi and Kramer used an EDANS fluorophore (donor) covalently attached to 5’ terminal phosphate of a DNA sequence and a DABCYL quencher (acceptor) joined to the 3’ terminal hydroxyl group to illustrate the molecular beacon principle.76 In the absence of a target DNA sequence that is complementary to the DNA probe with the two fluorophores, the DNA probe formed an intramolecular hairpin that brought the EDANS fluorophore in close proximity to DABCYL. The close proximity of the EDANS fluorophore to the DABCYL quencher resulted in nearly 100% energy-transfer, resulting in quenching of donor fluorescence. However, hybridization of the DNA probe with its complementary DNA sequence unwound the intramolecular hairpin, resulting in a larger distance between the donor and acceptor fluorophores and higher donor fluorescence intensity. Cardullo et al. measured the fluorescence intensity of a rhodamine dye labelled to 5’ end of a 12-mer dsDNA molecule in the absence and presence of the DNA intercalator acridine orange.77 Upon excitation of acridine orange bound to dsDNA, sensitized emission of the rhodamine acceptor at 577 nm occurred, due to energy transfer between acridine orange and the rhodamine dye. These examples demonstrate that FRET can act as a powerful structural probe for the analysis of dsDNA in solution. In Chapter 5, I use this technique to probe the conformation of gaseous dsDNA under a variety of mass spectrometry conditions.
1.6.4 DNA binders with “turn-on” fluorescence properties

An alternative approach to probing the structural properties of dsDNA using fluorescence is by monitoring the “turn-on” of fluorescence for non-covalent DNA binders. Typical non-covalent DNA binders attach to dsDNA by sandwiching between adjacent base pairs (ie., intercalation) or through minor-groove binding (see Figure 1.2). Two classic DNA stains that show significant turn-on fluorescence responses upon dsDNA binding is the DNA intercalator ethidium bromide, and the minor-groove binder DAPI (4,6-diamidino-2-phenylindole). The increase in fluorescence quantum yield for these two DNA binding fluorophores relative to their unbound forms is well characterized (~9-fold for ethidium and ~18-fold for DAPI in 0.01 M Tris buffer). Furthermore, the increase in fluorescence lifetime of these “turn-on” fluorophores upon dsDNA binding is well characterized. For example, the fluorescence lifetime of ethidium increases from ~1.8 ns in water to ~22.5 ns when bound to dsDNA. Olmstead and Kearns initially proposed that the short fluorescence lifetime of ethidium in water is due a non-radiative deactivation pathway arising from excited-state proton transfer between the –NH₂ groups of ethidium and water. They argued that the lifetime of ethidium increases when bound to dsDNA since ethidium is shielded from solvent and this proton transfer is inhibited, dramatically reducing the non-radiative deactivation rate. Other turn-on fluorophores such as SYBR Green I have gained considerable popularity due to their substantially larger fluorescence enhancements (~1500-fold) and higher quantum yields (Φ = 0.69) when bound to dsDNA. The fluorescence enhancement of SYBR Green I is thought to arise due to a restriction in its intramolecular vibrational motions when bound to dsDNA compared to the unbound form. Thus, the well-characterized fluorescence properties for DNA-binding fluorophores serve as a powerful tool for the structural characterization of dsDNA in solution. In Chapter 4, I examine whether the “turn-on” fluorescence properties for DNA-binding fluorophores is preserved in the gas phase, in an
effort to probe the conformation of dsDNA in the gas phase. The next section outlines efforts made to combine fluorescence spectroscopy with a quadrupole ion trap mass spectrometer, specifically for the characterization of dsDNA in the gas phase.

1.7 Instrument Overview

1.7.1 The Quadrupole Ion Trap Mass Spectrometer

The mass spectrometer used for the work described in this thesis is an Esquire 3000+ quadrupole ion trap (Bruker, Bremen, Germany). The ion trapping region of the QIT is made up of two types of hyperbolic metal electrodes: a ring electrode and two end-cap electrodes. Mass spectrometry analysis is performed by applying a high voltage potential operating at a frequency of 781.25 kHz to the ring electrode of the mass spectrometer, while a small (mV – V) oscillating potential is applied to the end-cap electrodes. This provides an upper limit of m/z 3000 for the mass scanning range in normal operating modes. Ions are trapped within the mass spectrometer by applying electric fields to the three electrodes. Ions feel restoring forces that alternately push them toward the center of the ion trap and pull them away, with their motion being described by the Mathieu Equations. Ultimately, one of the powerful features of the QIT is its ability to isolate and manipulate ions in the trapping region of the mass spectrometer. For example, ion isolation is performed by exploiting the fact that trapped ions oscillate at a known frequency (called its secular frequency) that is related to their m/z. By applying a frequency to the end-cap electrodes, ion motion is perturbed due to excitation resonant with the secular frequency. Therefore, ion isolation may be performed by applying a sweep of frequencies to eject all ions in the mass spectrometer except for the ion of interest. Furthermore, the size of the ion cloud can be perturbed through the use of the \( q_z \) trapping parameter, which is proportional to the potential applied to the ring electrode \( V_{RF} \) and m/z through the relationship described in equation 6.
Higher $q_z$ values reflect steeper potential wells for the trapped ions, reducing the size of the ion cloud. The $q_z$ trapping parameter is an important parameter for fluorescence measurements as it determines the extent of overlap between the laser beam and the cloud of trapped ions. In Chapter 2, I perform fluorescence measurements that visualize the cloud of trapped ions in the quadrupole ion trap and the dependence of ion cloud shape on various instrumental parameters, such as $q_z$.

1.7.2 Optical setup

The laser used to perform optical and fluorescence spectroscopy is a Nd:YVO$_4$-pumped Titanium:Sapphire laser (Ti:Sapphire Tsunami, Spectra-Physics, Mountainview, CA). A more detailed description of this laser setup has been outlined previously, so only a brief depiction of the experimental setup is provided here. A schematic depiction of the experimental setup is shown in Figure 1.5 and will be referred to throughout this section. The Ti:Sapphire is a tunable laser source that provides pulsed laser light in the 700 – 1080 nm range at a repetition rate of 80 MHz and ~100 fs pulse width. In order to perform spectroscopic studies in the UV and visible regions, frequency doubling of the Ti:Sapphire output is performed using a Beta-Barium Borate (BBO) crystal to generate 350 – 540 nm light. Typical bandwidths for fundamental light range from 10 – 25 nm and 2 – 4 nm for doubled light. Approximately 10 – 15% of the laser light is frequency doubled, therefore residual IR light is spatially separated from frequency doubled light using either a series of cold mirrors or a Pellin-Broca prism (Thorlabs Inc., Newton, New Jersey). Doubled light can then proceed through one of two optical paths depending on the spectroscopic study performed (this is indicated by dashed lines in Figure 1.5). To measure
Figure 1.5. A schematic depiction of the experimental setup employed for gas-phase fluorescence spectroscopy measurements. The dotted lines indicate that a removable mirror can be positioned such that light passes along one of two optical paths.
fluorescence lifetimes using TCSPC techniques, laser light passes through a pulse picker (Model 350-105, ConOptics Inc., Danbury, CT, USA) to increase the time between laser pulses. For photodissociation and steady-state fluorescence measurements laser light utilizes two separate mirrors that are collinear but translated relative to the optical path for fluorescence lifetime measurements. Laser light can be further attenuated using a neutral density filter (Thorlabs, Newton, New Jersey) and is synchronized with MS/MS events controlled in the Esquire 3000+ software (see below) using a shutter (model SH05 with SC10 controller, Thorlabs) that is triggered by the mass spectrometer.

1.7.3 Modifications to perform gas-phase fluorescence spectroscopy

Several modifications were made to the commercial QIT to enable gas-phase fluorescence and photodissociation spectroscopy. As shown in Figure 1.5, three windows have been fitted to the lid of the vacuum chamber, two that provide entrance and exit ports into and out of the vacuum chamber for the laser beam and a third that allows the collection of fluorescence light. To facilitate excitation of trapped gaseous ions, two 1.2 mm holes have been drilled into the ring electrode, such that laser light passes through the center of the ion trap. A home-built optics assembly that attaches to four horizontal rods on the QIT contains two mirrors that reflect the light through the holes in the ring electrode. The optics assembly also contains light baffles (1.0 mm aperture diameter) above the mirrors that minimize scattered laser light and other background from reaching the ions and the fluorescence detector. Fluorescence is collected through a third hole of 2.0 mm diameter that is orthogonal to laser excitation. Within this hole is a plano-convex lens that is ~1 cm from the center of the ring electrode and collects the fluorescence from trapped ions. Based on the distance of this lens from the centre of the trapping region and the fluorescence collection angle, it is estimated that 0.25% of all fluorescence light is
collected.\textsuperscript{66} Fluorescence then passes outside of the vacuum chamber, is refocused by a second
lens and reflected by the fluorescence collection mirror. To remove light arising from the
excitation beam, an appropriate long- or band-pass filter is chosen to selectively transmit
fluorescence. Fluorescence can then be sent to a spectrograph that contains an electron-
multiplied charge-coupled device (EM-CCD, Andor, Belfast, UK) to measure gaseous emission
spectra or ion cloud images.\textsuperscript{69,83} Alternatively, a removable mirror with a magnetic base can be
positioned along the optical path such that fluorescence light is directed to a single photon
avalanche photodiode (SPAD, Micro Photon Devices, Bolzano, Italy). The signal from the
SPAD is then directed to a TCSPC PC card to synchronize laser excitation and fluorescence
events to measure fluorescence lifetimes.\textsuperscript{84} Overall, it is estimated that the detection efficiency of
this setup is 0.15\%\textsuperscript{66}. Nevertheless, as the EM-CCD and SPAD are optically sensitive choices
engineered for low fluorescence light applications, this experimental setup is capable of probing
spectroscopic properties of gas-phase fluorophores. Next, the experimental sequence that is
followed to perform spectroscopic studies on gas-phase ions is outlined.

1.7.4 Experimental sequence

To perform a fluorescence experiment in the gas phase, ions are transferred from solution
into the gas phase via electrospray ionization. These ions then pass through a series of ion optics,
which include a heated glass capillary to promote solvent desolvation, a skimmer and two
octopoles that provide some mass discrimination. Ions are then accumulated in the trapping
region of the QIT. To remove excess internal energy and increase the trapping efficiency in the
QIT, the helium pressure is adjusted (0.6–2.3 x 10\textsuperscript{-3} mbar, depending on experiment). Following
ion thermalization, the precursor ion of interest can be mass selected and stored at a particular \( q_z \)
trapping parameter. Typical \( q_z \) values for fluorescence experiments are 0.59 to promote efficient
laser overlap with the cloud of trapped ions. As this experimental setup collects a small portion of the total fluorescence intensity, it is critical to maximize the number of trapped ions. This is measured through the ion charge control (ICC), a parameter that is provided by the MS software and reflects the number of secondary electrons produced at the electron multiplying detector. Irradiation of the trapped, mass-selected ions (discussed above) is synchronized with mass spectrometry events using a trigger that is controlled by the MS software. Following irradiation for a defined amount of time, the optical shutter will close and the MS/MS spectrum is recorded after the precursor/product ions are scanned out of the trap in a mass-selective manner. For fluorescence experiments, irradiation parameters and helium pressures are chosen such that <10% of the precursor ion photodissociates, ensuring that detected fluorescence does not arise to a significant extent from photofragments. Fluorescence emission spectra reflect the sum of repeated measurements with multiple ion populations. Emission spectra are then collected with no ions in the trap, but identical experimental conditions to enable appropriate background subtraction.

An alternative approach to investigating the spectroscopic properties of gas-phase fluorophores is through photodissociation “action” spectroscopy. Following the experimental sequence described above, trapped, mass-selected ions can be irradiated with laser powers sufficiently high to cause dissociation of covalent or non-covalent bonds. The extent of photodissociation (or photodissociation yield) is calculated by measuring the MS/MS intensity for the precursor ion during “laser on, laser off” periods. Then, the photodissociation yield is plotted as a function of wavelength to measure a pseudo-absorption spectrum for a fluorophore in the gas phase. In addition, photodissociation as a function of laser power (photodissociation power dependence) or as a function of irradiation time (dissociation kinetics) can be measured.
1.8 Scope of Work

The scope of this thesis is to report on the coupling of fluorescence spectroscopy with mass spectrometry, and the application of this technique for the structural characterization of dsDNA and dsDNA-dye complexes in the gas phase. Chapter 2 describes work that aims to visualize through fluorescence the ion cloud of trapped rhodamine 640 ions in the quadrupole ion trap mass spectrometer. The first image collected under normal mass spectrometry conditions is presented, as well as the effect of ICC and $q_z$ on the size of the ion cloud.

Chapter 3 reports on the intrinsic photophysics of gaseous ethidium ions, a classic DNA fluorophore which exhibits a well-known fluorescence enhancement upon binding dsDNA in solution. Gaseous emission spectra and time-resolved fluorescence are presented for isolated ethidium ions and ultimately compared to the spectral properties of ethidium in solution. Furthermore, in an effort to characterize the quantum yield of ethidium in the gas phase, relative brightness measurements are performed relative to a rhodamine dye that is well-characterized by our group. This work’s significance is that the spectroscopic properties of gaseous ethidium ions was found to be dependent on its environment, which in the future may provide some of the information needed to design more robust fluorophores for gas-phase applications.

Chapter 4 characterizes the spectroscopic properties of seven “turn-on” fluorophores both in isolation and when complexed to gaseous dsDNA to develop fluorescence tools for the structural characterization of dsDNA in the gas phase. While fluorescence is observed for six of the fluorophores when not bound to dsDNA in the gas phase, fluorescence is observed for only two dsDNA-dye complexes. This highlights that although several dsDNA-dye complexes exhibit well-characterized fluorescence “turn-on” responses in solution, the removal of solvent plays a role in the deactivation pathways for these non-covalent binders. Ultimately, this contribution provides an example where mass spectrometry and optical spectroscopy tools are combined to
characterize the fluorescence properties of dsDNA-dye complexes. In the future, the coupling of these techniques could characterize the conformation of dsDNA in the gas phase.

In Chapter 5, FRET is used to probe the conformation of a 20-mer dsDNA molecule with BODIPY fluorophores appended to the 5’ ends of complementary DNA sequences in the gas phase. Steady-state and time-resolved fluorescence measurements are performed on the 20-mer dsDNA complex in the 7’, 8’, and 9’ charge states. Furthermore, in an attempt to characterize changes in dsDNA conformation, FRET efficiencies are compared under a variety of mass spectrometry conditions. This work represents the first time that wavelength-dispersed and time-resolved FRET measurements are used to probe the conformation of dsDNA under a variety of mass spectrometry conditions. Moving forward, the development of gas-phase FRET techniques will provide highly accurate point-to-point distances that are capable of probing gaseous biomolecular conformation.

The goal of this thesis is to develop MS and optical spectroscopy tools to better characterize the conformation adopted by dsDNA in the gas phase. This will serve to investigate the degree to which gaseous dsDNA reflects its well-known solution-phase conformation. This is an important (and currently unanswered) question in the MS community, and has broader implications for the characterization of biomolecular ions in the gas phase. Thus, the work presented in this thesis aims to bridge the gap between the conformation of biomolecules in solution in the gas phase, increasing the applicability of MS for the structural characterization of biomolecules.
1.9 References

Chapter 2

Fluorescence Imaging for Visualization of the Ion Cloud in a Quadrupole Ion Trap Mass Spectrometer

This chapter has been adapted from:


This chapter has removed contributions from the primary author (F.O. Talbot) and only included experiments performed by S.V. Sciuto.

Abstract

Laser-induced fluorescence is used to visualize populations of gaseous ions stored in a quadrupole ion trap (QIT) mass spectrometer. Presented images include the first fluorescence image of molecular ions collected under conditions typically used in mass spectrometry experiments. Under these “normal” mass spectrometry conditions, the radial \( r \) and axial \( z \) full-width at half maxima (FWHM) of the detected ion cloud are 615 μm and 214 μm, respectively, corresponding to ~6% of \( r_0 \) and ~3% of \( z_0 \) for the QIT used. The effects on the shape and size of the ion cloud caused by varying the pressure of helium bath gas, the number of trapped ions and the Matthieu parameter \( q_z \) are visualized and discussed. Fluorescence images such as those presented here provide a useful reference for better understanding the collective behavior of ions in radio frequency trapping devices and how phenomena such as collisions and space-charge affect ion distribution.
2.1 Introduction

Quadrupole ion trap (QIT) instruments are a popular type of mass spectrometer due to their extensive multiple-stage tandem mass spectrometry capabilities, small footprint and reasonable costs. The analytical performance characteristics of any mass spectrometer, including QITs, ultimately depends on the behavior of ions within the instrument. In a QIT, ion behavior is influenced primarily by the overall electrodynamic field used to trap and manipulate ions. The impact of the electrodynamic field can also be modulated by the frequency and energy of collisions between the trapped ions with any other gaseous molecules present in the ion trapping region. For example, a key improvement in the performance of QIT mass spectrometers was the introduction of the mass-selective instability scan and realization that the presence of ~10^{-3} mbar helium bath gas substantially improved instrument sensitivity and mass resolution of the scan.\(^1\) The improvement in resolution was attributed to the collisional damping of ion motion, which results in a more homogeneous starting point for ion ejection.

The overall electrodynamic field experienced by a given ion depends not only on the geometry of the electrodes and the potential applied, but also on the presence of other ions within the trapping region through space-charge effects. The collective effect on ion cloud behavior by changing instrumental parameters is not always straightforward to predict or understand. For example, mass shifts of up to ~0.5 m/z units for some ions used to plague QIT mass spectrometers that used the “ideal” geometry, in which the radius of the ring electrode \((r_0)\) and spacing from the center of the trap to the closest point on the end-caps electrodes \((z_0)\) are related by \(r_0 = \sqrt{2}z_0\). The introduction of the “stretched” configuration by Finnigan MAT Co., in which the end-cap spacing was increased above the value in the “ideal” geometry, substantially alleviated the unexpected mass shifts. The modified hyperbolic angle trap employed by Bruker Daltonics has similar benefits.\(^2\) Several years after the unmasking of the stretched geometry,\(^3\) the
origin of chemical mass shifts were attributed by Cooks and coworkers primarily to the ion transport holes in the end-cap electrodes. Simulations and experiments show that these cause sufficient deviations from a quadrupolar potential to explain the appearance of the chemical mass shifts. Another example of complex collective ion behavior is evidence presented by Tolmachev and co-workers for ion cloud stratification in radio frequency (rf) multipole devices. Simulations show that upon simultaneous trapping of ions of disparate m/z values, the ions of lower m/z value are confined to the center of the trap while higher m/z ions are essentially excluded from the central volume. This result can be understood with the help of the Mathieu parameter $q_z$, which is proportional to $V_{RF} \cdot (m/z)^{-1}$, where $V_{RF}$ is the amplitude of the voltage on the ring electrode. Ions at higher $q_z$ can be thought of as experiencing a deeper potential well. Thus ions with a lower m/z are more strongly confined at a given $V_{RF}$.

Theoretical calculations have been a major resource to understand ion behavior in rf ion traps. For example, packages such as SIMION (currently marketed by Scientific Instrument Services, Ringoes NJ) and ITSIM have been widely used for the simulation of ion trajectories in mass spectrometers. While the effects of a known potential field on a single ion in vacuum is straightforward to simulate, effects due to the repulsion of multiple ions of like-charge (ie, space charge effects) and correct description of collisions present a significant challenge. One approach to incorporate these effects is their inclusion as perturbations (often damping and diffusion terms) to the trapping field. Very recently, large-scale simulations using parallel computing techniques have been used to include the effects of charge-charge interactions for up to $10^5$ ions in QITs and up to $10^6$ ions in Fourier-transform ion cyclotron resonance mass spectrometers.

One useful type of experiment to better understand the spatial distribution of ions within a QIT is based on measuring the extent of photodissociation as a laser beam is rastered across the
trapping volume. This has been termed “ion tomography.” In pioneering experiments, Cooks and collaborators monitored the axial distribution of ions in a stretched geometry Finnigan ion trap mass spectrometer by rastering a laser beam along a slot cut in the ring electrode. They showed that the axial dimension (from one end-cap to the other) of the ion cloud follows a Gaussian distribution under a variety of experimental conditions. The distribution appears narrowed upon the inclusion of \( \sim 10^{-3} \) mbar helium bath gas and upon increase of the amplitude of the trapping voltage on the ring electrode (ie, upon increasing the Mathieu parameter \( q_z \) of the ions). Later, these experiments were extended to monitor the radial distribution of the ion cloud, which was also found to follow a Gaussian distribution. When the number of ions stored in the trap was increased 35-fold, the extent of the radial distribution was observed to expand significantly (\( \sim 60\% \)) whereas the extent of the axial distribution remained approximately constant. Recent work by Remes and Glish explored the effect of \( q_z \) value on observed infrared multiple photon dissociation (IRMPD) efficiency. Higher IRMPD efficiency was observed at higher \( q_z \) values, which was rationalized as resulting from a smaller ion cloud, and hence more efficient overlap between the laser beam and ion cloud at higher \( q_z \) values.

Another fruitful type of experiment to monitor ion behavior in a QIT is based on optical spectroscopy. Advantages of optical spectroscopy over ion tomography experiments include the higher spatial resolution achievable and the relative lack of sensitivity to photodissociation and collisional cooling timescales. Early experiments by Wuerker et al. employed elastic light scattering to track the position of aluminum microparticles (\( \sim 20 \mu m \) diameter) in a QIT, producing now well-known photographs showing the Lissajous trajectory of a single particle. Simultaneous trapping of multiple microparticles formed “Coulomb crystals,” long-range crystals that possess extensive three dimensional order. Laser-induced fluorescence (LIF) has been used for decades in the physics community to study the behavior of small numbers of ions.
in traps.\textsuperscript{13, 21-27} Generally, these experiments are carried out for the purposes of high-resolution spectroscopy, optical clocks or the development of quantum computing, under conditions not employed in commercial QIT mass spectrometers. The vast majority of these experiments have monitored \textit{atomic} ions, which are highly fluorescent at low pressures and can be laser-cooled to temperatures of a few degrees Kelvin.\textsuperscript{25} In 1978, Neuhasuer, Dehmelt and co-workers published photographs showing the fluorescence from a few thousand barium ions confined in an “ideal” geometry quadrupole ion trap.\textsuperscript{21} Similar experiments were reported by other laboratories.\textsuperscript{22, 23} Ion clouds were observed to exhibit Gaussian distributions, with FWHM values that depend on the value of the trapping parameter $q_z$, generally falling within the range of $1/10 \rightarrow 1/5$ of the ring electrode radii. Another notable result was published in 1988 by Neuhauser and co-workers who made use of the Doppler effect to record photographs showing velocity dependent images of barium ion distribution.\textsuperscript{13} These images again illustrated the Gaussian nature of the spatial ion distribution and confirmed that the ions located at the exterior of the ion cloud have higher kinetic energies than those nearer the center. Doppler imaging also showed that ions trapped at higher $q_z$ have higher average velocities.

In this work, laser-induced fluorescence is used to visualize the behavior of ion clouds in a quadrupole ion trap mass spectrometer under a variety of experimental conditions of interest to mass spectrometrists. The dependence of ion cloud shape and dimension on the number of trapped ions, trapping parameter $q_z$ value and helium pressure is explored.

2.2 Experimental

2.2.1 \textit{Fluorescent dyes}

Several cationic fluorescent dyes were used in this work: rhodamine 640 (Rh640, also known as rhodamine 101, $m/z$ 491). The structures of this dye may be found in Appendix A.
Rh640 was purchased as a perchlorate salt from Exciton (Dayton, OH, USA). Solutions for ESI-MS were co-dissolved in equal volumes of LC-MS grade methanol (Sigma-Aldrich, St. Louis, MO) and ultra-pure water. One μM Rh640 solutions were used for all ion cloud imaging experiments presented here.

2.2.2 Instrumentation for Mass Spectrometry and Laser-induced Fluorescence

We have recently modified a commercial quadrupole ion trap (QIT) mass spectrometer (Esquire 3000+ from Bruker Daltonics) to enable fluorescence spectroscopy and imaging of the gaseous ions. This experimental set-up has been described in detail previously. Briefly, ions are generated by electrospray ionization, transmitted through a series of ion optics and accumulated, manipulated and stored in the trapping region of the QIT. The number of ions in the QIT can be assessed by monitoring the Ion Charge Control (ICC) value in the Esquire Control software, which reflects measured ion current and thus provides a relative measure of the number of trapped ions of a given m/z.

Fluorescence excitation of the trapped ions is enabled by two 1.2 mm diameter holes drilled through the ring electrode (r_o≈10 mm), such that light from a Ti:Sapphire (Spectra-Physics, Mountain View, CA) laser intersects the cloud of trapped ions. The Ti:Sapphire laser generates tunable light in the wavelength range of 360–540 nm upon second harmonic generation. The laser beam has a 1/e^2 diameter of 617 μm at the center of the trap where it intersects the ion cloud. A home-built optics assembly, which contains light baffles and mirrors, sits bridging over the ring electrode to facilitate fluorescence excitation using light that enters the vacuum chamber from above. Fluorescence excitation and concurrent detection of emitted light is enabled during selected MS/MS events using a trigger from the Esquire Control software. The trigger is sent to a Stanford Research Systems (SRS, Sunnyvale, CA) digital delay generator.
which signals the opening of a shutter, allowing laser irradiation to enter the trap. Fluorescence from trapped ions is collected through a third hole in the ring electrode of 2.0 mm diameter, which is orthogonal to the laser excitation beam. This hole contains a plano-convex lens (back focal length 11.3 mm) that is embedded ∼1.5 mm from the inner surface of the ring electrode. The collected fluorescence then passes outside of the vacuum chamber, where it is refocused by a second lens \((f = 100 \text{ mm})\) and reflected by a silver mirror oriented at a 45° angle. The reflected fluorescence is then passed through appropriate long- and/or band-pass filters (Chroma Technology Corp., Rockingham, VT, USA) to limit scattered light. Finally, fluorescence emission is focused by a third lens \((f = 100 \text{ mm})\) onto the slit of a spectrograph (Shamrock 303i, Andor Tech., Belfast, Ireland) which is coupled to a sensitive electron-multiplying charge coupled device (EM-CCD, Newton, Andor Technologies, Belfast, Ireland) for fluorescence detection. Fluorescence spectra were measured using a 300 line/mm grating, blazed at 500 nm. Images were recorded by using the zero-order diffraction (i.e., reflection) from a 300 line/mm grating, blazed at 300 nm. To generate high-quality images, the EM-CCD was operated using a readout rate of 50 kHz, 4x pre-amplification gain, electron-multiplication gain of 220, and temperature of 193 K. The combined collection optics produces a ∼1.6-fold magnification of the projected image, as estimated using an optical ray tracing software (Optalix, Optenso Co., Heerbrugg, Switzerland). The EMCCD camera has pixels of 16 μm x 16 μm. Thus, the images recorded with this configuration have 10 μm per pixel resolution.

2.2.3 Collecting an Image: Experimental Sequence

To generate an image of the ion cloud, ions are transferred into the gas phase via ESI and accumulated in the trapping region of the QIT. Gas-phase ions are then mass selected and stored at a specified \(q_z\). After ion accumulation, a delay period (≥ 30 ms) is implemented to ensure that
the ion cloud has been cooled to the center of the trap. Following this delay period, a shutter opens to irradiate the ion cloud for a specified length of time ($t_{irr}$) with light from the Ti:Sapphire laser during an MS/MS event defined in the Esquire Control software. Fluorescence is concurrently collected as described above. Ion cloud images shown are the difference between signals measured with and without ions in the trap. An example of the background may be found in Appendix A, Figure S1 (Figure A.1). A summary of the experimental conditions used for ion cloud imaging experiments is provided in Table 2.1. Experimental conditions are selected such that photodissociation is not observed in the mass spectra, unless otherwise noted.

2.2.4 Data Processing

Images were processed via horizontal and/or vertical binning using a program developed in-house, written in the Basic programming language supplied with the CCD camera software. The program scans an image in the horizontal (or vertical) direction, summing the fluorescent intensities for that given row (or column) of pixels. This results in radial (from horizontal binning) and axial (from vertical binning) profiles of the image. In order to assess the dimensions of the ion cloud, widths at 50% were calculated by fitting the vertical and horizontal profiles with a Gaussian function.
Table 2.1. Summary of experimental conditions used for ion cloud imaging.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>( \lambda_{\text{ex}} ) (nm)</th>
<th>ICC/10(^3)</th>
<th>( q_z )</th>
<th>( t_{\text{irr}} ) (s)</th>
<th>Power (mW)</th>
<th>( P_{\text{He}}/10^{-4} ) (mbar)</th>
<th>Image accumulation time (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal mass spectrometry conditions</td>
<td>510</td>
<td>65</td>
<td>0.27</td>
<td>1</td>
<td>12.5</td>
<td>5.7</td>
<td>4,800</td>
</tr>
<tr>
<td>Effect of ICC</td>
<td>510</td>
<td>65–1,500</td>
<td>0.59</td>
<td>1</td>
<td>15, 20</td>
<td>17</td>
<td>480</td>
</tr>
<tr>
<td>Effect of ( q_z )</td>
<td>510</td>
<td>65, 300</td>
<td>0.27–0.82</td>
<td>1</td>
<td>10</td>
<td>17</td>
<td>1200</td>
</tr>
</tbody>
</table>

2.3 Results and Discussion

Figure 2.1a shows a false color image of the fluorescence from an ensemble of rhodamine 640 ions, stored in a quadrupole ion trap mass spectrometer under normal MS operating conditions. This image was constructed by summing the fluorescence signal from 4800 trapped ion populations, each irradiated with 12.5 mW of 510 nm light for 1 second. Subtracted from these data was a background image, measured using the same experimental sequence and timing but no ions in the trap. The pressure of the helium buffer gas in the trap was set to Bruker’s recommended operating pressure (~6 \( \times \) 10\(^{-4} \) mbar)\(^{29}\) and ions were stored at the Matthew stability parameter \( q_z \) of 0.27, which results in a low mass cut-off (LMCO) of 30% of the parent \( m/z \). The number of ions stored in each population was the same as normally used for MS experiments with this instrument, characterized by an ICC value of 65 \( \times \) 10\(^3\). This ICC results in significantly better than unit mass resolution. Projecting the image along the axial or radial coordinate (i.e., binning the image shown in the vertical or horizontal direction) results in what we term “ion cloud profiles” in each dimension. The profiles measured for the normal MS condition ion cloud are also shown in Figure 2.1a. Each profile is well fit by a Gaussian distribution. The measured FWHM of the profiles are 214 \( \mu \text{m} \) in the axial dimension and 615 \( \mu \text{m} \).
Figure 2.1. (a) Image of the ion cloud under normal mass spectrometry conditions, together with radial (blue) and axial (red) profiles. (b) Ion cloud shown to scale within the electrodes of the QIT.
in the radial dimension. These values respectively correspond to ~3% of \( z_0 \) and ~6% of \( r_0 \) for this trap. Clearly, the trapped ions occupy only a very small portion of the trapping volume. This is illustrated in Figure 2.1b, which depicts an axial slice through the center of the electrode set with the image of the ion cloud shown to scale. We note that the FWHM measured from these fluorescence experiments are about one half the size of that predicted from earlier trajectory calculations performed in our lab, which used SIMION.\(^{29}\) This discrepancy may be due to the inadequacy of the simple hard-sphere collision model\(^{30}\) used in the trajectory calculations to describe ion behavior in the QIT. Another simplification present in the simulation was that space charge was ignored; however this cannot explain the smaller size of the actual ion cloud as the presence of space charge will act to increase the size of the cloud.

Measurement of the background-subtracted “normal MS condition” image shown in Figure 2.1 required a total of ~3 hours, a long experiment by mass spectrometry standards. This is longer than used to acquire images in subsequent experiments (Table 2.1) and also significantly longer than generally required (often 5 – 10 minutes) for measurement of a dispersed fluorescence spectrum with this instrument. The largest source of noise in this set-up is read-out noise from the EMCCD camera. Because the images require the transfer of 200-fold more data points than a dispersed spectrum (which benefit from on-chip full vertical binning), this lowers the overall signal-to-noise of an image, requiring longer acquisitions.

For fluorescence experiments we find it advantageous to operate at pressures 3 – 4 fold higher than the standard operating pressure recommended by Bruker. The increased pressure suppresses photodissociation, thus enabling the use of higher laser excitation power.\(^{28}\) We note that Rh640 was selected for the imaging experiments because it is more robust than many of the other rhodamines that we have examined, and has a higher photodissociation threshold.\(^{28, 31}\) For other small fluorophores, irradiation powers of 1 – 5 mW in our set-up is often optimal. No
observable change in the measured FWHM of the images results from this increase in pressure (Figure A.2). This result was unexpected, as it disagrees with conclusions from Cooks and coworkers who observed that higher pressures decreased the FWHM measured in ion tomography experiments.\textsuperscript{17} A likely explanation for this discrepancy is the higher background pressure present in our instrument ($1 \times 10^{-5}$ mbar compared to $3 \times 10^{-8}$ mbar in the work of Cooks). Alternatively, it may be that collisional damping of ion internal energy at the higher pressures, which might result in lower photodissociation yields in the ion tomography experiments and lower measured FWHM.

The effect of increasing the number of ions stored on the measured fluorescence image is shown in Figure 2.2. In Bruker traps, the ion charge control (ICC) value gives a relative read-out of the ion current measured for a given $m/z$. In our present set-up, ICC values of $30-65 \times 10^3$ are generally used as these ICC values result in mass spectra of reasonable signal-to-noise and better than unit mass resolution. We estimate that these ICC values correspond to $2-5 \times 10^3$ ions.\textsuperscript{28} Increasing the ion accumulation time results in significantly higher ion signal, at the cost of decreased mass resolution and accuracy due to space charge effects. At ICC $> 300 \times 10^3$ the isotopic peak of rhodamine 640 is no longer visible and at ICC = $10^6$, significant apparent mass shifts are evident (Figure A.3). Figure 2.2a shows images measured with ICC values ranging from $65 - 1,500 \times 10^3$. Panels b and c show radial and axial profiles derived from these images while the FWHM extracted from the profiles is plotted in panel d. All these data were measured using a $q_z$ of 0.59 in order to increase fluorescence signal (see below), which accounts for the differences between the $65 \times 10^3$ ICC image shown here and that of Figure 2.1.

Several trends are evident from the data shown in Figure 2.2. First, as an increasing number of ions are trapped, the cloud expands more quickly in the radial dimension than it does in the axial dimension. This results in an image that is significantly more oblong (aspect ratio of
Figure 2.2. (a) Ion cloud images measured with a range of numbers of trapped ions. The ICC value indicated below each image gives a relative value of the number of trapped ions (see text). (b) Radial and (c) axial binning profiles as a function of ICC. (d) Measured FWHM values for radial and axial dimensions as a function of ICC.
~3.1) at the higher ion numbers than at the lowest (aspect ratio of ~2.5). At normal ion numbers, the measured axial and radial profiles are well-fit by Gaussians. This is true in the axial dimension for all probed ICC values, but in the radial dimension, an asymmetry becomes apparent at ICC ≥ 300 × 10³. The radial profiles change a little upon realignment of the fluorescence collection path. Thus, we believe that the observed lack of symmetry is due to clipping of the fluorescence image, rather than an actual asymmetry in the ion cloud, though in theory the latter could be caused by an asymmetry in the ring electrode. We also note that the cloud rapidly expands in size (~1.4 fold radially and ~1.1 fold axially) as the ICC value increases from 65 to 300 × 10³, a point at which isotopic peaks coalesce (Figure A.3). The expansion in ion cloud size slows down dramatically as the number of ions is increased another five-fold from 300 to 1,500 × 10³, and is accompanied by a large mass shift (Figure A.3). The data shown in Figure 2.2 is reminiscent of observations from ion tomography experiments of Cooks and coworkers, who also noted that the radial dimension of the ion cloud expanded rapidly at low ions numbers but that ion cloud size levels off as the ion number was further increased. However, no expansion in the axial dimension upon increasing ion number was apparent in the ion tomography experiments, while the LIF results reported here clearly show a measurable effect (Figure 2.2d).

Another parameter that significantly affects ion behavior is the Matthieu stability parameter \( q_z \). Increased \( q_z \), as results from either increased voltage on the ring electrode and/or lower \( m/z \), should result in ions being trapped in a deeper pseudo-potential well, thus compressing the ion cloud. Consequently, for an ion of a given \( m/z \), an increase in the \( q_z \) value will result in a greater overlap between the ion cloud and the Gaussian excitation beam from the laser. Under normal operating conditions, the ion cloud is somewhat larger than the focused irradiation beam (617 µm 1/e² beam diameter according to knife edge measurements vs. ~615
μm radial FWHM ion cloud, which corresponds to a 1/e^2 diameter of 1,045 μm as the radial profile has a Gaussian shape. Figure 2.3 shows the effect of increasing ion \( q_z \) value on the shape and size of the ion cloud. The images shown in panel a were recorded at \( q_z \) values ranging from 0.27 (30% LMCO) to 0.82 (90% LMCO) with a normal number of ions (ICC = 65 × 10^3) stored in the trap. Panel c shows a series of images recorded over the same \( q_z \) range, but with a large number of ions in the trap (ICC = 300 × 10^3). Panels b and d show radial and axial profiles derived from these images, with the FWHM extracted from the profiles plotted in e. The size of the ion cloud decreases dramatically as the ion \( q_z \) is increased. The decrease is largest in the radial dimension, which is 40% smaller at \( q_z \) 0.82 than at \( q_z \) 0.27 when a normal number of ions are present in the trap. The decrease in the axial dimension is much less, just 22% over the same range. For the higher number of stored ions, the decrease in FWHM is 25% and 17% in the radial and axial dimensions, respectively. We note that the integrated fluorescence intensity increases as \( q_z \) (and the LMCO) is increased. We attribute the apparent decrease in fluorescence intensity of the LMCO 90% data at ICC = 65 × 10^3 to photodissociation. Other series of experiments, taken with a lower laser powers, show the expected increase in fluorescence intensity at LMCO 90% (see for example Figure A.4). The trend towards increased brightness is in qualitative agreement with the expectation of a better overlap between the ion cloud and excitation beam at higher \( q_z \). However, the magnitude of the observed effect is significantly larger than expected. As the LMCO is raised from 30% to 65% for the normal number of ions, the measured fluorescence intensity doubles. In contrast, the calculated overlap of the measured ion cloud sizes with a 700 μm beam only increases by 23% (t). For the larger ion cloud (ICC = 300 × 10^3), the discrepancy is even larger, with a measured fluorescence intensity increase of 233% while the calculated overlap increases by just 13%. Another possible explanation for the large increase in fluorescence intensity as the ion cloud is compressed is a
Figure 2.3. Ion cloud (a) images and (b) profiles measured with increasing $q_z$ value (specified as LMCO% = $q_z/0.908 \times 100\%$) from left to right with a normal number of ions in the trap (ICC = $65 \times 10^3$). Images (c) and profiles (d) measured with the same LMCOs as in (a), but with more ions in the trap (ICC = $300 \times 10^3$). (e) Measured FWHM values of radial (blue) and axial (red) profiles at ICC of $65 \times 10^3$ (filled symbols) and $300 \times 10^3$ (open symbols).
difference in collection efficiency of light originating from the center vs. the edge of the field of view of the collection optics. However, the collection solid angle differs by <1% for a point in the center of the trap and a point 1 mm away from the center. Thus, the magnitude of this effect is extremely small. Another factor that can contribute to the increase in brightness with $q_z$ is the higher velocity of ions at higher $q_z$.\textsuperscript{13} Higher ion velocity will result in an increased number of collisions, which can relax ions stranded in a long-lived dark (presumably triplet) state,$^{33}$ returning them to the ground state. This will increase the number of ions available for excitation and fluorescence, thereby increasing the number of fluorescence cycles each ion will undergo during the course of the irradiation time.

2.4 Conclusions

As the saying goes, “seeing is believing.” Here, fluorescence is used to visualize the cloud of ions stored in a quadrupole ion trap mass spectrometer under a variety of conditions. This includes the first fluorescence image reported of an ion cloud under typical mass spectrometry conditions. Other reported images show the increase in size of the ion cloud as more ions are held in the trap and the decrease in ion cloud size as the ion $q_z$ value is increased. While the vast majority of the phenomena observed in this work can be or have been predicted from simulations, it is always valuable to have pictures from experiments. Moreover, significant challenges to accurate modeling of ensembles of ions in rf devices remain. These include the implementation of realistic descriptions of collisions and the ability to account for space charge effects. Images, such as the ones reported here, should provide a basis against which to calibrate results from simulations.
A long-term goal of this imaging work is the creation of a movie showing the evolution of an ion cloud during an MS experiment. To achieve adequate time resolution for the construction of such a movie, substantial improvement in the sensitivity of these experiments is essential. Routes to increase sensitivity include, as ever for fluorescence experiments, the need to decrease background signal and to increase the solid angle for fluorescence collection. In our current set-up, at most 0.20% of the light is collected through the 2.0 mm diameter hole in the ring electrode. Increasing the size of this aperture will substantially increase the fluorescence collection efficiency.

2.5 Acknowledgements

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Appendix A

For information that is supplementary to the main text of this article, see Appendix A.

2.6 References


Chapter 3

The intrinsic photophysics of gaseous ethidium ions


Abstract

Ethidium is a cationic dye with fluorescence that is enhanced ~9-fold upon binding DNA. In order to better understand how the local environment modulates the behavior of this dye, we measured the photophysical properties of gaseous ethidium ions, using a quadrupole ion trap mass spectrometer that has been modified for fluorescence spectroscopy. The photodissociation maximum of gaseous ethidium measured through action spectroscopy is 485 nm and the emission maximum is 548 nm. The Stokes shift (2370 cm\(^{-1}\)) of gaseous ethidium is marginally larger than that of ethidium in non-polar solvents, and significantly less than that in polar solvents. Time-resolved fluorescence measurements of gaseous ethidium ions show two components with lifetimes of 21.4 ± 1.5 and 5.1 ± 0.7 ns, which suggest the presence of multiple conformations in the gas phase. Both lifetimes are significantly longer than that of aqueous ethidium, while the longer of the two lifetimes is remarkably similar to that of ethidium in complex with double-stranded DNA in solution. In line with this, the estimated quantum yield of gaseous ethidium is ~30% lower than that of ethidium in complex with DNA in solution, and ~10-fold higher than that of aqueous ethidium. These benchmark results provide a reference from which to better understand the factors that modulate the fluorescence of phenanthridine-based dyes by the local environment.
3.1 Introduction

Ethidium bromide (Scheme 3.1) is a classic DNA stain whose fluorescence is enhanced 9-fold upon binding to double-stranded DNA (dsDNA). The enhancement of ethidium fluorescence is thought to arise due to an intercalative mode of binding, where the ethidium molecule lies stacked between hydrogen-bonding base pairs. Intercalation reduces the non-radiative deactivation rate constant \( k_{nr} \), evident from the significant lengthening of the fluorescence lifetime of the dsDNA-ethidium complex (\( \tau = 22.5 \text{ ns} \)) relative to aqueous ethidium (\( \tau = 1.8 \text{ ns} \)). While the dsDNA-ethidium complex has been much-studied in solution, the mechanism of the fluorescence enhancement upon DNA binding is still not well understood. An understanding of ethidium’s intrinsic photophysical properties, which can be attained by removing solvent interactions and performing measurements in the gas phase, will provide a baseline from which to better understand the mechanism of fluorescence enhancement upon binding DNA and aid the design of more efficient turn-on fluorophores.

Scheme 3.1. The structure of ethidium.

The underlying photophysics of ethidium bromide in solution and its fluorescence enhancement upon binding to dsDNA have been extensively investigated, although no consensus has been reached. In an important early contribution, Olmstead and Kearns concluded that the quenching of ethidium in polar solvents results from excited-state proton
transfer from the exocyclic –NH₂ groups (which becomes more acidic in the excited state) of ethidium to solvent. The significance of this quenching mechanism was supported by the short fluorescence lifetime in water (~1.8 ns) in comparison to that measured in ethanol, dimethyl sulfoxide (DMSO), and glycerol (5.0 – 6.0 ns), suggesting a substantially larger non-radiative deactivation rate in water. Furthermore, the fluorescence lifetime of ethidium in deuterated water (D₂O; 6.5 ns) was dramatically longer than that in H₂O. Olmsted and Kearns construed that when ethidium is intercalated in dsDNA, it is shielded from the solvent and this proton transfer is inhibited, reducing the non-radiative deactivation rate constant and resulting in a longer fluorescence lifetime and higher quantum yield. Pal et al. provided additional evidence in support of the excited-state proton transfer quenching mechanism upon noting the decreased fluorescence intensity and shorter lifetime of ethidium in acetone compared to that in acetonitrile, which is a less effective hydrogen-bond acceptor than acetone. Furthermore, analysis of acetonitrile/water mixtures provided evidence of a dynamic quenching of ethidium by water. However, Phukan and Mitra recently investigated the absorption and fluorescence of ethidium in a wider variety of solvents and found no correlation between fluorescence quantum yield (φ) and hydrogen bonding accepting ability of the solvent. For example, the quantum yield of ethidium bromide in 1,4-dioxane (φ = 0.0061) is significantly lower than that in water (φ = 0.022), despite 1,4-dioxane’s being a worse hydrogen bond acceptor. The researchers also found no correlation between solvatochromism of ethidium and solvent polarity. However, they did note that Stokes shifts in non-polar solvents are significantly smaller (2051-2273 cm⁻¹) than those found in polar solvents (2743-4139 cm⁻¹). Luedtke et al. explored the importance of the exocyclic amines implicated in the excited-state proton transfer mechanism by evaluating the fluorescence of a series of ethidium derivatives featuring substitutions of these groups. In several cases, an enhancement of the derivatives’ quantum yield relative to ethidium was
observed. For example, substituting both –NH₂ groups on ethidium for the NH-functional group found in urea resulted in a 57-fold enhancement in the quantum yield for the urea derivative relative to ethidium. However, some derivatives with N-based functional groups that are not acidic, such as the derivative with two dimethylamino (-N(CH₃)₂) groups, had reduced quantum yields relative to ethidium, indicating the importance of deactivation pathway(s) other than excited-state proton transfer. In particular, the authors suggested that the electron donating ability of the exocyclic amino groups plays an important role in non-radiative decay.

Deactivation pathways that involve rotation of the peripheral phenyl substituent on ethidium have been proposed as an alternative to the excited-state proton transfer mechanism. Sommer et al. proposed the significance of the phenyl group rotation upon measurement of time-resolved fluorescence spectra of ethidium in glycercol, which shifts ~40 nm to the red on the subnanosecond timescale. Prunkl et al. made a case for the contribution to quenching of a charge transfer (CT) state, accessed by twisting of the phenyl ring upon electronic excitation. This suggestion was made based on the comparison of ethidium to derivatives with differing electron-donating capabilities in place of the phenyl ring and supported by calculations. The authors proposed that upon intercalation into dsDNA, the CT state becomes less accessible, in part perhaps because the rotation of the pendant phenyl ring on ethidium is hindered, resulting in a reduction of the non-radiative decay rate constant and an enhancement of fluorescence.

In this work, the photophysical properties of gaseous ethidium are reported. These are measured making use of a modified quadrupole ion trap (QIT) mass spectrometer equipped for fluorescence spectroscopy. By performing gas-phase fluorescence measurements, any deactivation due to solvent proton transfer or collisional deactivation are eliminated. This study of ethidium’s intrinsic behavior lays the groundwork to better understand the factors that drive the fluorescence enhancement of this fluorophore upon binding dsDNA.
3.2 Material and Methods

3.2.1 Mass spectrometry and gas-phase fluorescence spectroscopy

Ethidium bromide was purchased from Sigma (Oakville, ON, Canada) at a concentration of 25 mM in H₂O and diluted to a concentration of 5 μM in 50/50 (v/v %) methanol-water. Rhodamine 575 (Rh575) was obtained from Exciton Corporation (Dayton, OH, USA) and was diluted to a concentration of 1 μM in 50/50 (v/v %) methanol-water. Ions were transferred from solution into the gas phase via electrospray ionization (ESI) operated in positive ion mode using spray voltages between 3-4 kV.

Gaseous ions were accumulated in the trapping region of a quadrupole ion trap (QIT, Bruker, Esquire 3000+) mass spectrometer that has been modified to enable photodissociation and fluorescence measurements of gaseous ions. This experimental set-up has been described in detail previously.²⁰, ²¹ Ions are accumulated in the trapping region of the QIT at an adjustable pressure (0.6-2.3 × 10⁻³ mbar, depending on the experiment) of room temperature helium for 10-1000 ms. The desired ion (ethidium m/z 314 or rhodamine 575 m/z 415) is mass selected. Irradiation of trapped mass-selected ions is synchronized with mass spectrometry (MS)/MS events defined in the MS control software using a trigger from the mass spectrometer that opens a shutter after ion isolation. After the shutter is closed, an MS/MS spectrum is recorded upon scanning the product ions out of the trap in a mass-selective manner. For fluorescence experiments, irradiation parameters and helium pressure were adjusted such that <5% of the ethidium photodissociated, while these parameters were re-adjusted to favor photodissociation for the other experiments (see Table B.1). The data shown is the sum of repeated measurements of multiple ion populations. For the fluorescence measurements, these were interspersed with
measurements made without ions in the trap (see Figure B.2), which were used for background subtraction.

The light source used for photodissociation and fluorescence excitation experiments is a Tsunami Titanium:Sapphire laser (Ti:Sapph; Spectra-Physics, Mountain View, CA) pumped by a Nd:YVO$_4$ laser (Millenia 10s, Spectra-Physics, Mountain View, CA). This laser system generates pulsed tunable IR light in the wavelength range of 700 – 1080 nm, which is then frequency doubled. To enable irradiation of the ions in the QIT, two 1.2 mm holes have been drilled in the ring electrode such that the frequency doubled output of the Ti:Sapph intersects the trapped, gaseous ions. Fluorescence from trapped ions is collected through a third hole (2.0 mm diameter) in the QIT ring electrode (~1 cm internal diameter), which is orthogonal to the optical excitation axis. A lens inserted into the ring electrode assists in the collection of the fluorescence light, which then passes outside of the vacuum chamber through a UV-fused silica window with an anti-reflective coating. The collected fluorescence light then passes through a long-pass filter (Chroma Technology Corp., Rockingham, VT, USA) in order to reduce scattered laser light. The filtered light can then be sent to one of two detectors: a single-photon avalanche photodiode (SPAD) that enables measurement of time-resolved fluorescence using time-correlated single photon counting (TCSPC) techniques,$^{21}$ or a spectrograph coupled to an electron-multiplying charge coupled device (EM-CCD) for the measurement of fluorescence emission spectra. For TCSPC measurements, the repetition rate of the laser was reduced from 80 MHz down to 10 MHz by passing the laser beam through a pulse picker (Conoptics, Danbury, CT, USA). The EM-CCD was operated using a 50 kHz readout rate, 4x pre-amplified gain, an electron-multiplication gain of 210, and at a temperature of 198 K. To measure accurate emission maxima, the spectrograph/EM-CCD system was externally calibrated with gaseous rhodamine 640 ($\lambda_{em}^{max} = 561$ nm).$^{22}$ Fluorescence lifetimes were determined by fitting the background-
subtracted time-resolved fluorescence data with the convolution of an instrument response function approximated as a Gaussian of adjustable width (~330 ps) with exponential decays of adjustable lifetime and relative amplitude, as detailed elsewhere.\textsuperscript{21}

3.2.2 Electronic action spectroscopy

Photodissociation (PD) MS/MS was performed by injecting ions into the mass spectrometer, mass-selecting the ion of interest, irradiating these at the selected wavelength for 5200 ms with 7 mW irradiation power and measuring the resultant mass spectrum. The photodissociation yield is calculated using Eq. 1, where $I_{\text{laser on precursor}}$ was the intensity of the precursor ethidium ion with the laser on and $I_{\text{laser off precursor}}$ was the intensity of the ethidium ion measured using the same experimental sequence, but without opening the shutter, so the laser beam is blocked. Thus, if the precursor ethidium ion does not absorb the excitation wavelength and thus does not photodissociate, the PD yield is 0. If the ethidium ion absorbs light and the entire population undergoes PD, the PD yield is 1. Intensities were calculated from the average of approximately 15 mass spectra. $I_{\text{laser off precursor}}$ was measured both before and after laser irradiation to ensure stability of the electrospray.

$$ PD \text{ Yield} = 1 - \frac{I_{\text{laser on precursor}}}{I_{\text{laser off precursor}}} $$

To generate an electronic action spectrum, the photodissociation yield under a defined set of experimental conditions was repeatedly measured using a series of excitation wavelengths.

3.2.3 Relative brightness determination

The relative brightness of cationic rhodamine 575 to that of ethidium was assessed by comparing the fluorescence intensities of each ion under similar mass spectrometry and
excitation conditions. These parameters include equivalent helium pressure, ion irradiation time, and fluorescence accumulation times. A single excitation wavelength (485 nm) was used for the relative brightness measurement. Therefore, measured fluorescence intensities were corrected by the excitation efficiencies at this wavelength relative to the maximum excitation efficiency, obtained from gas-phase action spectra of ethidium (1.00) and rhodamine 575 (~0.75). Fluorescence intensity was obtained by integration of emission spectra, using a program developed in-house. Data were also normalized for the number of each ion present in the mass spectrometer. This is controlled by changing the ion accumulation time and assessed by an Ion Charge Control (ICC) value, which reflects the ion current at the detector of the mass spectrometer. The measured ICC is proportional (but not equal to) the number of trapped ions of a given mass and charge. The relative brightness was calculated by comparing the integrated fluorescence signals of each fluorophore, normalized for excitation power, number of ions, and excitation efficiency relative to maximum. A summary of the parameters used for the relative brightness measurement are listed in Table B.1, together with further details about the relative brightness measurements.

3.2.4 Solution-phase characterization

Fluorescence measurements of 5 μM ethidium solutions were performed on a LS50B spectrofluorometer (Perkin Elmer, Waltham, MA, USA). Emission spectra were recorded using the excitation maxima in each solvent (475 nm in water, 505 nm in acetonitrile, and 523 nm in methanol). The excitation slit bandwidth was 2.5 nm whereas the emission slit bandwidth was 5 nm.
3.3 Results and Discussion

3.3.1 ESI mass spectrum and photodissociation of ethidium

Figure 3.1a shows the positive mode ESI mass spectrum of ethidium bromide, illustrating the appearance of the ethidium cation at m/z 314. Figure 3.1b shows the mass spectrum after ethidium is monoisotopically isolated, and Figure 3.1c shows the photodissociation mass spectrum of ethidium ions that have been mass-selected and subsequently irradiated with 7 mW of 480 nm light for 5.2 seconds. Fragments appear at m/z 299, 298, 286, 285, 284 and 269. Due to the relatively low mass resolving power of this instrument, an unambiguous assignment of fragment ions is difficult from QIT data. Instead, tandem mass spectrometry in a high-resolution Fourier transform ion cyclotron resonance (FTICR) mass spectrometer was performed in order to identify more accurately the elemental composition of these fragments. Dissociation in the FTICR mass spectrometer was performed using the technique of sustained off-resonance irradiation collision-induced dissociation (SORI-CID), which is a low-energy collision-induced dissociation method. The fragments that are generated by 480 nm photodissociation appear to be the same as those generated by SORI-CID (Figure B.1). Shown in Table B.2 is a summary of the fragment ions induced upon SORI-CID, as well as their likely identities.

3.3.2 Electronic action and emission spectra

Figure 3.2 compares spectra of gaseous ethidium (top) with those measured in several solvents (bottom). The solid black squares shown in Figure 3.2a represent a photodissociation action spectrum measured to probe the intrinsic absorption spectrum of the gaseous ion, while the red trace is the smoothed emission spectrum. The gas-phase emission spectrum is a near-mirror image of the measured action spectrum. The excitation maximum of gaseous ethidium
Figure 3.1. (a) ESI mass spectrum of ethidium bromide solution in positive ion mode, showing predominantly the 1+ charge state of ethidium (m/z 314). (b) Monoisotopic isolation of the ethidium cation. (c) Photodissociation MS/MS of monoisotopically isolated ethidium with $\lambda_{ex} = 480$ nm.
Figure 3.2. (a) Electronic action (black squares) and smoothed fluorescence emission (red trace) spectra for ethidium in the gas phase. The blue trace through the action spectrum was constructed from the smoothed fluorescence emission spectrum, by reflection, and 9% horizontal magnification. (b) Ethidium absorption and emission spectra measured in water (solid blue), acetonitrile (dashed green) and methanol (dotted orange) at a concentration of 5 μM. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
measured through action spectroscopy is 485 nm and the emission maximum is 548 nm, with a resultant Stokes shift of ~2370 cm\(^{-1}\). The measured gas-phase Stokes Shift is somewhat larger than previously found for solution-phase ethidium in non-polar solvents (2051-2273 cm\(^{-1}\)), but significantly smaller than that found in polar solvents (2743-4139 cm\(^{-1}\)).\(^{14}\) Strikingly, the measured Stokes Shift for gaseous ethidium is substantially larger than Stokes Shifts previously published for gas-phase rhodamines (~400-800 cm\(^{-1}\)).\(^{23,24}\) Moreover, the FWHM of the emission spectrum (~2500 cm\(^{-1}\)) is also much broader than gas-phase emission spectra previously measured for rhodamines (~1200 cm\(^{-1}\)).\(^{23,24}\) The large gas-phase Stokes Shift and broad spectral profiles for ethidium support the idea that ethidium undergoes a significant geometry change upon electronic excitation. This is consistent with the postulated rotation of the phenyl substituent upon excitation,\(^{13,19}\) or may reflect some other distortion of the structure.

Absorption and emission spectra of ethidium measured in water, methanol and acetonitrile are shown below in Figure 3.2b for comparison to the gas-phase data. In contrast to the gas-phase data, the absorption and emission spectra for ethidium in these three solvents are not mirror images, featuring relatively broad absorption bands that appear to have some underlying structure and significantly narrower emission profiles that lack the same structure. The emission profiles measured in the three solvents are similar to each other, both in the relative narrowness of their main band and in the position of the maxima. The gas-phase emission profile is somewhat broader than those measured in these solutions, and the maximum lies substantially to the blue of that in each of water, methanol, and acetonitrile (615 nm, 613 nm, and 599 nm, respectively). The gas-phase photodissociation maximum lies ~800 cm\(^{-1}\) and ~1500 cm\(^{-1}\) to the blue of the absorption maxima in acetonitrile and methanol, respectively, whereas it is ~200 cm\(^{-1}\) to the red of the absorption maximum in water. These differences are not due solely to solvent
polarity or polarizability: the excitation and emission maxima in the non-polar 1,4-dioxane lies to the red of all of these, at 544 nm and 625 nm, respectively.\textsuperscript{14}

We estimate the 0-0 transition in gaseous ethidium, from the bisector between the action and emission spectra, to lie at ~19400 cm\textsuperscript{-1}. For ethidium bromide in polar/H-bonding solvents including water, methanol, and acetonitrile (Figure 3.2b), the absorption and emission spectra are not good mirror images, which makes it difficult to estimate the energy of the 0-0 transition in these solvents for comparison. By calculating the bisector between the absorption and emission maxima in 1,4-dioxane (a solvent of low polarity, often thought of as being close to that of the gas phase) from Phukan and Mitra,\textsuperscript{14} the 0-0 transition can be approximated as ~17000 cm\textsuperscript{-1}. The ~2400 cm\textsuperscript{-1} difference between the 0-0 transition suggests significant stabilization of the excited state relative to that of the ground state by even the relatively non-polar 1,4-dioxane solvent.

3.3.3 Time-resolved measurements

Time-resolved fluorescence of trapped gaseous ethidium ions upon excitation at 485 nm is shown in Figure 3.3. The measured fluorescence decay was well fit by the convolution of a double exponential decay with a Gaussian instrument response function. The fitted lifetimes and relative amplitudes of the two components are: $\tau_1 = 5.1 \pm 0.7$ ns ($42 \pm 2\%$) and $\tau_2 = 21.4 \pm 1.5$ ns ($58 \pm 3\%$). The longer of these lifetimes is strikingly similar to the 22.5 ns lifetime of ethidium bound to double-stranded DNA.\textsuperscript{2} The uncertainties in lifetime given here are three times the calculated standard error. We have shown previously that this is a reasonable estimate for uncertainty of monoexponential decays with S/N > 8-fold higher than that measured here.\textsuperscript{21} However, combining the challenges of multi-exponential fitting and relatively low S/N of these data, the stated uncertainty may be larger than the estimates given above. We note that the time-resolved measurements of gaseous ethidium shown here represent data collection time of ~5.5
Figure 3.3. Time-resolved fluorescence of gaseous ethidium ions measured at $\lambda_{ex} = 485 \, nm$. Panel (a) shows the time-resolved decay fit with a double-exponential function. Panels (b) and (c) show the residuals for the double- and single-exponential fits, respectively.
hours, so we did not feel that longer measurements with the current instrumental configuration were merited.

Several factors make these gas-phase fluorescence measurements difficult. These include low ion densities \((10^5 - 10^8 \text{ ions/cm}^3)\),\(^{20}\) short effective path length \((\sim 700 \mu\text{m}, \text{limited by the size of the ion cloud})\),\(^{25}\) low fluorescence collection efficiencies \((0.15\% \text{ of all fluorescence light collected})\),\(^{20}\) and a low fluorescence quantum yield of ethidium \((\text{reported values range from } \phi = 0.01^{13} \text{ to } \phi = 0.022^{14} \text{ to } \phi = 0.04^{1} \text{ in aqueous solution, and is likely } \sim 10\text{-fold higher in the gas phase, see below})\). The presence of two components in the fluorescence lifetime of gas-phase ethidium suggests that two conformations of ethidium exist in the gas phase. The time-resolved fluorescence of ethidium bromide in most solvents, including water, methanol and acetonitrile is well fit by a single exponential decay; however, the presence of a double-exponential decay has been noted previously in 1-butanol and tetrahydrofuran solvents.\(^{14}\) Double exponential decays in the time-resolved fluorescence of ethidium bromide have also been noted in trehalose sugars,\(^{26}\) membranes,\(^{27}\) and polymer films\(^{28}\) and interpreted to be an indication of the presence of ethidium in different microenvironments. This may be correct; however, the gas-phase results on ethidium presented here show that ethidium exhibits a double-exponential decay when in isolation. The two components may reflect conformations of gaseous ethidium with different angles of the pendant phenyl ring relative to the phenanthridium core and/or different orientations of the ethylamine group. Early computational work from Le Bret and Chalvet\(^{4}\) predicted that the maximum of the absorption would depend strongly on the angle between the phenanthridium core and the peripheral phenyl ring; a change in angle from \(30^\circ\) to \(90^\circ\), shifted the computed maximum from 480 nm to 450 nm. However, NMR and X-ray crystallography measurements all find that phenyl ring is nearly orthogonal to the phenanthridium core, the dihedral angle varying from \(83^\circ - 85^\circ\).\(^{29,30}\)
The lifetime of gaseous ethidium can be used to estimate its fluorescence quantum yield in the gas phase if its radiative deactivation rate can be assessed. In practice, the radiative rate might be extrapolated from solution-phase data, if one assumes that the ratio of radiative rate to the square of the index of refraction \( \frac{k}{n^2} \) is relatively constant. This is a strategy that we have used previously to estimate the quantum yield in the gas phase for rhodamine 6G. We note that we are not able to measure the quantum yield of gaseous ions trapped in our set-up directly because the low ion density and short path length preclude direct measurements of the absorbance of the trapped ions. Using lifetimes and quantum yields provided by Phukan and Mitra, it can be shown that there is little correlation between the radiative rate and the square of the index of refraction for ethidium. Therefore, we believe that this method is unable to provide useful estimates for ethidium’s quantum yield in the gas phase. As a result, we chose to use a different method to estimate ethidium’s quantum yield in the gas phase: measurement of its brightness relative to another dye. For this, we chose to use protonated rhodamine 575, which we have previously characterized in the gas phase.

### 3.3.4 Relative brightness and quantum yield estimation

The brightness of gaseous ethidium was compared to that of protonated rhodamine 575 (Rh575H+) in order to estimate the quantum yield of gaseous ethidium. Brightness is the product of a fluorophore’s quantum yield (\( \phi \)) and its molar absorptivity coefficient (\( \varepsilon \)); thus, the quantum yield of gaseous ethidium was estimated using the following:

\[
\phi_{\text{ethidium}}^{\text{gas phase}} = \left( \frac{\phi_{\text{ethidium}}^{\text{gas phase}}}{\phi_{\text{Rh575H}^+}^{\text{gas phase}}} \right) \left( \frac{\varepsilon_{\text{Rh575H}^+}^{\text{gas phase}}}{\varepsilon_{\text{ethidium}}^{\text{gas phase}}} \right)
\]

where \( I \) is the normalized integrated fluorescence intensity of each fluorophore. Rh575H+ was selected as a reference because the previous characterization of its photophysical properties in...
solution\textsuperscript{32} and in the gas phase\textsuperscript{21,24} enable a reasonable estimate of its quantum yield in the gas phase. Moreover, its gas-phase excitation maximum (495 nm)\textsuperscript{24} is similar to that of ethidium, simplifying the experiment.

Upon subjecting the two gaseous fluorophores to comparable mass spectrometry and fluorescence excitation conditions, the brightness of ethidium was found to be just 2\% of that of Rh575H\textsuperscript{+}. While the quantum yield of gaseous Rh575H\textsuperscript{+} has not been measured directly, it can be estimated to be \~0.66 by extrapolating a gaseous radiative deactivation rate from solution values\textsuperscript{32} (employing the assumption described above of the dependence of radiative rates on the square of the index of refraction, which has been examined for Rh575H\textsuperscript{+} in solution)\textsuperscript{32} and its measured lifetime in the gas phase.\textsuperscript{21} This calculation is detailed in Appendix B. We estimate the ratio of the molar extinction coefficients of these two dyes in the gas phase is similar to the ratio of their maxima in aqueous solution (\(\varepsilon_{\text{Ethidium}} \sim 5420 \text{ M}^{-1}\text{cm}^{-1}\) at 479 nm\textsuperscript{13} and \(\varepsilon_{\text{Rh575H}^+} \sim 100,000 \text{ M}^{-1}\text{cm}^{-1}\) at 525 nm).\textsuperscript{32} Combining the measured relative brightness of 0.02 with the relative absorption efficiency, we roughly estimate that the quantum yield of gaseous ethidium is approximately 37\% that of Rh575H\textsuperscript{+}, or \~0.24. This is significantly higher than the aqueous solution quantum yield of 0.022 \(\pm 0.002\) recently reported by Phukan and Mitra.\textsuperscript{14} The apparent increase in quantum yield and lengthening of fluorescence lifetime in ethidium reflects a significant reduction in nonradiative deactivation rate constant, potentially due to the elimination of quenching via excited-state proton transfer. Our estimated gas-phase quantum yield is similar to that reported for acetonitrile (0.23) and dichloromethane (0.20) solution,\textsuperscript{14} which are the highest reported quantum yields we found for ethidium free in solution. Notably, the gas-phase quantum yield for ethidium estimated here is \~30\% lower than the quantum yield of ethidium in the presence of double-stranded DNA (0.35).\textsuperscript{1}
3.3.5 Power dependence and dissociation kinetics

In order to assess the mechanism of photodissociation of ethidium in the mass spectrometer, and to see if there is additional evidence of multiple conformations of this chromophore in the gas phase, the dissociation power dependence and kinetics of the gaseous ions were investigated. Figure 3.4a shows a plot of the PD yield as a function of laser power (P) upon irradiation at 485 nm. The fit to the data clearly does not follow a simple exponential decay, which would be expected if the absorption of a single 485 nm (2.6 eV) photon resulted in PD. Instead, the data are reasonably well fit by a third order polynomial in power, indicating that the dissociation of ethidium is driven by multiple photon processes in the QIT.

The dissociation kinetics of the ethidium cation (Figure 3.4b) follow pseudo-first order behaviour. The presence of only a single dissociation rate constant (equal to the negative slope of the kinetic plot) down to 96% depletion of the parent ion, provides no evidence for the presence of two conformations of the gaseous ion (as suggested by the time-resolved fluorescence data). It may be that multiple conformations exist, but that they have similar dissociation rate constants upon photo-excitation. This would not be surprising, considering the nature of these two measurements. Photodissociation of ethidium occurs from vibrationally excited ions in their ground electronic state, after the photon energy is redistributed throughout the ion via internal conversion and intramolecular vibrational redistribution (IVR) of energy. The similarity in the photodissociation mass spectrum and the SORI-CID mass spectrum strongly supports this picture. Photodissociation thus breaks the weakest chemical bond(s) in the molecule, the energy threshold for which is likely not highly sensitive to conformation. On the other hand, fluorescence decays are an exquisitely sensitive probe of the excited potential energy surface of the molecule and are much more sensitive to the conformation of the molecule. Fluorescence decay measurements are able to reveal that different conformers decay back to the ground state.
Figure 3.4. (a) Photodissociation of gaseous ethidium measured as a function of laser power using $\lambda_{ex} = 485$ nm and an irradiation time of 5.20 seconds. The dashed red line illustrates a third order polynomial fit to the power dependence (i.e. $PD \text{ Yield} = e^{(-aP-bP^2-cP^3)t}$). Fitted parameters were $a = 5 \times 10^{-22}$, $b = 0.01261$, $c = 0.00431$. (b) Photodissociation kinetics measured using $\lambda_{ex} = 490$ nm and 25 mW irradiation power. The linear fit of the kinetics data (red dashed line) shows pseudo first-order behavior.
with different rates even though these conformers have similar rates of photo-fragmentation. Ultimately, this emphasizes the need for multiple techniques to better understand molecular properties including conformation.

3.4 Conclusions

In this work, the intrinsic photophysics of trapped ethidium ions was probed in a quadrupole ion trap mass spectrometer equipped for gas-phase fluorescence spectroscopy. Photodissociation action spectroscopy yielded a maximum of 485 nm in the gas phase, which lies within the wide range of absorption maxima reported for ethidium in different solvents. The emission maximum is 548 nm, which lies significantly to the blue of reported condensed-phase ethidium emission maxima. The ~2370 cm\(^{-1}\) Stokes shift and the broad excitation and emission profiles (FWHM of ~2500 cm\(^{-1}\)) measured for gaseous ethidium are consistent with the occurrence of a significant geometry change upon electronic excitation. Analysis of time-resolved measurements suggest two components, with lifetimes of ~5.1 (~42%) and ~21.5 ns (~58%). Both lifetimes are significantly longer than that measured for ethidium in water (\(\tau \sim 1.8\) ns), consistent with overall substantial reduction in the non-radiative decay rate for ethidium in the gas phase. While we are unable to measure fluorescence quantum yields using our current instrumentation, comparing the brightness of gaseous ethidium and protonated rhodamine 575 provide a rough estimate of 0.2 for the quantum yield of gaseous ethidium.

In some ways, the photophysics of gaseous ethidium resembles that of ethidium in complex with DNA. In particular, the longer fitted lifetime of gaseous ethidium and its lifetime when bound to double-stranded DNA in solution are strikingly similar. Moreover, the brightness of ethidium is substantially enhanced when bound to DNA\(^1\) and when transferred from aqueous solution into the gas phase. Why is this? One proposed quenching mechanism for aqueous
Ethidium is clearly eliminated in the gas-phase studies: excited-state proton transfer.\textsuperscript{11} However, we cannot discount the significance of a twisted intramolecular charge state type phenomenon;\textsuperscript{13} in the gas-phase studies, quenching due to charge transfer states should be reduced, as these are stabilized by interactions with polar (or polarizable) solvent molecules and thus will be less energetically accessible upon the removal of solvent. Future studies of ethidium and its derivatives, in complex with well-defined numbers of solvent molecules, should help elucidate the reasons for the extreme sensitivity of this fluorophore to its local environment. We are also examining the response of ethidium to complexation with DNA in the gas phase. The results presented here clarify the intrinsic photophysics of ethidium. These benchmark results thus lay the groundwork to elucidate the main driving forces and mechanisms for its sensitivity to molecular interactions, and should help to tailor the molecular design of fluorophores with improved “turn-on” responses upon binding double-stranded DNA.

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3.6 References

Chapter 4

Developing combined optical spectroscopy and mass spectrometry tools for the characterization of double-stranded DNA in the gas phase: examining DNA-binders fluorescence “turn-on” responses

Stephen V. Sciuto, Rebecca A. Jockusch. This chapter is the draft of a manuscript to be submitted for publication.

Abstract

It has been known for over 20 years that intact double-stranded DNA (dsDNA) ions in the gas phase can be detected by electrospray ionization mass spectrometry. However, the removal of solvent and counter ions, coupled with increased electrostatic repulsion from negatively charged phosphate groups may perturb the conformation adopted by dsDNA upon transfer to the gas phase, destabilizing helical conformations present in solution. Here, we use the fluorescence enhancement of several non-covalent DNA-binding fluorophores in solution to develop tools for characterization of dsDNA in the gas phase. Coupling trapping mass spectrometry with selected-ion laser-induced fluorescence (SILIF), fluorescence emission spectra for the DNA-binding fluorophores are measured when isolated and complexed to dsDNA in the gas phase. Fluorescence is observed for complexes of dsDNA with ethidium and acridine orange in the gas phase. However, relative brightness measurements on complexes of dsDNA with ethidium and acridine orange in comparison to their unbound forms indicate that ethidium and acridine orange do not exhibit “turn-on” fluorescence responses when complexed to dsDNA in the gas phase. This study represents the first time fluorescence from a non-covalent dsDNA-dye complex is detected in the gas phase, in an effort to determine whether DNA-binding fluorophores that exhibit fluorescence enhancements in solution can be used for the characterization of dsDNA structure in the gas phase.
4.1 Introduction

Both mass spectrometry (MS) and fluorescence-based tools have become key technologies in the biosciences. When used in tandem, the unparalleled selectivity of MS and sensitivity of fluorescence can be combined to elucidate key interactions in complex systems, such as the role solvent plays in biomolecular structure.¹ For example, our laboratory has recently used the technique of Förster Resonance Energy Transfer (FRET) to probe the gaseous structure of protein GB1 using selected ion (SI) laser induced fluorescence (LIF). Gaseous, mass-selected GB1 ions showed lower FRET efficiencies than solution-phase single-molecule FRET measurements.² The results indicate that in the gas phase, the conformation(s) of the gaseous protein become increasingly more extended with increasing charge, in contrast to the protein’s notable conformational stability over a wide pH range in solution. This highlights the stabilizing role of solvent on protein structure. Therefore, an important question for those who use MS to investigate biomolecules and their interactions is the degree to which biomolecules retain their solution-phase structures in the gas phase. Here, we apply the combination of electrospray ionization (ESI-) MS and SILIF to probe the conformation of double-stranded DNA (dsDNA) in the absence of solvent and counter ions, i.e., when transferred to the gas phase via electrospray ionization. In particular, we examine whether the “turn-on” fluorescence response upon binding dsDNA in solution of several well-known non-covalent probes is preserved in the gas phase.

Several decades after the detection of dsDNA by MS, it is still unclear what the structure of dsDNA is in the gas phase. In solution, a delicately balanced interplay between intra- and inter-molecular interactions, including hydrogen-bonding, base stacking, and electrostatic interactions gives rise to three well-known conformations of dsDNA: the A-, B-, and Z-forms.³ However, upon transfer of the dsDNA complex into the gas phase, solvent and counterions are
removed. This could result in disruption of the structure of dsDNA in the gas phase. Increased repulsive interactions among the negatively charged phosphate groups along the DNA backbone will favor extended structures. Alternatively, gaseous dsDNA may compact as the phosphate groups seek to share the limited number of protons, resulting in increased intramolecular hydrogen-bonding.

Researchers have used dissociation, ion mobility, and molecular dynamics to probe the conformation of oligonucleotide sequences up to 30 base-pairs in length. Using collisional cross sections (CCS) obtained from ion mobility, Bowers and coworkers first proposed that poly(CG)$_n$ sequences underwent a transition from the well-known B-form in solution to the A-form upon transfer into the gas phase. Charge densities of the poly(CG)$_n$ sequences investigated by Bowers and coworkers were 0.5–0.64 charges per base pair (bp), i.e. 7–9 for 14-mer dsDNA. More recent work by Gabelica and coworkers have shown that the structure of dsDNA in the gas phase is dependent on several factors, such as GC content, charge state, and instrumental conditions that effect the kinetics and energetics of ESI-MS experiments. In contrast to Bowers’ findings, the authors noted that CCS distributions for 12-mer dsDNA ions in the 5$^\text{th}$ charge state (~0.42 charges/bp) were broader than rigid G-quadruplexes, consistent with the presence of multiple conformations for 12-mer dsDNA. Other 12-mer dsDNA ions electrosprayed under “native” conditions show CCS distributions for the 5$^\text{th}$ charge state that are consistent with compact “zipped” structures, which potentially arise from intramolecular hydrogen-bonds between phosphate groups. Molecular dynamics simulations have also provided some development in our understanding of gaseous dsDNA structure. Early work by Orozco and coworkers proposed a significantly elongated double helix for 16-mer sequences in the 8$^\text{th}$ charge state. However, recent molecular dynamics simulations have indicated that charge state plays an important role when comparing the simulated CCS with the aqueous structure for 7-mer dsDNA.
ions. For example, the simulated CCS of a 7-mer dsDNA ion in the 2−, 3−, and 4− charge states was found to be identical to the CCS of the aqueous structure only for the 3− charge state. Thus, while many experimental and theoretical reports suggest the preservation (of sometimes different aspects) of helical structures in gaseous dsDNA, an overall consensus has not been reached. Here, we explore the use of combined SILIF techniques to address this challenging question.

Several optical and spectroscopic approaches in combination with MS have been used to probe the structure of dsDNA in the gas phase. Early work by Danell and Parks used BODIPY fluorophores covalently attached to the 5’ and 3’ ends of 14-mer complementary single-stranded DNA to probe the “unravelling” of heated dsDNA ions using FRET. Later, Gabelica et al. interrogated intact 12-mer dsDNA ions in the 5− charge state ([ds-5H]5−) in a quadrupole ion trap (QIT) mass spectrometer. Upon 260 nm irradiation, which in solution is near the absorption maximum of DNA bases, [ds-5H]5− ions formed a charge-reduced radical species ([ds-5H]4−), indicating that the preferred dissociation channel under their experimental conditions was electron photodetachment (ePD). The authors noted an increase in the intensity of the [ds-5H]4− ion as duplex GC percentage increased, suggesting that the low ionization potential of guanosines is a key determinant in the gaseous deactivation pathway of dsDNA ions upon 260 nm excitation. In a subsequent study, Gabelica et al. reported on photoactivation measurements for complexes of 12-mer dsDNA ions with covalently and non-covalently bound chromophores that absorb at different wavelengths than the DNA bases. Two kinds of non-covalent chromophores were investigated, those that bind to dsDNA in solution via minor-groove binding (Hoechst 33258, DAPI, netropsin, and berenil) and intercalation (ethidium, doxorubicin, and m-amsacrine). Several dsDNA-dye complexes showed ePD using UV-visible (>350 nm) wavelengths where the non-covalent chromophore absorbs but the DNA bases do not, suggesting
a coupling of energy levels between the chromophore and the DNA. The authors postulated that energy transfer resulting in ePD for selected dsDNA-dye complexes depends on the chromophore possessing a sufficiently long excited-state lifetime such that two-photon excitation can pass the dissociation threshold for the DNA excited states. Though well characterized in solution, the fluorescence of these DNA-binding dyes in the gas phase, neither in isolation nor in dsDNA complexes, has been characterized until now.

Here we examine non-covalent fluorophores that exhibit well-characterized changes in fluorescence properties when bound to dsDNA in solution\(^{20-28}\) to develop tools for characterization of dsDNA and its complexes in the gas phase. The fluorescence dyes that we chose to investigate are ethidium bromide (EB), acridine orange (AO), SYBR Green I (SG), DAPI (4,6-diamidino-2-phenylindole), and three Hoechst dyes (H33258, H33342, and H34580). The structures of these dyes are shown in scheme 4.1, together with their fluorescence “turn-on” responses (increase in fluorescence quantum yield, \(\Phi_{FL} \uparrow\)) when bound to dsDNA in aqueous solution.\(^ {23,27,28}\) These dyes bind to dsDNA through three types of interactions: (i) intercalation, where planar dye molecules are “sandwiched” between stacking bases (ii) minor/major groove binding, and/or (iii) external electrostatic attachment. Association equilibrium constants (\(K_a\)) for the interaction of the “turn-on” fluorophores with DNA span several orders of magnitude; \(K_a\)’s for ethidium and acridine are \(~10^5 \text{ M}^{-1}\) compared to \(~10^8 \text{ M}^{-1}\) for the Hoechst dyes, SG, and DAPI.\(^ {24,29-32}\) Furthermore, the type of binding plays a significant role in the fluorescence “turn-on” response observed. For example, recent work by Sayed et al. reported on the fluorescence enhancement of protonated AO at various [dsDNA]/[AOH\(^+\)] ratios.\(^ {33}\) The authors argue that at high AOH\(^+\) concentrations relative to dsDNA, AOH\(^+\) dimers predominantly bind to the exterior of dsDNA via electrostatics, resulting in an initial decrease in fluorescence intensity of the dsDNA-AOH\(^+\) complex relative to AOH\(^+\). At higher dsDNA concentrations, AOH\(^+\) binds via
intercalation resulting in a fluorescence enhancement. Another classic DNA probe is ethidium, which exhibits a 9-fold increase in fluorescence quantum yield in solution (Φ = 0.039 → 0.35) upon binding to dsDNA via intercalation. Our lab has previously characterized the fluorescence of both ethidium and acridine orange ions in isolation (and for AOH when bound to cucurbit[7]uril) using siLIF. DAPI is another classic DNA-binding dye. It exhibits an 18-fold increase in fluorescence quantum yield (Φ = 0.019 → 0.34) upon binding the minor groove of dsDNA. Other minor-groove binding fluorophores, such as the Hoechst dyes studied here, have been shown to have selectivity for DNA sequences with AT-regions, also exhibiting dimeric association to specific dsDNA sequences. More recently, SYBR Green I and PicoGreen have gained popularity due to their substantially larger fluorescence enhancements upon binding dsDNA in solution (SYBR Green I and PicoGreen have ~1700-fold and ~1100-fold fluorescence enhancements, respectively). Ultimately, comparing the fluorescence properties of these dyes in solution and the gas phase, and how DNA binding alters the properties in both phases will enable improved understanding of the role solvent plays in the fluorescence enhancement for these “turn-on” fluorophores. This may also provide insight into the conformation of gaseous dsDNA.

For the gas-phase work we employ a quadrupole ion trap (QIT) mass spectrometer that has been modified for fluorescence measurements of mass-selected gaseous ions. The spectroscopic properties of the unbound dyes and their dsDNA-dye complexes in solution and in the gas phase are compared. This includes relative brightness measurements to assess whether dsDNA-dye complexes show fluorescence enhancements in the gas phase. In addition, photodissociation as a function of irradiation power is employed to explore photodissociation pathways.
Scheme 4.1. Structures of the DNA-binding dyes studied in this work. (a) Hoechst 33342, (b) Hoechst 34580, (c) Hoechst 33258, (d) DAPI, (e) ethidium, (f) acridine orange, (g) SYBR Green I. The fluorescence “turn-on” response relative to the unbound dye ($\Phi_{Fi} \uparrow$) is indicated next to the dye.

ss1: 5’-GCGGGTTAAGGGCG-3’
ss2: 3’-CGCCCAATTCGGC-5’

Scheme 4.2. dsDNA, made from complementary oligonucleotides ss1 and ss2
4.2 Experimental Methods

4.2.1 Fluorescent Dyes and DNA

Ethidium bromide was purchased from Sigma at a concentration of 25 mM in H$_2$O. DAPI, Hoechst 33258, Hoechst 33342, Hoechst 34580, acridine orange, and Rhodamine 123 (Rh123) were purchased from Aldrich (Oakville, ON, Canada). SYBR Green I was purchased from Life Technologies (Grand Island, NY, USA) as a 10,000x concentrate in DMSO. The 14-mer DNA sequences $d(GCGGGAATTGGGCG)$ [ss1; avg. mass 4384.9 Da], $d(CGCCCTTAACCCGC)$ [ss2; avg. mass 4144.7 Da], $d(G_4T_4G_4)$ [ss3; avg. mass 3788.5 Da], and $d(TAAGCGGCCGGAAT)$ [ss4; avg. mass 4214.8 Da] were purchased from Integrated DNA Technologies (Coralville, Iowa, USA) as ammonium salts and used without further purification. To form dsDNA ($ds = ss1 + ss2$; avg. mass 8529.6 Da), equimolar amounts of complementary DNA in 25 mM ammonium acetate were mixed together to form a stock solution which was 2 mM each in ss1 and ss2. The stock solution was annealed by heating to 90°C for 10 minutes and then allowed to cool to 10°C over a period of 8 hours at a rate of -10°C/h. CD spectroscopy confirmed the presence of the B-DNA conformation in solution (Figure C.1). To form the bimolecular G-Quadruplex [$d(G_4T_4G_4)_2$], a solution of 1 mM ss3 was subjected to the above annealing procedure in 150 mM ammonium acetate. For use as a brightness control, a fluorescently-labeled version of ss1 with rhodamine green-X (RhoGX) covalently bound to its 5’ end, purified through reversed-phase HPLC, was also purchased from Integrated DNA Technologies. A second fluorescently-labeled DNA sequence with BODIPY Fl covalently bound to its 5’ end (the sequence was BODIPY Fl-ATTCCGGGCCGCTTA) also purified through reverse-phased HPLC, was purchased from ThermoFisher Scientific (Waltham, Massachusetts, USA). To form dsDNA complexes with rhodamine green-X and BODIPY Fl covalently bound, 1 mM of the complementary DNA sequence (ss4) was added in an equimolar amount and annealed...
as described above. DNA stock solutions were placed in DNA LoBind tubes purchased from Fisher Scientific (Ottawa, ON, CA) and stored in the freezer at -40°C. Final DNA-dye concentrations used for ESI-MS analysis contained 25 μM of each DNA strand and 25 μM dye (except for AOH+, which was 50 μM) in 25 mM ammonium acetate.

4.2.2 Mass Spectrometry and Gas-Phase Fluorescence Spectroscopy

We have modified a commercial quadrupole ion trap (Bruker, Esquire 3000+, QIT) mass spectrometer to enable fluorescence spectroscopy of gaseous ions. This experimental set-up has been described in detail previously.39, 40 Briefly, ions and complexes are transferred into the gas phase using nanoelectrospray tips (outer diameter 1.0 mm, inner diameter 0.75 mm) that are pulled to a fine (~5 μm) opening using a Sutter P-97 Flaming/Brown Micropipette Puller (Novato, California, USA). Capillary voltages to generate the spray ranged from 850 – 1100 V. The ions are then transmitted through a heated glass capillary (capillary exit = -325 V, T = 150°C; dry gas flow = 1.5 L/min) and a series of ion optics (skimmer = -70 V; Oct 1 DC = -12 V; Oct 2 DC = -1 V; Oct RF = 200 Vpp; Trap drive = 90 - 105) to the trapping region of the QIT. MS parameters were optimized to maximize survival of dsDNA upon nanoESI of annealed dsDNA solution, in order to facilitate gentle transfer of dsDNA ions. The gentleness of source conditions was assessed by the survival of the non-covalent complex of G-quadruplex d(G₄T₄G₄)₂ with three bound NH₄⁺ ions.41 With the most gentle conditions used (referred to below as “gentle conditions”) the capillary exit voltage was changed from -325 V to -275 V, Oct 2 DC from -1 V to -6 V, and the skimmer potential from -70 V to -50 V. Under these conditions, MS signal decreased ~2-3 fold, which is why these gentle conditions were not used for the majority of fluorescence experiments. Ions are accumulated in the trapping region of the QIT for 10-1000 ms and can subsequently be manipulated during a reaction period of up to 20 s.
Manipulations include mass selection, collision-induced dissociation and/or irradiation by visible light produced from a tunable laser system. Room-temperature helium is used as a trapping gas in the QIT. The relative number of trapped ions inside the QIT is assessed by an Ion Charge Control (ICC) value, which reflects the ion current at the detector of the mass spectrometer. The measured ICC is proportional to the number of trapped ions of a given mass and charge.

The light source used for photodissociation and fluorescence excitation experiments is a Titanium:Sapphire laser (Ti:Sapph; Tsunami Spectra-Physics, Mountain View, CA) pumped by a Nd:YVO$_4$ diode laser (Millenia Pro 10s; Spectra-Physics). This laser system generates tunable IR light in the wavelength range of 700 – 1080 nm. Upon second harmonic generation, this creates tunable light between 350 and 540 nm. To enable fluorescence excitation of the ions in the QIT, two 1.2 mm holes have been drilled in the ring electrode such that the frequency-doubled output of the Ti:Sapph laser intersects the ensemble of trapped, gaseous ions. Fluorescence excitation is synchronized with MS/MS events in the mass spectrometry control software using a trigger from the mass spectrometer that opens a shutter, thus allowing irradiation of the trapped gaseous ions. Fluorescence from trapped ions is collected through a third hole in the QIT ring electrode (inner radius ~ 1 cm) of 2.0 mm diameter, which is orthogonal to the optical excitation axis. A lens inserted into the ring electrode assists in the collection of the fluorescence light, which then passes outside of the vacuum chamber through a window. The fluorescence light then passes through a long-pass filter (Chroma Technology Corp., Rockingham, VT, USA) in order to reduce scattered laser light. The filtered light can then be sent to one of two detectors: a single-photon avalanche photodiode (SPAD; Micro Photon Devices, Bolzano, Italy, model PDM-100) in order to measure fluorescence lifetimes using time-correlated single photon counting (TCSPC), or a spectrograph coupled to an electron-multiplying charge coupled device (EM-CCD; Andor, Belfast, UK) in order to measure fluorescence emission spectra. The EM-CCD was
chosen for this work because light levels are extremely low (due to limited number of trapped ions and poor solid angle for collection) and fluorescence light is amplified prior to signal readout in this camera, thus limiting the effects of readout noise. The EM-CCD was operated using a 50 kHz readout rate, 4x pre-amplified gain, an electron-multiplication gain of 210-250, and at a temperature of 198 K. The spectrograph/EM-CCD system was wavelength calibrated with gaseous rhodamine 640 ($\lambda_{em}^{max} = 561$ nm). A summary of the experimental parameters used for fluorescence emission and relative brightness experiments is listed in Table C.1 for the unbound dyes and Table C.2 for the dsDNA-dye complexes.

4.2.3 Relative Brightness Determination

The relative brightness of gas-phase fluorophores was assessed by comparing the fluorescence intensities of each ion under the same mass spectrometry and excitation conditions. For [ds-9H+3Et$^+$]$^6$- and [ds-6H+AOH$^+$]$^5$ complexes relative to ethidium and acridine orange, respectively, these parameters include equivalent current at the ion detector (ICC), helium pressure, and fluorescence accumulation times. The laser powers and excitation wavelengths employed for relative brightness of Et$^+$ and the [ds-9H+3Et$^+$]$^6$- complex was 3 mW at 480 nm and 20 mW at 520 nm, respectively. For AOH$^+$ and the [ds-6H+AOH$^+$]$^5$ complex, the laser powers for each were 1 mW and the excitation wavelength was 470 nm. Further experimental details regarding relative brightness measurements of the dsDNA-dye complexes and the unbound dyes can be found in Table C.2. Relative brightness measurements of dsDNA-dye complexes were compared to three rhodamine brightness controls (Rh123, ss1-RhoGX, and ds-RhoGX). The laser powers employed for Rh123, ss1-RhoGX, and ds-RhoGX brightness controls were 1.0 mW (Rh123) and 2.0 mW (ss1-RhoGX and ds-RhoGX complexes), respectively. The same wavelength (450 nm) was used for fluorescence excitation of Rh123, ss1-RhoGX and ds-
RhoGX. We believe that this should result in ~equal excitation efficiency for each fluorophore, because of the similarity of the fluorophore structures and emission spectra (see below). Fluorescence intensities of the rhodamine controls were determined by integration of the measured emission spectra over 480 – 624 nm. The relative brightness was calculated by comparing the integrated fluorescence signals, normalized for laser power, of each fluorophore.

4.2.4 Solution-phase characterization

Solution-phase fluorescence measurements were performed on a LS50B spectrofluorometer (Perkin Elmer, Waltham, MA, USA). Emission spectra of the fluorophores listed in scheme 1 were recorded in H₂O at concentrations of 1 μM. For DAPI, the pH was adjusted to 11.6 using NH₃ to ensure that the 1⁺ charge state was investigated. DsDNA-dye solutions were dissolved in 25 mM ammonium acetate, as was employed for ESI-MS experiments. For EB and the ds-EB solution, the same concentrations were employed: 25 μM of each DNA strand and 25 μM ethidium. The ds-AOH⁺ solution was 0.5 μM dsDNA and 1 μM AOH⁺ because concentrations used for ESI-MS analysis saturated the fluorescence detector. Emission spectra for dyes in aqueous solution and when bound to dsDNA were measured at their respective excitation maxima, using identical excitation (ex.) and emission slit (em.) bandwidths. For EB and the ds-EB solution, these were 475 nm and 515 nm, respectively (ex. 2.5 nm, em. 5 nm). For AOH⁺ and the ds-AOH⁺ solution, the excitation wavelength was 490 nm (ex. 5 nm, em. 5 nm).
4.3 Results and Discussion

4.3.1 Gas-phase emission spectra of isolated dyes

The fluorescence emission spectra for three Hoechst dyes ([H33342+H]^+, [H34580+H]^+, and [H33258+H]^+), [DAPI+H]^+, ethidium (Et^+), and acridine orange (AOH^+) in the gas phase are compared with their spectra in aqueous solution in Figure 4.1. Measured emission maxima and FWHM are summarized in Table 4.1 for each fluorophore and compared with available literature values for the solvated forms. No fluorescence from gaseous SYBR Green I in the 1^+ charge state (m/z 510) was detected under optimized fluorescence conditions, which perhaps is not surprising because its quantum yield in solution when not bound to dsDNA is extremely low (Φ = 0.0004). Water was chosen as the solvent for solution-phase fluorescence in order to investigate 1^+ charge states for the solvated fluorophores. The only exception to this was for DAPI, where reports have proposed that DAPI exists in its doubly protonated form at pH 7.0. Thus, solution spectra for DAPI (Figure 4.1d) were measured at both pH 7 and 11.6.

The shifts in emission spectra for the fluorophores in their gaseous and aqueous environments were compared. Emission spectra of like charge states for gaseous ethidium and acridine orange show blue-shifts relative to emission in water, as noted in our previous reports examining these dyes, and also observed for a series of cationic rhodamine dyes. This indicates a larger spacing between S_0 and S_1 electronic levels for gaseous ions relative to their solvated counterparts, as would be expected for molecules for which the excited states are preferentially stabilized by interaction with the solvent. Conversely, the red-shift in emission spectra for the gaseous Hoechst dyes relative to their aqueous counterparts (and gaseous [DAPI+H]^+ relative to solution at pH 11.6) (Figs. 4.1a-d) suggests a spacing between S_0 and S_1 that decreases upon removal of solvent. The measured pK_a values for the 1^+ and 2^+ charge states of H33258 are 7.94 and 5.68, respectively (and are likely similar for H33342 and H34580),
Figure 4.1. Gas- (dots and solid line generated with Savitzky-Golay smoothing with 25 points) and solution-phase (dashed line) fluorescence emission spectra for (a) Hoechst 33342, (b) Hoechst 34580, (c) Hoechst 33258, (d) DAPI, (e) Ethidium, and (f) acridine orange in the 1+ charge state. Solution-phase emission spectra were measured for 1 μM solutions in H₂O, except for DAPI at pH 11.6 (large dots), which was made from 0.9 M NH₃. A summary of experimental conditions used to measure gas-phase fluorescence emission spectra can be found in Table C.1.
Table 4.1. A summary of the fluorescence characteristics in solution and the gas phase for DNA-binding fluorophores examined in this work. ($m/z$: mass-to-charge ratio; $\lambda^\text{gas}_{em,max}$ and $\lambda^\text{soln}_{em,max}$: gas and solution-phase emission maxima; $FWHM^\text{gas}_{em}$ and $FWHM^\text{soln}_{em}$: full-width at half-maximum for gas and solution-phase emission spectra; $\tilde{\nu}^\text{gas}_{em} - \tilde{\nu}^\text{soln}_{em}$: energetic spacing between gas and solution-phase emission maxima).

<table>
<thead>
<tr>
<th>Dye</th>
<th>$m/z$ (g)</th>
<th>$\lambda^\text{gas}_{em,max}$ (nm)</th>
<th>$\lambda^\text{soln}_{em,max}$ (nm) (this work)</th>
<th>Literature $\lambda^\text{soln}_{em,max}$ (nm)</th>
<th>$FWHM^\text{gas}_{em}$ (cm$^{-1}$)</th>
<th>$FWHM^\text{soln}_{em}$ (cm$^{-1}$)</th>
<th>$\tilde{\nu}^\text{gas}<em>{em} - \tilde{\nu}^\text{soln}</em>{em}$ (cm$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H33342+</td>
<td>455</td>
<td>506</td>
<td>500</td>
<td>471$^{[a]}$, 510$^{[b]}$, 457$^{[c]}$</td>
<td>3400</td>
<td>3300</td>
<td>-240</td>
</tr>
<tr>
<td>H33258+</td>
<td>427</td>
<td>509</td>
<td>499</td>
<td>508$^{[a]}$, 505$^{[b]}$, 486$^{[c]}$, 500$^{[d]}$</td>
<td>3500</td>
<td>3400</td>
<td>-400</td>
</tr>
<tr>
<td>H34580+</td>
<td>456</td>
<td>491</td>
<td>471</td>
<td>490$^{[b]}$, 495$^{[c]}$</td>
<td>2600</td>
<td>4200</td>
<td>-860</td>
</tr>
<tr>
<td>DAPI+</td>
<td>277</td>
<td>453, 484</td>
<td>440 (pH 11.6), 466 (pH 7)</td>
<td>496$^{[a]}$, 453$^{[c]}$, 470$^{[d]}$, 460$^{[f]}$</td>
<td>3500</td>
<td>3400 (pH 11.6), 4500 (pH 7)</td>
<td>-650, -2000</td>
</tr>
<tr>
<td>AOH+</td>
<td>266</td>
<td>490$^{[g]}$</td>
<td>522</td>
<td>522$^{[g]}$, 525$^{[c]}$</td>
<td>1000</td>
<td>1700</td>
<td>1200</td>
</tr>
<tr>
<td>Et+</td>
<td>314</td>
<td>548$^{[h]}$</td>
<td>600 (H$_2$O), 617 (NH$_4$OAc)</td>
<td>632$^{[a]}$, 599$^{[i]}$</td>
<td>2500</td>
<td>2200</td>
<td>1600</td>
</tr>
<tr>
<td>SG+</td>
<td>510</td>
<td>not detected</td>
<td>521</td>
<td>530$^{[j]}$</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

thus we estimate that ~5-10% of the Hoechst solution in water (pH 7) is in the $2^+$ charge state. As our mass spectrometry experiments isolate individual charge states, our gas-phase measurements provide a more straightforward method of investigating the spectroscopic properties of individual charge states.

The emission profiles of several of the gaseous dyes show more features than their solution-phase counterparts. This is perhaps most striking for [DAPI+H]$^+$, where two major features at ~453 nm and ~485 nm are apparent in the gas-phase data. These may correspond to protonation at distinct sites (see also Scheme 4.1d). Early work reported by Barcellona et al. showed dispersed emission spectra and fluorescence lifetimes of DAPI as a function of pH.\textsuperscript{20} Emission spectra and time-resolved measurements of DAPI at pH 0.5 and 12 showed two distinct components with emission maxima of 445 nm (pH 12) and 482 nm (pH 0.5). The authors proposed that these components corresponded to two different sites of protonation on DAPI. We also note that the S/N of DAPI’s gas-phase emission spectrum is noticeably lower than that of the dyes (except SYBR Green I). This is likely due to the combination of DAPI’s low Φ and the ease with which DAPI photodissociates relative to the other dyes (see below). After this assessment of the intrinsic fluorescence of these dyes in isolation, we proceeded to investigate the fluorescence of these dyes when complexed with dsDNA in the gas phase.

4.3.2 Fluorescence measurements of dsDNA-dye complexes

Fluorescence emission of the gaseous dsDNA-dye complexes was monitored for all seven dyes listed in Scheme 4.1. Under conditions where fluorescence was observed for a control of similar size and charge density (a negatively charged complex of BODIPY Fl bound to the 5’ end of dsDNA), no fluorescence was detected for complexes in the 6’ charge state of dsDNA with four SYBR Green I, and a single DAPI, H33258, H33342, H34580, and acridine orange.
Thus, the strong “turn on” fluorescence response (see scheme 4.1 for $\Phi_{FL} \uparrow$ values) present in solution upon dsDNA binding is clearly not reproduced for these dyes in the gas phase. This may be the result of conformational changes in dsDNA resulting in altered binding geometries. Alternatively, the binding mode of these dyes (which range from intercalation to minor-groove binding in solution) may be different in the gas phase, perhaps leading to smaller $\Phi$ values in comparison to the isolated dyes. The lack of detectable fluorescence for these complexes may be due in part to a dissociation deactivation pathway (such as electron photodetachment observed for the Hoechst dyes and DAPI or strand separation observed for SYBR Green I, see Figure C.7) that competes with fluorescence.

Fluorescence was detected from the $[\text{ds-9H}+3\text{Et}^+]^6^-$ and $[\text{ds-6H}+\text{AOH}^+]^5^-$ complexes (Figure 4.2a-b). The $[\text{ds-9H}+3\text{Et}^+]^6^-$ complex was chosen for gas-phase fluorescence characterization as this complex had the highest signal intensity in the mass spectrum, simplifying the gaseous fluorescence measurement. However, our analysis (see below) suggests that the dyes in these complexes also do not exhibit “turn-on” fluorescence responses upon complexation with dsDNA in the gas phase. For comparison with the gas phase data, the observed turn-on fluorescence response for the ds-EB solution is shown in Figure 4.2c. The measured increase in fluorescence shown is ~5-fold, somewhat smaller than the literature value of ~9-fold$^{23}$ due to incomplete ethidium intercalation at the low concentrations (25 μM each of dye and dsDNA) used. Surprisingly, the solution-phase data shown in Figure 4.2d illustrates a 20% decrease for the ds-AOH$^+$ solution relative to AOH$^+$. While AOH$^+$ is traditionally thought to exhibit a fluorescence “turn-on” response of more than 2-fold$^{27,33}$ upon binding dsDNA, a decrease in fluorescence of acridine orange upon introduction of dsDNA was also recently reported by Sayed et al.$^{33}$ The researchers postulated that at high acridine orange concentrations (23 μM) relative to dsDNA (1 – 30 μM), acridine orange dimers bind electrostatically to
Figure 4.2. Savitz-Golay smoothed fluorescence emission spectra measured for (a) gaseous ethidium (orange), [ds-9H+3Et]^6^- (red), and [ss1-5H+Et]^4+ (brown) complexes and for (b) gaseous acridine orange (blue) and the [ds-6H+AOH]^5+ (light blue) complex. (c) – (d) Solution-phase emission spectra in 25 mM NH₄OAc for (c) 25 μM ethidium (orange) and 25 μM each of dsDNA and EB solution (red) and (d) 1 μM acridine orange (blue) and 0.5 μM of dsDNA and 1 μM acridine orange solution (light blue). (e) – (f) Relative brightness measurements for low ICC (~25k to ensure linearity) populations of Rh123^+ (green), [ds-RhoGX+5H]^6+ and [ds-RhoGX+4H]^5+ complexes (pink). Fluorescence intensities in (a) and (b) have been normalized to illustrate their relative brightness in comparison to rhodamine-labeled controls.
phosphates in the dsDNA backbone, and that this externally-bound complex has a lower Φ than intercalated AOH⁺ monomers. Thus, due to the higher concentrations used for ESI-MS analysis of the [ds-6H+AOH⁺]⁵⁻ complex (50 μM AOH⁺ and 25 μM dsDNA), we think it is likely that acridine orange dimers bind electrostatically to dsDNA in solution prior to subsequent analysis in the gas phase. However, mass spectra obtained under these conditions showed no evidence of dimeric association of AO to dsDNA. We also note that the solution used for ESI-MS analysis likely has a higher population of AO dimers than the fluorescence spectrum shown in Fig. 4.2d. A control experiment examining the fluorescence turn-on response at higher dsDNA concentrations (1.5 μM) relative to acridine orange (0.1 μM) illustrated a 2.5-fold increase in fluorescence intensity for the ds-AOH⁺ complex relative to AOH⁺ (Figure C.2). We plan to pursue a comparison of the fluorescence from [ds-6H+AOH⁺]⁵⁻ complexes formed by varying dsDNA and AOH⁺ concentrations in the future.

Gaseous ethidium and acridine orange show significant red-shifts in emission maxima upon complexation to dsDNA. The measured emission maximum for the [ds-6H+AOH⁺]⁵⁻ complex is ~522 nm, a ~1300 cm⁻¹ red-shift relative to the emission maximum of isolated AOH⁺ in the gas phase (λ_em^max = 490 nm), and nearly identical to the emission maximum of the ds-AOH⁺ solution (λ_em^max = 520 nm). Similarly, the measured emission maximum for the [ds-9H+3Et⁺]⁶⁻ complex in the gas phase is ~628 nm, a ~2300 cm⁻¹ red-shift in emission maximum relative to isolated gaseous ethidium (λ_em^max = 548 nm). In contrast, in aqueous solution, the measured emission maxima for ethidium shifts to the blue, from 617 nm to 607 nm upon introduction of dsDNA. We note that the emission maximum of ethidium appears to vary considerably when measured in H₂O (600 nm) versus ammonium acetate (617 nm), as other reported literature values measured in aqueous solutions for ethidium do.⁶⁻ Intriguingly, the emission maxima of the [ds-9H+3Et⁺]⁶⁻ and [ds-6H+AOH⁺]⁵⁻ complexes are similar to the
emission maxima of the corresponding dsDNA-dye solutions. This analysis suggests that while the electronic environment felt by gaseous Et\(^+\) and AOH\(^+\) is quite different from that of ethidium or acridine orange in water, the electronic environment of these dyes when bound to dsDNA in solution or the gas phase is more similar. This seems to be consistent with the picture that interactions present in solution (such as intercalation and/or electrostatic attachment) are preserved in the gas phase for both acridine orange and ethidium. However, it may be that ethidium and acridine orange bind to dsDNA through an alternative interaction. In particular, the electrostatic attraction between the positive charges on ethidium and acridine orange with phosphates in the DNA backbone means that the formation of externally bound dsDNA-dye complexes in the gas phase cannot be ruled out.

The apparent lack of fluorescence “turn-on” response for any dsDNA-dye complexes prompted us to question whether our mass spectrometry conditions were disrupting the double helix in the gas phase. As we employed “harsh” mass spectrometry conditions for all fluorescence measurements of the dsDNA-dye complexes, we measured the fluorescence intensity of the [ds-9H+3Et\(^+\)]\(^6\)-complex under “gentle” conditions. Using “gentle” source conditions, as assessed by survival of the DNA G-quadruplex \[d(G_4T_4G_4)_2\] with three NH\(_4\)^+ ions\(^{53}\) and in line with suggestions by Gabelica and coworkers,\(^{54}\) the fluorescence intensity of the [ds-9H+3Et\(^+\)]\(^6\)-complex was unchanged. The mass spectra obtained for the G-Quadruplex under “harsh” and “gentle” conditions can be found in Figure C.3. Intriguingly, a control experiment examining fluorescence from the single stranded [ss1-5H+Et\(^+\)]\(^4\)-complex upon 490 nm excitation (brown in Figure 2a) at ion currents similar to the [ds-9H+3Et\(^+\)]\(^6\)-complex showed no detectable fluorescence, despite electron photodetachment signifying absorption of light at this wavelength. A summary of experimental parameters used for this experiment is summarized in Table C.2. The lack of fluorescence for the [ss1-5H+Et\(^+\)]\(^4\)-complex is likely due to a different
mode of binding to the single strand (e.g. a non-intercalative electrostatic interaction to the phosphate groups) that opens a large nonradiative decay channel, such as (possibly) intra-complex excited state proton transfer.

4.3.3 Relative brightness of dyes versus dsDNA-dye complexes

To determine how well measured ion current reflects the actual number of ions of different m/z, and therefore obtain a better estimate of brightness of isolated Et\(^+\) and AOH\(^+\) compared to that of the [ds-9H+3Et\(^+\)]\(^6\) and [ds-6H+AOH\(^+\)]\(^5\) complexes, the relative fluorescence response of two controls, each containing similar rhodamine fluorophores, were interrogated using identical mass spectrometry and optical excitation conditions. For this, an isolated rhodamine (Rh123 [m/z 345]) and double stranded DNA with a covalently attached fluorophore (ds-RhoGX [m/z 1532 and 1838]) (Figure C.4) were employed\(^a\).

Fig. 4.2e compares fluorescence emission spectra of our brightness controls: cationic Rh123 (green) and the [ds-RhoGX+5H]\(^6\) complex (pink). Corresponding fluorescence emission spectra of Rh123 and the [ds-RhoGX+4H]\(^5\) complex are shown in Fig. 4.2f. The emission spectra were all measured in positive ion mode using the same experimental parameters, including the same ion current (ICC) for each species. The most striking feature of these data is that the integrated fluorescence intensity decreases substantially as the molecular size and charge increase: the integrated fluorescence intensity of the [ds-RhoGX+5H]\(^6\) and [ds-RhoGX+4H]\(^5\) complexes is just 14\% and 58\% of that of Rh123, respectively. The brightness controls shown were measured on the same day, with data within each panel measured back-to-back, in an effort

\(^a\)Rh123 and RhoGX contain the same central xanthene chromophore, however Rh123 has a methyl ester group on the pendant moiety while RhoGX contains a carboxylic acid group. Fluorescence emission spectra of gaseous rhodamine 110 (carboxylic acid group, RhoGX analog) and Rh123 (methyl ester group) confirmed that these substitutions did not change the fluorescence profiles of the fluorophores significantly (emission maxima: 481 nm and 483.5 nm,\(^5\) respectively). Rh110 and Rh123 also show equal fluorescence intensities under identical mass spectrometry and fluorescence excitation conditions.
to ensure reliability. However, in a similar experiment more than one year before, we found that the [ds-RhoGX+5H]6+ complex was just 7% as bright as Rh123+ (Figure C.5). This ~2-fold discrepancy may reflect differences in mass spectrometry conditions or problems in the 1st set of measurements (which were reproduced with old and new samples of ds-RhoGX or reflect problems with measurements made over two days). Therefore, our brightness estimates for the [ds-9H+3Et+]6- and [ds-6H+AOH+]5- complexes relative to the isolated dyes are only rough, and highlight the need to develop tools for accurate measurement of a fluorophores’ quantum yield in the gas phase.

Assuming that the brightness of the rhodamine fluorophore does not change significantly when in isolation vs. when covalently bound to dsDNA, the data shown in Fig. 4.2e-f indicate that the ICC registered by the ion detector depends on both the total mass and charge of the precursor ion. This result is reminiscent of the findings of Axelsson et al., who noted that the number of secondary electrons produced from an Al2O3 conversion dynode/Si-based ion detector depends on the mass and charge of a given ion. In particular, the data shown in Figure 4.2e indicates that approximately 6-fold fewer [ds-RhoGX+5H]6+ ions (m/z 1532) are needed to generate the same current at the ion detector as Rh123+ ions (m/z 345). Thus, it is not surprising then that the fluorescence intensity of the [ds-9H+3Et+]6- (m/z 1577) and [ds-6H+AOH+]5- (m/z 1760) complexes recorded with the same ion currents as Et+ (m/z 314) and AOH+ (m/z 266) is dramatically lower, since both dsDNA-dye complexes require fewer trapped ions to generate the same ion current as the bare dyes. By using the reduction in integrated fluorescence intensity for Rh123+ and the [ds-RhoGX+5H]6+ complex (1 → 0.14) as reference values and comparing this to the apparent relative brightness between Et+ and the [ds-9H+3Et+]6- complex (1 → 0.04), the relative brightness of Et+ versus the [ds-9H+3Et+]6- complex is ~3:1. In other words, the tertiary complex is ~1/3 as bright as the isolated dye, despite its inclusion of three dye molecules.
Similarly, using the integrated fluorescence intensity for Rh123\(^+\) and the [ds-RhoGX+4H]\(^5+\) complex (1 → 0.58) in comparison to the apparent relative brightness between AOH\(^+\) and the [ds-6H+AOH\(^+\)]\(^5-\) complex (1 → 0.27), we estimate that AOH\(^+\) is roughly twice as bright as the [ds-6H+AOH\(^+\)]\(^5-\) complex. Note that this analysis assumes that the brightness of the rhodamine dye does not change between its unbound and dsDNA bound forms. We believe this assumption is reasonable because this fluorophore is quite robust in solution (\(\Phi\) ranges from 0.74 in DMF to 1.0 in CH\(_3\)CN)\(^57\) and the measured emission maxima for each fluorophore in the gas phase were within 5 nm of one another (Figure C.6).

This analysis indicates that ethidium and acridine orange do not “turn-on” when bound to dsDNA in the gas phase. For AOH\(^+\), this contrasts results of a previous study from our laboratory in which a large (six- to nine-fold) enhancement in gas-phase brightness was observed upon complexation of this dye with the macrocyclic host molecule cucurbit[7]uril (CB7).\(^34\) We attributed this result in part to the relative rigidity of the dye upon (perhaps partial) encapsulation within the macrocyclic host. The lack of enhancement in fluorescence of AOH\(^+\) upon interaction with the dsDNA examined here then suggests that the dye is not in as rigid an environment as that provided by the CB7 host. This, in turn suggests that AOH\(^+\) is not intercalated in dsDNA in the gas phase, as it is in many solution-phase experiments. Below we discuss evidence that at least two conformations of the [ds-6H+AOH\(^+\)]\(^5-\) complex exists in the gas phase. This may reflect two types of binding present in solution, such as intercalative and electrostatic binding, that is preserved in the gas phase.

For Et\(^+\), the lack of turn-on response upon dsDNA binding is in line with our previous work characterizing the intrinsic photophysics of ethidium in isolation.\(^35\) There, we provide evidence that ethidium fluorescence is already “turned-on” in the gas phase, likely due to elimination of an excited-state proton transfer between solvent and ethidium which quenches its
fluorescence in protic solvents. The low brightness of the \([\text{ds-9H+3Et}^+]^6\) complex measured here suggests that Et\(^+\) in this gaseous dsDNA complex in fact has a lower \(\Phi\) than our estimate for gaseous Et\(^+\) (~0.2)\(^{35}\) or Et\(^+\) in dsDNA in solution (~0.35).\(^{23}\) Therefore, ethidium’s turn-on response is due to isolation from the solvent (via sequestration within dsDNA or upon transfer to the gas phase) rather than conformational restriction.

4.3.4 Power dependence of Hoechst dyes, DAPI, acridine orange, SG, and ethidium photodissociation

To explore the mechanisms that are in play upon fluorophore excitation when bound to dsDNA, the photodissociation power dependence of all seven dsDNA-dye complexes was examined. This data is included in Figure C.7, as are MS/MS spectra for the dsDNA-dye complexes (Figure C.8). Complexes of dsDNA with the Hoechst dyes, DAPI, and acridine orange form fragments generated through ePD, whereas SG and ethidium complexes dissociate to form ssDNA-dye complexes through strand separation. A few key takeaways are apparent from the photodissociation power dependence data. For the \([\text{ds-7H+DAPI}^+]^6\) complex, the primary dissociation pathway is ePD and the fit to the data is linear with respect to power (inset of Figure C.7d), indicating that absorption of a single photon is sufficient to cause ePD. All three Hoechst dyes also dissociate primarily by ePD (in line with comparison by Gabelica and coworkers)\(^{58}\), but fits suggest that multiple populations are present. One of these populations is depleted linearly with increasing power, suggesting single photon ePD. The fit to the data for the complex of acridine orange with dsDNA (Figure C.f) also requires the inclusion of two components with different power dependencies. This provides evidence of two populations for the \([\text{ds-6H+AOH}^+]^5\) complex, which may reflect the presence of AOH\(^+\) binding to dsDNA via different modes.\(^{33}\) We note that at certain laser powers, the \([\text{ds-10H+4SG}^+]^6\) and \([\text{ds-9H+3Et}^+]^6\)
complexes show an *increase* in precursor ion intensity, which we believe is due to these complexes unique dissociation pathways through strand separation (see Appendix C for more details).

### 4.4 Conclusions

This work examined a large subset of DNA-binding dyes that exhibit well-characterized fluorescence properties upon binding dsDNA in solution in an effort to develop tools for characterization of dsDNA in the gas phase. For the first time, fluorescence from two gaseous dsDNA-dye complexes was characterized. Though detectable, the relative brightness measured from complexes containing ethidium and acridine orange show that these fluorophores do not “turn on” when complexed to dsDNA in the gas phase. Previous work performed by our group proposed that ethidium’s quantum yield is already enhanced in the gas phase, likely due to elimination of an excited-state proton transfer between ethidium and solvent in the gas phase. This results in less of a “turn-on” response for ethidium when complexed to dsDNA in the gas phase, though our analysis indicates that the [ds-9H+3Et+]⁶⁻ complex has a lower Φ than when isolated in the gas phase or when ethidium is complexed to dsDNA in solution. Similarly, we estimate that AOH⁺ is two-fold brighter than the [ds-6H+AOH⁺]⁵⁻ complex, despite previous work suggesting that rigidification of AOH⁺ increases its Φ substantially. This result may be due to the formation of an electrostatically-bound AOH⁺ complex in solution that is preserved upon transfer to the gas phase. However, taken together, the drop in brightness for the [ds-9H+3Et⁺]⁶⁻ and [ds-6H+AOH⁺]⁵⁻ complexes (as well as lack of detectable fluorescence from complexes of H33258, H33342, H34580, SG, DAPI, and acridine orange with dsDNA in the 6⁻ charge state) support the idea that the rigid dsDNA double helix is not preserved in the gas phase.
Despite there being examples of non-covalent binders that exhibit “turn-on” fluorescence responses in the gas phase, an equivalent fluorophore that exhibits this response when bound to dsDNA continues to be elusive. From the survey of well-known dsDNA-binding dyes reported here, acridine orange appears to be a promising candidate for further examination under varied conditions. The results presented also offer a baseline from which to better understand fluorescence “turn-on” probes, in particular the role played by isolation from solvent interactions, while potentially providing insight into the conformation of gaseous dsDNA.

Currently, we are utilizing Förster Resonance Energy Transfer (FRET) techniques to probe the conformation of dsDNA in the gas phase under a variety of mass spectrometry conditions. An improved understanding of biomolecular conformation (such as dsDNA examined here), and the degree to which conformations present in the gas phase reflect the solution-phase species promises to aid in the use of MS for drug discovery applications.

4.5 Acknowledgements

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4.6 References


Chapter 5

Förster Resonance Energy Transfer measurements probe the conformation adopted by double-stranded DNA ions in the gas phase

S.V. Sciuto and R.A. Jockusch. This chapter is an early draft of a manuscript (to be submitted).

Abstract

Double-stranded DNA (dsDNA) ions can be detected intact in the gas phase of a mass spectrometer. However, an overall consensus on the conformation(s) adopted by dsDNA ions has not been reached. Here, we use gas-phase Förster Resonance Energy Transfer (FRET) to probe the conformation of a 20-mer dsDNA ion in the gas phase of a quadrupole ion trap (QIT) that has been modified for gas-phase fluorescence spectroscopy. Steady-state fluorescence spectra obtained for the dsDNA ions in a charge-state resolved manner indicate decreasing FRET efficiencies with increased charge density. Furthermore, time-resolved fluorescence measurements for dsDNA ions in the 7⁺ and 8⁺ charge states provide evidence of a compact conformation with FRET efficiencies of 0.92 and 0.86, respectively. This agrees with previous reports suggesting that gaseous dsDNA with low charge densities adopts a compact conformation rather than well-known solution-phase conformations. Finally, we examine the FRET efficiency as a function of increasing capillary exit voltage, using a bimolecular G-Quadruplex to report on the degree of “harshness” inside the QIT. We observe a decrease in FRET efficiency as the capillary exit voltage increases, consistent with elongation of dsDNA ions when harsher source conditions are employed in the mass spectrometer. This highlights that gas-phase FRET can be used as a complementary technique to characterize the conformational transition(s) that occur when biomolecular ions are transferred from solution to the gas phase.
5.1 Introduction

The first mass spectrum illustrating the appearance of intact double-stranded DNA (dsDNA) ions was first reported by Smith and coworkers in 1993 using an electrospray ionization (ESI) source. Since then, the development of ESI-mass spectrometry (MS) for the gentle transfer of large biomolecular assemblies has fueled growth in the characterization of dsDNA in the gas phase. Transferring dsDNA from solution into the gas phase is no small feat; negatively charged phosphate groups along the DNA backbone that are screened by counterions and polar solvent in solution have stronger electrostatic repulsion in the gas phase, which can disrupt the DNA double helix. Furthermore, collisions in the ionization and source regions of a mass spectrometer can break apart inter- and intramolecular interactions brought on via hydrogen-bonding and base stacking in dsDNA. Thus, identifying the factors that drive conformational changes for biomolecular ions is important as it can aid in our understanding of drug targeting by MS analysis.

One informative route to characterizing gas-phase structure is to examine the correlation between Watson-Crick (G–C and A–T) hydrogen-bonding in solution and the dissociation energetics of dsDNA ions in the gas phase. Early work by Schnier et al. used blackbody infrared radiative dissociation to calculate the activation energy required for dissociation of complementary and non-complementary DNA duplexes. The activation energy for dissociation of the complementary A7–T7 dsDNA in the 3− charge state was significantly higher than that for noncomplementary A7–A7 and T7–T7 dsDNA, suggesting that gas-phase dsDNA stability reflects the well-known Watson-Crick base pairs. Similar work by Pan et al. assessed the stability of dsDNA anions with varying GC content. Sequences with higher GC content (which have more hydrogen bonds in solution) required higher dissociation thresholds in the gas phase, suggesting that dsDNA ions retain a memory of their hydrogen bonding network in the absence of solvent.
However, dsDNA ions with the same GC content but different sequences showed no particular relationship between solution-phase stability and dissociation threshold, suggesting that other factors (base stacking, position of GC hydrogen-bonds) also play a role in the gas-phase stability of dsDNA ions.

Collisional cross section (CCS) distributions from ion mobility (IM) MS are useful to distinguish elongated structures, such as those associated with dsDNA helices, from more compact forms. Early reports from Bowers and coworkers indicated that for poly(CG)$_n$ sequences with fewer than 20 base-pairs, CCSs were consistent with dsDNA ions transitioning from the B-DNA form in solution to the A-DNA form in the gas phase. However, the charge densities of the dsDNA ions studied by Bowers and coworkers (-0.5 charge/base pair) are at the high-end of charge densities typically observed when dsDNA solutions are electrosprayed from “native-like” (25 – 150 mM) ammonium acetate solutions. For example, Rosu et al. showed that mass spectra of 12-mer dsDNA ions electrosprayed from ammonium acetate solutions predominantly formed the 5$^+$ charge state, ie. [ds-5H]$^{5+}$, a charge density of -0.4 charges per bp. Furthermore, CCS distributions for the rigid DNA G-quadruplex d(TG$_4$T)$_4$ were much narrower than CCS distributions for [ds-5H]$^{5+}$ ions, providing evidence of multiple conformations of dsDNA ions in the gas phase. While IM-MS is one of the premiere techniques for overall structural characterization of gaseous biomolecules, structural interpretation at an atomistic level of detail relies on comparison with computations and sufficient resolving power in the ion mobility region. Furthermore, the possibility of interconversion of conformers within the separation region hamper extraction of CCSs. For example, Burmistrova et al. investigated the CCS of numerous 18-mer dsDNA ions upon collisional activation. For several of the dsDNA ions unique conformers with distinct CCS could be assigned. However, sequences with short AT-tracts at the 5’ position had broad CCS distributions that suggested the presence of multiple
conformers, but were not easily resolved. This highlights that the gaseous structure of dsDNA is dependent on numerous factors, such as charge state, sequence, and likely ion source conditions employed on the mass spectrometer.

Our laboratory has coupled mass spectrometry with fluorescence-based tools to develop powerful new probes for gaseous biomolecular ion conformations and interactions.\textsuperscript{16, 17} In particular, the implementation of Förster Resonance Energy Transfer (FRET) may provide new insights into gas-phase biomolecular structure.\textsuperscript{18} FRET is a distance-dependent fluorescence technique that reports on the non-radiative energy transfer between a donor and acceptor fluorophore which are typically covalently attached to a biomolecule of interest.\textsuperscript{19} FRET rates are strongly dependent on the donor-acceptor distance ($r_{DA}$) with an inverse $r_{DA}^{-6}$ dependency, which arises because the energy transfer results from dipole-dipole coupling between a donor and acceptor fluorophore. FRET is particularly useful for systems on the order of 10 – 100 Å, making this technique an informative tool for biomolecular characterization.

While the idea to use FRET\textsuperscript{20-23} in combination with trapping MS to probe biomolecular conformation is not new, its implementation to extract reliable FRET efficiencies (which requires time- or wavelength-dispersed data) is recent.\textsuperscript{16, 17} Early work by Danell and Parks used FRET to probe the structural transitions that occur when dsDNA is heated in a quadrupole ion trap (QIT) mass spectrometer.\textsuperscript{20} The authors labelled BODIPY fluorophores to the 5’ and 3’ ends of mutually complementary single-stranded DNA (ssDNA) sequences (the sequences were BODIPY-TMR-5’-AATTAATCCGCGG-3’ and BODIPY-TR-3’-TTAATTAGCGGC) and induced heating within the QIT on the 7’ dsDNA ion (-0.5 charge/bp). As the temperature increased, increased donor fluorescence was observed, indicating that the distance between donor and acceptor labels had increased. Danell and Parks postulated that the increased donor fluorescence at elevated temperatures was due to an “unravelling” of the AT stretch prior to
dissociation. Related methodologies that utilize photodissociation of an acceptor molecule following non-radiative energy transfer from a donor fluorophore (termed “action FRET”) have also been developed recently to characterize the conformation of gas-phase ions. Very recently, our laboratory has examined the gaseous structure of protein GB1 using FRET techniques. Lower FRET efficiencies in the gas phase than in solution indicated that the gaseous protein is more extended than the solution-phase counterpart, and that the protein readily unfolds in the gas phase at charge states which are folded in solution.

Here, we employ FRET in combination with trapping mass spectrometry to probe the conformation of dsDNA in the gas phase. A 20-mer dsDNA sequence with BODIPY fluorophores appended to the 5’ ends of mutually complementary ssDNA (Scheme 5.1) are characterized in the gas phase of a quadrupole ion trap (QIT) mass spectrometer using FRET. Steady-state and time-resolved fluorescence measurements report on the conformations of 7’, 8’, and 9’ charge states of this dsDNA sequence. In addition, changes in FRET efficiency with ion source conditions are examined to delineate the effect of individual MS parameters on biomolecular structure.

5.2 Experimental

5.2.1 DNA Sequences and formation of the FRET complex

Mutually complementary 20-mer ssDNA sequences with BODIPY Fl (donor, D) and BODIPY 576/589 (acceptor, A) appended to the 5’ ends via C₃ linkers and purified through dual reversed-phase HPLC were purchased from Integrated DNA Technologies (IDT, Coralville, Iowa, USA). The sequences were BODIPY Fl-5’-ATTCCCGCCGCTTAGGCAGT-3’ (ss1) and BODIPY 576/589-5’-ACTGCCTAAGCGGGCCGGAAT-3’ (ss2). All other ssDNA sequences used in this work were also purchased from IDT and used without further purification. To form
Scheme 5.1. (a) Structures of the donor, BODIPY Fl (blue) and the acceptor, BODIPY 576/589 (green). The attachment to the 5' ends of ss1 and ss2 are indicated. (b) The 20-mer dsDNA complex made from complimentary oligonucleotides ss1 and ss2.
the FRET complex, equal amounts (20 nmoles) of ss1 and ss2 were mixed together in 25 mM ammonium acetate. The mixture was then annealed at 80°C for 10 minutes, followed by controlled cooling (-4°C/30 minutes) until room temperature was reached. To form donor and acceptor-only dsDNA controls, equal amounts (14 nmoles) of the BODIPY Fl or BODIPY 576 sequences were mixed with the unlabeled complementary sequence and subjected to the annealing procedure described above. The bimolecular G-Quadruplex \([d(G_4T_4G_4)_2]\) was formed by annealing 1 mM ss3 \([d(G_4T_4G_4)]\) in 150 mM ammonium acetate, as described above. Stock solutions for all DNA solutions were stored in DNA LoBind tubes purchased from Fisher Scientific (Ottawa, ON, CA) and stored in the freezer at -20°C. Final DNA concentrations used for ESI-MS analysis contained 10 μM DNA in 25 mM (150 mM for \([d(G_4T_4G_4)_2]\)) ammonium acetate.

5.2.2 Mass Spectrometry and Gas-Phase Fluorescence Spectroscopy

We have modified a commercial quadrupole ion trap (Bruker, Esquire 3000+, QIT) mass spectrometer to enable selected-ion laser induced fluorescence (SILIF) measurements. This experimental set-up has been described in detail previously.\(^\text{26, 27}\) Below, we outline the experimental conditions pertaining to this current study.

DsDNA is transferred into the gas phase via nanoelectrospray ionization (nanoESI) using capillary voltages between 800 – 1100 V. The size of the emitter tip was ~5 μm and the flow rate ~10-20 nL/min. The ions are then transmitted through a heated glass capillary and a series of ion optics before entering the trapping region of the QIT. In order to facilitate gentle transfer of the FRET complex, MS parameters were varied to enable survival and mass spectral detection of dsDNA ions. Ions are accumulated in the trapping region of the QIT for 100 - 200 ms using helium as trapping gas and are subsequently manipulated during a reaction period of up to 20 s.
Manipulations include mass selection, collision-induced dissociation and/or irradiation by visible light produced from a tunable laser system. The relative number of trapped ions inside the QIT is assessed by an Ion Charge Control (ICC) value which reflects the ion current at the detector of the mass spectrometer. The measured ICC is proportional to the number of trapped ions of a given mass and charge. Therefore, fluorescence intensity comparisons of ions with similar masses and identical charge states (eg. FRET-labeled dsDNA and donor only dsDNA controls) are normalized to ICC values in order to assess meaningfully differences in measured fluorescence intensity.

The light source used for photodissociation and fluorescence excitation experiments is a Titanium:Sapphire laser (Ti:Sapph; Tsunami Spectra-Physics, Mountain View, CA) pumped by a Nd:YVO₄ diode laser (Millenia Pro 10s; Spectra-Physics). This laser system operates at an 80 MHz repetition rate and ~100 fs pulse width, generating tunable IR light in the wavelength range of 700 – 1080 nm. Upon second harmonic generation, this creates tunable light between 350 and 540 nm. Laser light can then pass through one of two optical paths, depending on the fluorescence measurement made. For fluorescence lifetime measurements using time-correlated single photon counting (TCSPC) techniques, laser light passes through a pulse picker (Model 350-105, ConOptics Inc., Danbury, CT, USA) to increase the time between laser pulses in order to prevent the effects of photon pile up. Light then passes through two 1.2 mm holes that have been drilled in the ring electrode such that the frequency doubled output of the Ti:Sapph laser intersects the ensemble of trapped, gaseous ions. Measurements performed by our group estimate that the laser beam has a 1/e² diameter of ~617 μm. Fluorescence excitation is synchronized with MS/MS events in the mass spectrometry control software using a trigger from the mass spectrometer that opens a shutter, thus allowing irradiation of the trapped \( m/z \)-selected gaseous ions. Fluorescence from trapped ions is collected through a third hole in the QIT ring electrode.
(inner radius ~ 1 cm) of 2.0 mm diameter, which is orthogonal to the optical excitation axis. A lens inserted into the ring electrode assists in the collection of the fluorescence light, which then passes outside of the vacuum chamber through a window. The fluorescence light then passes through a long-pass filter (Chroma Technology Corp., Rockingham, VT, USA) in order to reduce scattered laser light. The filtered light can then be sent to one of two detectors: a single-photon avalanche photodiode (SPAD; Micro Photon Devices, Bolzano, Italy, model PDM-100) capable of measuring fluorescence lifetimes using the technique of time-correlated single photon counting (TCSPC), or a spectrograph coupled to an electron-multiplying charge coupled device (EM-CCD; Andor, Belfast, UK) capable of measuring fluorescence emission spectra. The EM-CCD was operated using a 50 kHz readout rate, 4x pre-amplified gain, an electron-multiplication gain of 250, and at a temperature of 198 K.

5.2.3 Calculation of FRET efficiencies: steady-state and time-resolved fluorescence

To calculate the FRET efficiency of a given charge state for steady-state and time-resolved measurements, the same charge states of DA-dsDNA and D-dsDNA complexes were subjected to identical mass spectrometry and fluorescence excitation conditions. The FRET efficiency ($E$) was calculated in three different ways (ratiometric, decrease in donor intensity, and decrease in donor lifetime), as described using equation 1:

$$E = \frac{1}{1 + \frac{\eta_A}{\eta_D} \frac{I_A}{I_D}} = 1 - \frac{I_{DA}}{I_D} = 1 - \frac{\tau_{DA}}{\tau_D}$$

where $I_D$, $I_A$, and $I_{DA}$ represent integrated fluorescence intensities for D-dsDNA, A-dsDNA, and DA-dsDNA complexes, respectively, and $\eta_A/\eta_D$ is a correction factor to account for quantum yield and wavelength detection efficiency differences for donor and acceptor. To obtain FRET efficiencies using the ratiometric method, donor and acceptor intensities were integrated from
490–525 nm and 568–603 nm, respectively. The FRET efficiency was also calculated by measuring time-resolved fluorescence of the donor in the presence ($\tau_{DA}$) or absence ($\tau_D$) of the acceptor chromophore. Fluorescence measurements for DA- and D-dsDNA complexes utilized a 480 nm long-pass filter (480LP, Chroma Technology Corp., Rockingham, VT, USA) in the optical detection path. The only exception to this was for time-resolved measurements for the DA-dsDNA complex, where a 505 nm (ET505/20X, Chroma) or 520 nm band-pass filter (520/44 nm, Semrock, Inc., Rochester, NY, USA) was used to spectrally isolate fluorescence arising from the donor fluorophore.

5.3 Results and Discussion

5.3.1 Mass Spectrometry and Electron Photodetachment of FRET-labeled dsDNA

Complementary 20-mer ssDNA sequences with fluorescent BODIPY dyes labeled for gas-phase FRET experiments (Scheme 5.1) appended to 5’ ends were annealed in solution to form dsDNA. Figure 5.1a shows the nanoESI mass spectrum illustrating the appearance of DA-dsDNA complexes in the 7, 8, and 9 charge states, as well as peaks arising from ssDNA ions. Each DA-dsDNA charge state appears as a distribution of 1-5 Na$^+$ adducts, compared to 0-2 Na$^+$ adducts for ssDNA ions. Figure 5.1b shows mass-selection and storage for 3.10 s of the [DA-dsDNA]$^7$ complex ($m/z$ 1880), while Figure 5.1c shows that 2.5 mW of 470 nm light for 3.10 s results in ~5-10% precursor ion loss due to electron photodetachment (ePD) and the appearance of the charge-reduced [DA-dsDNA]$^{6-}$ ($m/z$ 2193) complex. ePD has been observed previously as the dominant dissociation pathway for dsDNA with covalently and non-covalently bound fluorophores.
Figure 5.1. (a) NanoESI mass spectra for the 10 μM double-stranded DNA FRET complex in 25 mM ammonium acetate illustrating the appearance of [DA-dsDNA], [D-ss1], and [A-ss2] ions. (b) Storage of the [DA-dsDNA]$^7^-$ complex for 3.10 seconds and (c) irradiation of the [DA-dsDNA]$^7^-$ complex with 470 nm light for 3.10 seconds using 2.5 mW.
5.3.2 Charge-state resolved steady-state FRET spectra

Figure 5.2 shows steady-state FRET spectra for the [DA-dsDNA]$^7$, [DA-dsDNA]$^8$, and [DA-dsDNA]$^9$ complexes upon 470 nm excitation under similar conditions to the photodissociation mass spectra shown in Figure 1. 470 nm was chosen as the excitation wavelength for all FRET experiments as it preferentially excites the donor over the acceptor. This was confirmed by measuring the fluorescence intensity as a function of wavelength for [D-ssDNA]$^4$ and [A-ssDNA]$^4$ complexes. This data is shown in the Figure D.1 in addition to experimental conditions used for the FRET complexes (Table D.1). Fluorescence emission spectra for the singly labeled D-dsDNA and A-dsDNA controls measured under the same experimental and fluorescence excitation conditions are shown for comparison in Figure 5.2. Steady-state emission spectra were not collected for the [A-dsDNA]$^9$ complex due to the substantially longer time required (~1.5 hours) to obtain S/N similar to [DA-dsDNA]$^9$ and [D-dsDNA]$^9$ ions. The measured emission maxima for donor (BODIPY Fl) and acceptor (BODIPY 576/589) moieties are 506 nm and 578 nm, respectively. This corresponds to a small ~200 cm$^{-1}$ and ~300 cm$^{-1}$ blue-shift in emission maxima for BODIPY Fl and BODIPY 576/589 relative to its emission maximum in H$_2$O, respectively.$^{29,30}$ BODIPY Fl’s emission measured here is also nearly identical to the emission maximum measured previously for gaseous [BODIPY Fl + K$^+$] ($\lambda_{em}^{max} = 503$ nm). Furthermore, time-resolved fluorescence lifetimes of the [BODIPY Fl + K$^+$] complex (~12.0 ns) are nearly identical to lifetimes measured for BODIPY Fl covalently attached to 14-mer (~11.6 ns) and 20-mer (~11.5 ns) dsDNA. This highlights the robustness of the BODIPY fluorophores examined here, suggesting that the overlap integral (a key component of the Förster distance, see below) for this donor-acceptor pair in solution may be similar in the gas phase.
Figure 5.2. Smoothed gas-phase fluorescence emission spectra for (a) 7⁻ (b) 8⁻ and (c) 9⁻ charge states of FRET labeled DA-dsDNA (red) and singly-labeled D-dsDNA (blue) and A-dsDNA (green) controls upon 470 nm excitation. Fluorescence intensities have been normalized to the measured ion current (ICC). Smooths used Savitz-Golay averaging with a 10-point window. The dashed lines show that the measured emission maxima of the donor and acceptor do not change significantly as a function of charge state.
The FRET efficiency of the DA-dsDNA ions decreases as the charge density increases. The measured FRET efficiencies, obtained through comparison with D-dsDNA control, for the [DA-dsDNA]$^7^-$, [DA-dsDNA]$^8^-$, and [DA-dsDNA]$^9^-$ complexes are 0.83, 0.54, and 0.46, respectively. This is somewhat larger than FRET efficiencies obtained through the ratiometric method for the [DA-dsDNA]$^7^-$, [DA-dsDNA]$^8^-$, and [DA-dsDNA]$^9^-$ complexes (measured FRET efficiencies are 0.81, 0.42, and 0.36, respectively). A summary of the measured FRET efficiencies obtained through comparison of donor and acceptor band intensities in the FRET complex (ratiometric method), donor-control measurements, and time-resolved fluorescence (Figure 5.3) are listed in Table 5.1. The decrease in FRET efficiency with increasing charge density indicates that the distance between donor and acceptor increases with charge, consistent with elongation of the 20-mer FRET complex as might be expected from simple electrostatics. Ultimately, FRET efficiencies measured from the steady-state spectra of the dsDNA ions (shown in Figure 5.2) reflect a weighted-average of conformations present. Therefore, in order to delineate the FRET efficiency of distinct conformations, changes in donor fluorescence lifetimes arising from energy transfer to the acceptor was measured.

5.3.3 Time-resolved fluorescence lifetimes for 7- and 8- DA-dsDNA complexes

Time-resolved fluorescence of the donor band from [DA-dsDNA]$^7^-$ (collected from 495–514 nm) and [DA-dsDNA]$^8^-$ (collected from 498–542 nm) complexes upon 470 nm excitation is shown in Figure 5.3. The measured fluorescence decays were well fit by the convolution of a bi-exponential decay with a Gaussian instrument response function. The residuals from the single exponential fit can be seen in Figure D.2. The fitted lifetimes and relative amplitudes were $\tau_1 = 0.97 \pm 0.12 \text{ ns (69 \pm 3\%)}$ and $\tau_2 = 11.6 \pm 1.1 \text{ ns (31 \pm 1\%)}$ for the [DA-dsDNA]$^7^-$ complex and $\tau_1 = 1.5 \pm 0.35 \text{ ns (46 \pm 3\%)}$ and $\tau_2 = 10.2 \pm 0.8 \text{ ns (54 \pm 3\%)}$ for the [DA-dsDNA]$^8^-$.
Table 5.1. Gas-phase FRET efficiencies ($E$) for different charge states of the 20-mer DA-dsDNA complex computed from multiple methods.

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<th>Time-resolved donor fluorescence</th>
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</tr>
<tr>
<td>9-</td>
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</table>

$^a$FRET efficiency obtained through comparison of donor and acceptor bands in [DA-dsDNA] complex. $^b$FRET efficiency measured through decrease in donor intensity in [DA-dsDNA] complex relative to [D-dsDNA] control. $^c$FRET efficiency measured through decrease in donor fluorescence lifetime in [DA-dsDNA] complex relative to [D-dsDNA] control. $^d$Obtained through time-resolved measurements and Förster distance estimate of 73 Å, see Appendix D.
Figure 5.3. Time-resolved fluorescence for the (a) [DA-dsDNA]$^7$- and (b) [DA-dsDNA]$^8$- complexes upon 470 nm excitation. To collect spectrally-resolved fluorescence from the donor in the FRET complex, an appropriate band-pass filter (505BP for panel a and 520BP for panel b, see experimental) was used to reject fluorescence arising from the acceptor molecule.
complex. Unfortunately, the fluorescence signal from the donor band of the 9⁻ complex was too weak to measure time-resolved fluorescence with adequate S/N within a reasonable period of time (cf. ~5 and 6 hours for [DA-dsDNA]⁸⁻ and [DA-dsDNA]⁷⁻ complexes, respectively). Time-resolved data for [DA-dsDNA]⁷⁻ and [DA-dsDNA]⁸⁻ complexes suggests the presence of multiple populations, though time-resolved fluorescence from [D-dsDNA] controls are required to obtain accurate FRET efficiencies for these conformations.

Time-resolved fluorescence of the [D-dsDNA]⁷⁻ and [D-dsDNA]⁸⁻ complexes were well fit by a single exponential decay with lifetimes of 11.5 ± 0.15 ns and 10.9 ± 0.27 ns, respectively. This data is shown in Figure D.3. We note that the uncertainties in lifetime for [DA-dsDNA] and [D-dsDNA] complexes are three times the calculated standard error. We have shown previously that this is a reasonable estimate for the uncertainty of monoexponential decays arising from protonated rhodamine dyes with S/N > 10-fold higher than that measured here.²⁷ However, it is likely that the uncertainty in these multi-exponential decays is larger than the estimate given above due to the low S/N of the data. Thus, the measured lifetimes for the ~10.9 ns and ~11.5 ns components in the [D-dsDNA] controls are likely more similar than presented above.

Time-resolved fluorescence from [DA-dsDNA] complexes indicates at least two populations for the 7⁻ and 8⁻ charge states: a relatively extended conformation and a compact conformation (see Table 5.1 for measured FRET efficiencies). The FRET efficiencies for these populations can be obtained through comparison of time-resolved donor fluorescence in the [DA-dsDNA] complex relative to the [D-dsDNA] control. For example, the fluorescence lifetime of the [D-dsDNA]⁷⁻ control is ~12.6 ns compared to ~1.0 ns for the high-amplitude component in the [DA-dsDNA]⁷⁻ complex, resulting in a FRET efficiency of ~0.92. Similarly, the FRET efficiency of the short component (~1.5 ns) in the [DA-dsDNA]⁸⁻ complex is ~0.86. The high
FRET efficiency for the [DA-dsDNA] complexes indicates a short donor-acceptor distance, consistent with a compact conformation of dsDNA in the gas phase. The high FRET efficiency could potentially be explained through a quenching interaction between the donor and the DNA backbone. However, the large shift in relative amplitude for the short components in the [D-dsDNA]\(^7\) and [DA-dsDNA]\(^7\) complexes (~15% → ~69%) strongly suggests a quenching mechanism that proceeds via FRET. Next, we compare the calculated donor-acceptor distances obtained from time-resolved measurements of [DA-dsDNA] complexes with computed distances for A-dsDNA and B-dsDNA conformations.

To determine the extent that donor-acceptor distances \(r_{DA}\) obtained from time-resolved measurements of the [DA-dsDNA] complexes compare to distances for A- and B-dsDNA conformations, we utilized a back-of-the-envelope Förster distance estimate of 73 Å. Details of this Förster distance estimate can be found in Appendix D. Using the FRET efficiencies of the short components in the [DA-dsDNA]\(^7\) \((E = 0.92)\) and [DA-dsDNA]\(^8\) \((E = 0.86)\) complexes, and the estimated Förster distance (73 Å), \(r_{DA}\) distances are ~49 Å and ~54 Å for the short components of the [DA-dsDNA]\(^7\) and [DA-dsDNA]\(^8\) complexes, respectively. This is smaller than the distance range obtained through the Maestro software package for A-dsDNA (62–82 Å) and B-dsDNA (72–92 Å), suggesting a smaller conformation of dsDNA in the gas phase.

Previous work by Gabelica and coworkers used ion mobility to propose that for 12-mer DNA duplexes in the 5\(^-\) charge state, CCS values were consistent with a compact “zipped” DNA duplex that arises from hydrogen-bonding between adjacent phosphate groups along the DNA backbone.\(^{11}\) The charge densities for the 7\(^-\) and 8\(^-\) charge states studied here are similar to that investigated by Gabelica and coworkers (-0.35/bp and -0.4/bp cf. -0.42/bp), suggesting that a compact zipped structure may also be observed for substantially larger DNA duplexes. Indeed, a recent report by Gabelica and coworkers proposed the “zipped” DNA duplex based on ion
mobility measurements of 18-mer dsDNA ions with differing GC content. However, we note that our Forster distance estimate may be dramatically different in the gas phase, potentially leading to inaccurate donor-acceptor distances. Next, in an effort to identify changes in the conformation of dsDNA under different experiment conditions, we measured the FRET efficiency of the [DA-dsDNA]− complex at different capillary exit voltages.

5.3.4 Variation of source conditions results in changes to FRET efficiency of [DA-dsDNA]−

Figure 5.4 illustrates the dramatic effect that conditions in the ion source region have on the measured efficiencies of energy transfer for the lowest charge state dsDNA examined. Panels a-c compare steady-state fluorescence spectra for the FRET-labeled [DA-dsDNA]− complex and [D-dsDNA]− control at three capillary exit voltages (-85 V, -275 V, -360 V), while panels d-f show mass spectra measured for the DNA G-Quadruplex d(G4T4G4)2 in the 5− charge state under each of these conditions. This DNA G-Quadruplex has been shown through NMR to bind three NH₄⁺ ions in solution and successful MS detection of the complex with three bound ammonium ions has been reported to be a useful indication of gentle MS source conditions. The mass spectrum measured at the lowest capillary exit voltage (-85 V) agrees well with the mass spectrum obtained by Balthasart et al. under their gentlest conditions, and suggests that the [DA-dsDNA]− FRET spectrum also reflects gentle conditions. However, as the capillary exit voltage is decreased to -275 V and -360 V, the G-quadruplex mass spectra show that source conditions become harsher. FRET efficiencies obtained by monitoring the change in fluorescence intensity in the donor band of the FRET labeled [DA-dsDNA]− complex and [D-dsDNA]− control decrease from ~0.92 (-85 V) → ~0.8 (-275 V) → ~0.76 (-360 V). This indicates that as the capillary exit voltage increases, the average donor-acceptor distance
Figure 5.4. Gas-phase fluorescence emission spectra for the [D-dsDNA]$^7^-$ (blue) and [DA-dsDNA]$^7^-$ complexes measured with the capillary exit held at (a) -85 V, (b) -275 V, and (c) -360 V. Fluorescence intensities have been normalized to the measured ion current (ICC) of the [D-dsDNA]$^7^-$ complex in each panel. Panels d-f show the ESI mass spectrum of the DNA G-Quadruplex d(G$_4$T$_4$G$_4$)$_2$ in the 5$^-$ charge state when capillary exit is held at (d) -85 V, (e) -275 V, and (f) -360 V. The number of bound ammonium ions is indicated in red.
increases, consistent with elongation of dsDNA ions. Interestingly, the FRET efficiency of the
[DA-dsDNA]$^-$ complex under the gentlest conditions is identical to the FRET efficiency for the
short component in the [DA-dsDNA]$^-$ complex measured through time-resolved fluorescence
(Table 5.1). This may suggest that a single compact conformation “unravels” at higher capillary
exit voltages to form a more elongated conformation. Unfortunately, the low signal intensity of
the donor band under the gentlest conditions preclude measurement of donor lifetimes in our
current setup.

A trend that can be observed from the data presented in Figure 5.4a-c is that the total
fluorescence intensity from [DA-dsDNA]$^-$ increases with higher capillary exit voltages. For
example, the total integrated fluorescence intensity increases ~2-fold from $\sim 5.0 \times 10^7$ at -85 V to
$\sim 1.0–1.2 \times 10^8$ at -275 V and -360 V. Over this range of capillary exit voltages, the intensity of
the donor control [D-dsDNA]$^-$ was the same at -85 V and -360 V, though we measured a ~30%
relative increase in fluorescence intensity for the [D-dsDNA]$^-$ control at -275 V. Taken together,
these data strongly suggest that the quantum yield of the acceptor increases under harsher source
conditions. Thus, it seems likely that the BODIPY 576 fluorophore used as acceptor is quenched
by interaction with DNA bases at lower capillary exit voltages. We cannot entirely rule out the
lower possibility that some portion of the donor fluorescence is quenched at lower capillary exit
voltages in the [DA-dsDNA]$^-$ complex. However, it seems unlikely that this quenching
mechanism would be present in the [DA-dsDNA]$^-$ complex and not the donor-only control.

5.4 Conclusions

This work describes the use of FRET to probe the conformation of dsDNA ions in the gas
phase. FRET efficiencies obtained from steady-state spectra for dsDNA ions suggest
Coulombically-driven expansion as charge density increases. Furthermore, time-resolved
measurements for the 7\(^-\) and 8\(^-\) charge states indicate the presence of a compact conformation of dsDNA, which may reflect the presence of “zipped” dsDNA ions that have intramolecular hydrogen bonds between adjacent phosphate groups as suggested by Gabelica and coworkers.\(^{11,12}\) Finally, measured FRET efficiencies decrease significantly as the capillary exit voltage increases, consistent with elongation of dsDNA ions under “harsher” mass spectrometry conditions.

The combination of gas-phase FRET with trapping mass spectrometry promises to be a powerful tool for the characterization of gaseous biomolecular ion structure. FRET is capable of Å-level distance determination; however, a better understanding of the Förster distance \(R_0\) and the properties of fluorophores that are used for donor and acceptor labels is required. The apparent quenching of the acceptor under certain conditions highlights that additional development is required to realize the full promise of gas-phase FRET as a structural tool. However, our use of an appropriate donor-only control allowed us to extract FRET efficiencies through alternative means (via decrease in donor fluorescence intensity and lifetime) in the gas phase, rather than the ratiometric approach. Ultimately, these studies aim to characterize the structural transition(s) that occur when dsDNA ions are transferred from solution into the gas phase. This will aid in the use of MS as a discovery tool for drug targeting, while helping workflows that aim to characterize the conformation of biomolecular ions in the gas phase.

5.5 Acknowledgments

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5.6 References


Chapter 6

6.1 Conclusions and Future Considerations

In this dissertation I outlined how electrospray ionization (ESI) and trapping mass spectrometry (MS) can facilitate spectroscopic investigations on mass-selected ions using action spectroscopy and laser-induced fluorescence. Highlights of this work include the first measurement visualizing the cloud of trapped ions in a quadrupole ion trap under normal MS conditions (Chapter 2), the first spectroscopic investigation of the classic DNA intercalator, ethidium bromide in the gas phase (Chapter 3), the first report of fluorescence from two noncovalent dsDNA-dye complexes in the gas phase (Chapter 4), and the use of gas-phase FRET to probe the conformation of dsDNA under a variety of experimental conditions (Chapter 5). A few simple conclusions can be extracted from these investigations. First, visualization of the ion cloud via fluorescence imaging represents a relatively facile method of investigating the effect of various instrument parameters (ICC, $q_z$, and helium pressure) on ion cloud shape. Second, monitoring the spectroscopic properties of ethidium bromide can report on the intrinsic photophysical properties of ethidium without interfering solvent effects. Then in a controlled manner, the photophysical properties of ethidium (and acridine orange) can be compared when unbound versus when complexed to dsDNA in solution and the gas phase. Third, the work presented in Chapter 5 shows that gas-phase FRET can elucidate the effect of various instrument parameters while a non-covalent dsDNA ion travels from solution into the gas phase. While techniques such as ion mobility are capable of probing similar changes in conformation, gas-phase FRET is capable of providing Å-scale distance measurements for gas-phase ions. As this type of resolution is not explicitly available from other gas-phase techniques, FRET serves as a valuable technique for probing the conformation of gaseous ions.
A common theme that presents itself throughout this thesis is that a fluorophores’ spectroscopic properties are heavily modulated by solvent interactions. For example, it is well-known that ethidium exhibits a classic “turn-on” fluorescence response when bound to dsDNA in solution. However, the work presented in Chapter 3 provides evidence that ethidium exhibits this “turn-on” response when solvent interactions are absent. It is proposed that ethidium undergoes an excited-state proton transfer with solvent in solution. Therefore, ethidium cannot proceed with this deactivation pathway in the gas phase due to the absence of solvent, reducing its non-radiative deactivation rate and enhancing its quantum yield. This result is reminiscent of the behaviour of gaseous rhodamine B ions, whose enhanced quantum yield in the gas phase was proposed to be due to the removal of solvent interactions.\(^1\) However, not all fluorophores exhibit enhancements in fluorescence quantum yield upon transfer into the gas phase. Previous work by our group on the photophysical properties of the fluorescein dianion illustrate that despite its high fluorescence quantum yield in solution (~0.92), the dianion does not fluoresce significantly in the gas phase because electron photodetachment becomes the dominant relaxation pathway.\(^2\) This highlights that a fluorophore’s fluorescence properties can be strongly dependent on solvent interactions. Thus, gas-phase studies provide the advantage of investigating a fluorophore’s *intrinsic* photophysical properties, while reporting on the influence of deactivation pathways that arise due to solvent interactions. Gas-phase studies can also help with the rational design of robust fluorophores and useful fluorescence sensors, which will aid in the applicability of fluorescence-based techniques for the characterization of biomolecular ions.

There were a few surprising results observed for fluorophores that bind non-covalently to dsDNA in the gas phase. Chapter 4 proposed that ethidium and acridine orange do not exhibit “turn-on” fluorescence responses when complexed to dsDNA in the gas phase. It was rationalized that due to ethidium’s intrinsic enhancement in quantum yield in the gas phase, the
binding of ethidium to dsDNA results in less of a “turn-on” fluorescence response in the gas phase. Furthermore, our analysis of the [ds-9H+3Et+]⁵⁻ complex indicated that that the gaseous dsDNA complex is less bright than isolated ethidium. For acridine orange, we speculated that due to the high concentrations of AOH⁺ relative to dsDNA employed, a complex where AOH⁺ dimers bind electrostatically to dsDNA is formed in solution.³ Thus, upon transfer of the [ds-6H+AOH⁺]⁵⁻ complex into the gas phase, an electrostatically-bound complex is present, resulting in a decrease in fluorescence intensity for [ds-6H+AOH⁺]⁵⁻ complex relative to AOH⁺ in the gas phase. An interesting future direction for the complex of dsDNA with AOH⁺ is examining the potential “turn-on” fluorescence response for the gaseous [ds-6H+AOH⁺]⁵⁻ complex under solution conditions that favor intercalative binding.

The work presented in Chapter 5 represents an important milestone for the characterization of dsDNA using gas-phase Förster Resonance Energy Transfer (FRET). While changes in integrated donor fluorescence has been used previously to characterize conformational changes of a gaseous 14-mer dsDNA,⁴ this was the first time wavelength- and time-resolved fluorescence were used on gaseous dsDNA to extract FRET efficiencies. The results provide evidence that dsDNA ions with low charge densities (~0.4/bp) can adopt a compact conformation that may arise due to intramolecular hydrogen-bonding between adjacent phosphate groups.⁵ Furthermore, measured FRET efficiencies at varying capillary exit voltages provide evidence that the conformation of dsDNA can change as source conditions within the mass spectrometer become more “harsh”. However, in order to obtain accurate point-to-point distances, more accurate determinations of the Forster distance (R₀) is necessary. Synthesis of a suitable, rigid ladder with conjugated donor and acceptor fluorophores and a well-defined donor-acceptor distance would yield an accurate determination of R₀. One suitable system that employs an array of 6-membered cyclohexene rings with double bonds at the 1’ and 4’ positions could
provide an appropriate rigid ladder. Overall, the development of FRET for the characterization of dsDNA in the gas phase opens up several opportunities for probing gas-phase conformation and stability. One adventurous direction is examining the FRET efficiency with and without non-covalent binders, in an effort to determine the change in conformation that occurs upon non-covalent binding. Other investigations that combine FRET with variable-temperature studies can probe the stability of dsDNA ions in the gas phase compared to solution.

An additional requirement to perform gas-phase FRET that is sometimes overlooked in solution-phase measurements relates to donor and acceptor charge state. In the gas phase, electrostatic attraction between the negatively charged phosphate groups on DNA and the positive charge of a donor and/or acceptor fluorophore may result in deactivation pathways that quench donor and/or acceptor fluorescence. These electrostatic interactions can be less pronounced in solution as counterions and polar solvents are typically effective at screening charged groups. Thus, choosing dyes that are neutral (such as the BODIPY dyes used in Chapter 5) and/or do not interact to a significant extent with negatively charged phosphate groups is critical for the characterization of dsDNA using gas-phase FRET. Fluorophore charge may also play a role in the characterization of other biomolecules in the gas phase.

One design limitation with the current fluorescence setup is the total amount of fluorescence light collected from trapped ions, which is estimated to be ~0.25%. The work presented in Chapter 4 provided evidence that as the mass and charge of a precursor ion increases, fewer trapped ions are able to be accumulated in the QIT due to inherent space-charge effects. Thus, depending on the combination of a precursor ion’s quantum yield, absorption cross-section, and the number of trapped ions, a limit will be reached in the QIT for the maximum biomolecular mass (and minimum Φ) that can be studied. One approach to circumvent this limitation is to increase the total amount of fluorescence light collected. Increasing the
optical sensitivity of our setup would also improve S/N for time-resolved measurements, thereby improving the accuracy of FRET efficiencies and changes in radiative and non-radiative decay constants. One modification that can increase sensitivity is drilling a larger hole in the ring electrode that can accommodate a larger fluorescence collection lens.

The coupling of optical spectroscopy with ion mobility mass spectrometry would provide an extremely powerful readout of gas-phase biomolecular conformation. Introducing an ion mobility region before the trapping region of the QIT would allow biomolecular ions to be separated based on size prior to photodissociation and/or fluorescence characterization. Furthermore, results obtained with ion mobility selection could be compared to fluorescence measurements performed on individual charge states in the trapping region to examine structural transitions in the ms timescale. Another promising methodology is the trapping of biomolecular ions with a well-defined number of water molecules. The possibilities with this methodology are endless. Some examples include examining the number of water molecules required to “reproduce” solution-phase environments, and whether biomolecular ions in a mass spectrometer can revert back to their solution-phase conformations. This will aid in our understanding of the degree to which biomolecular ions resemble their solution-phase conformations in the gas phase, a current and active area of research in the mass spectrometry field.

6.2 References


Appendix A: Supporting information for Chapter 2

Fluorescence Imaging to Visualize the Ion Cloud in a Quadrupole Ion Trap Mass Spectrometer
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Supplementary Information to the manuscript published in Journal of the American Society for Mass Spectrometry

Scheme A.1. The structure of rhodamine 640 (Rh640) used for ion cloud imaging.
Figure A.1. Example of background subtraction for fluorescence images. Images are shown with the same intensity scale. (a) Total fluorescence signal measured with ions present in the trap. (b) Signal measured with no ions in the trap. (c) Background-subtracted image. Background arises both from scattered laser light as well as from ambient light in the laboratory. The circular halo seen in (a) and (b) results from a lens tube in the fluorescence collection line. The vertical lines in (a) and (b) result from the entrance slit of the spectrograph.
Figure A.2. Effect of added helium bath gas (additional pressure specified) on measured ion cloud images (top), radial (middle) and axial (bottom) profiles. The background pressure (without any added helium) is $1 \times 10^{-5}$ mbar.
Figure A.3. Images and corresponding mass spectra at increasing ICC.
Figure A.4. Ion cloud profiles in the (a) radial and (b) axial dimensions measured with increasing $q_z$ value (specified as LMCO% = $q_z/0.908 \times 100\%$) with a normal number of ions in the trap (ICC = $65 \times 10^3$). Data were measured with a different alignment of the fluorescence collection optics than used to collect data in Figures 2 and 3 of the main manuscript, which accounts for the different shape of the observed profiles. The data shown above was measured with a lower laser power than that used to collect the data in Figure 3 of the main manuscript and no photodissociation was observed in the measured mass spectra.

Table A.1. Measured ion cloud sizes, measured relative intensities and calculated fractional overlaps with a 617 μm e⁻² diameter excitation beam.

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<tr>
<td>0.590</td>
<td>204</td>
<td>604</td>
<td>3.35 x 10^8</td>
<td>3.33</td>
<td>1.14</td>
</tr>
<tr>
<td>0.817</td>
<td>191</td>
<td>515</td>
<td>3.70 x 10^8</td>
<td>3.68</td>
<td>1.30</td>
</tr>
</tbody>
</table>
Appendix B: Supplementary information for Chapter 3

The intrinsic photophysics of gaseous ethidium ions – Extra Supplementary Material
Stephen V. Sciuto, Rebecca A. Jockusch

Table B.1. Summary of the experimental parameters used for measurement of action spectrum, fluorescence emission spectrum, and fluorescence lifetime for gaseous ethidium.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Action Spectroscopy</th>
<th>Fluorescence Emission</th>
<th>Fluorescence Lifetime</th>
<th>Relative brightness</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ion</td>
<td>Ethidium</td>
<td>Ethidium</td>
<td>Ethidium</td>
<td>Ethidium</td>
</tr>
<tr>
<td>( \lambda_{\text{ex}} ) (nm)(^a)</td>
<td>400 – 520</td>
<td>490</td>
<td>485</td>
<td>485</td>
</tr>
<tr>
<td>ICC(^b)</td>
<td>( 5.0 \times 10^4 )</td>
<td>(1.5 – 3) ( \times ) ( 10^6 )</td>
<td>( 6.0 \times 10^5 )</td>
<td>( 1.2 \times 10^6 )</td>
</tr>
<tr>
<td>( P_{\text{He, trap}} ) (mbar)(^c)</td>
<td>( 5.7 \times 10^{-4} )</td>
<td>( 2.0 \times 10^{-3} )</td>
<td>( 2.0 \times 10^{-3} )</td>
<td>( 1.7 \times 10^{-3} )</td>
</tr>
<tr>
<td>( t_{\text{ex}} ) (s)(^d)</td>
<td>5.2</td>
<td>5.2</td>
<td>4.15</td>
<td>4.15</td>
</tr>
<tr>
<td>( P ) (mW)(^e)</td>
<td>7</td>
<td>6 – 8</td>
<td>8</td>
<td>3.7</td>
</tr>
<tr>
<td>( t_{\text{tot}} ) (min)(^f)</td>
<td>2</td>
<td>180</td>
<td>320</td>
<td>40</td>
</tr>
<tr>
<td>( q_z )(^g)</td>
<td>0.27</td>
<td>0.59</td>
<td>0.82</td>
<td>0.59</td>
</tr>
<tr>
<td>Filter(^h)</td>
<td>--</td>
<td>500 LP</td>
<td>550 BP</td>
<td>500 LP</td>
</tr>
</tbody>
</table>

Mass Spectrometry abbreviations:

\(^a\) \( \lambda_{\text{ex}} \) – excitation wavelength
\(^b\) ICC – ion charge control
\(^c\) \( P_{\text{He, trap}} \) – estimated helium pressure inside the trap
\(^d\) \( t_{\text{ex}} \) – laser excitation time
\(^e\) \( P \) – laser power
\(^f\) \( t_{\text{tot}} \) – total laser on times
\(^g\) \( q_z \) – trapping parameter
\(^h\) Filter – long/band pass filter used
Table B.2. Comparison of fragment ions generated from monoisotopically isolated ethidium (Et; \( m/z \) 314.168) upon photodissociation and low energy (Sori) CID. The intensities relative to the precursor ion and their likely identities are shown.

<table>
<thead>
<tr>
<th>( m/z ) (PD-MS) from QIT</th>
<th>Intensity relative to precursor ion</th>
<th>( m/z ) (Sori-CID) from FTICR-MS</th>
<th>Intensity relative to precursor ion</th>
<th>Mass loss relative to precursor / Da</th>
<th>Likely identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>299</td>
<td>0.028</td>
<td>299.144</td>
<td>0.013</td>
<td>15.024</td>
<td>([\text{Et} – \text{CH}_3]^+)</td>
</tr>
<tr>
<td>298</td>
<td>0.037</td>
<td>298.136</td>
<td>0.017</td>
<td>16.032</td>
<td>([\text{Et} – \text{CH}_4]^+)</td>
</tr>
<tr>
<td>286</td>
<td>0.55</td>
<td>286.136</td>
<td>0.41</td>
<td>28.032</td>
<td>([\text{Et} – \text{C}_2\text{H}_4]^+)</td>
</tr>
<tr>
<td>285</td>
<td>0.41</td>
<td>285.128</td>
<td>0.26</td>
<td>29.039</td>
<td>([\text{Et} – \text{C}_2\text{H}_5]^+)</td>
</tr>
<tr>
<td>284</td>
<td>0.22</td>
<td>284.120</td>
<td>0.14</td>
<td>30.047</td>
<td>([\text{Et} – \text{C}_2\text{H}_6]^+)</td>
</tr>
<tr>
<td>269</td>
<td>0.07</td>
<td>269.109</td>
<td>0.05</td>
<td>45.056</td>
<td>([\text{Et} – \text{C}_2\text{H}_5\text{NH}_2]^+)</td>
</tr>
</tbody>
</table>

Figure B.1. Sustained off-resonance irradiation collision-induced dissociation (Sori-CID) MS/MS of monoisotopically isolated ethidium (\( m/z \) 314.168) measured in a 7 T Fourier transform ion cyclotron resonance (FTICR) mass spectrometer.
Figure B.2. Overlay of 15 lifetime measurements for gaseous ethidium (red → darker red) and 15 interspersed background scans (blue → darker blue) upon $\lambda_{ex} = 485$ nm.
Estimation of protonated Rh575 quantum yield in the gas phase

This section provides an explanation on how we estimated protonated rhodamine 575’s (Rh575H+) quantum yield in the gas phase using its fluorescence lifetime in solution, and the assumption that the radiative rate depends on the square of the index of refraction.

The quantum yield of gaseous Rh575H+ depends on the radiative and non-radiative deactivation rate constants:

\[ \phi_{\text{Rh575}}^{\text{gas}} = \frac{k_{r}^{\text{gas}}}{k_{r}^{\text{gas}} + \sum k_{\text{nr}}^{\text{gas}}} = k_{r}^{\text{gas}} \tau_{\text{Rh575}} \] (1)

We have previously reported that the fluorescence lifetime (\( \tau \)) of gaseous R575H+ is 5.65 ns [1]. The value of \( k_{r}^{\text{gas}} \) was estimated from its radiative decay ray in ethanol using the Strickler-Berg relationship (Eq. 2):

\[ k_{r}^{\text{gas}} \approx \frac{n_{\text{gas}}^{2}}{n_{\text{ETOH}}^{2}} k_{r}^{\text{ETOH}} \] (2)

where \( n \) is the index of refraction of the medium (taken to be 1.0 for the vacuum and 1.36 for ethanol). The radiative decay rate of Rh575H+ in ethanol can be determined from literature values of its fluorescence lifetime (3.80 ns) and non-radiative decay rate constant (4.7 \( \times \) 10\(^7\) s\(^{-1}\)) [2] using the relationship

\[ k_{r}^{\text{ETOH}} = \frac{1}{\tau_{\text{ETOH}}} - \sum k_{\text{nr}}^{\text{ETOH}} \] (3).

Substituting Eq. 3 into Eq. 2 gives

\[ k_{r}^{\text{gas}} \approx \frac{n_{\text{gas}}^{2}}{n_{\text{ETOH}}^{2}} \left( \frac{1}{\tau_{\text{Rh575}}} - \sum k_{\text{nr}}^{\text{ETOH}} \right) \] (4).

Substituting the expression for \( k_{r}^{\text{gas}} \) into Eq. 1 and using experimental values from \(^{83, 83, 107}\) we estimate

\[ \phi_{\text{Rh575}}^{\text{gas}} \approx \left[ \frac{n_{\text{gas}}^{2}}{n_{\text{ETOH}}^{2}} \left( \frac{1}{\tau_{\text{ETOH}}} - \sum k_{\text{nr}}^{\text{ETOH}} \right) \right] \tau_{\text{Rh575}}^{\text{gas}} \]

\[ \phi_{\text{Rh575}}^{\text{gas}} \approx \left[ \frac{1}{1.36^{2}} \left( \frac{1}{3.8 \times 10^{-9} \text{s}} - 4.7 \times 10^{7} \text{s}^{-1} \right) \right] (5.65 \times 10^{-9} \text{s}) \]  

\[ \phi_{\text{Rh575}}^{\text{gas}} \approx 0.66. \]
### Appendix C: Supplementary information for Chapter 4

**Developing combined optical spectroscopy and mass spectrometry tools for the characterization of double-stranded DNA in the gas phase – Extra Supplementary Information**

Stephen V. Sciuto, Rebecca A. Jockusch

**Table C.1.** A summary of the experimental parameters used for fluorescence emission measurements of the unbound dyes.

<table>
<thead>
<tr>
<th>Unbound dyes</th>
<th>H33258</th>
<th>H33342</th>
<th>H34580</th>
<th>DAPI</th>
<th>Et⁺</th>
<th>AOH⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICC</td>
<td>7.5 × 10⁵</td>
<td>7.0 × 10⁵</td>
<td>7.5 × 10⁵</td>
<td>2.5 × 10⁵</td>
<td>1.5 × 10⁶</td>
<td>6.5 × 10⁵</td>
</tr>
<tr>
<td>Charge</td>
<td>1+</td>
<td>1+</td>
<td>1+</td>
<td>1+</td>
<td>1+</td>
<td>1+</td>
</tr>
<tr>
<td>( P_{He,\text{trap}} ) (mbar)</td>
<td>2 × 10⁻³</td>
<td>2 × 10⁻³</td>
<td>2 × 10⁻³</td>
<td>2 × 10⁻³</td>
<td>2 × 10⁻³</td>
<td>2 × 10⁻³</td>
</tr>
<tr>
<td>( t_{\text{ex}} ) (s)</td>
<td>2.05</td>
<td>2.05</td>
<td>2.05</td>
<td>2.05</td>
<td>5.2</td>
<td>2.05</td>
</tr>
<tr>
<td>( t ) (min)</td>
<td>40</td>
<td>50</td>
<td>40</td>
<td>90</td>
<td>180</td>
<td>20</td>
</tr>
<tr>
<td>( \lambda_{\text{ex}} ) (nm)</td>
<td>400</td>
<td>400</td>
<td>400</td>
<td>400</td>
<td>490</td>
<td>450</td>
</tr>
<tr>
<td>P (mW)</td>
<td>2.0</td>
<td>1.25</td>
<td>0.75</td>
<td>0.3</td>
<td>6-8</td>
<td>3.0</td>
</tr>
<tr>
<td>Filter</td>
<td>410LP</td>
<td>410LP</td>
<td>410LP</td>
<td>410LP</td>
<td>500LP</td>
<td>460LP</td>
</tr>
</tbody>
</table>

**Mass spectrometry abbreviations**

- ICC – ion charge control
- \( P_{He,\text{trap}} \) – helium trap pressure
- \( t_{\text{ex}} \) (s) – laser excitation time
- \( t \) (min) – fluorescence collection time
- \( \lambda_{\text{ex}} \) - excitation wavelength
- P (mW) – laser power

**Molecule abbreviations**

- H33258: Hoechst 33258
- H33342: Hoechst 33342
- H34580: Hoechst 34580
- DAPI: 4,6-diamidino-2-phenylindole
- Et⁺: ethidium
- AOH⁺: acridine orange
### Table C.2
A summary of the experimental parameters used for fluorescence emission and relative brightness measurements for the dsDNA-dye complexes and the rhodamine controls.

**dsDNA-dye complexes**

<table>
<thead>
<tr>
<th>Fluorescence emission – dsDNA-dye complexes</th>
<th>[ds-9H+3Et]^6-</th>
<th>[ss1-5H+Et]^4-</th>
<th>[ds-6H+AOH+]^5-</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICC</td>
<td>3-4×10^5</td>
<td>3-4×10^5</td>
<td>2.5×10^4</td>
</tr>
<tr>
<td>Charge</td>
<td>6-</td>
<td>4-</td>
<td>5-</td>
</tr>
<tr>
<td>$P_{He,,trap}$ (mbar)</td>
<td>1.2-2.0×10^-3</td>
<td>1.0×10^-3</td>
<td>1.0×10^-3</td>
</tr>
<tr>
<td>$t_{ex}$ (s)</td>
<td>4.15 – 5.2</td>
<td>5.2</td>
<td>1.0</td>
</tr>
<tr>
<td>$t$ (min)</td>
<td>560</td>
<td>130</td>
<td>190</td>
</tr>
<tr>
<td>$\lambda_{ex}$ (nm)</td>
<td>520</td>
<td>490</td>
<td>470</td>
</tr>
<tr>
<td>$P$ (mW)</td>
<td>10.5 – 12</td>
<td>15</td>
<td>0.5</td>
</tr>
<tr>
<td>Filter</td>
<td>532LP</td>
<td>500LP</td>
<td>480LP</td>
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</tbody>
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<table>
<thead>
<tr>
<th></th>
<th></th>
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<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>ICC</td>
<td>8.0×10^4</td>
<td>8.0×10^4</td>
<td>2.5×10^4</td>
<td>2.5×10^4</td>
</tr>
<tr>
<td>Charge</td>
<td>6-</td>
<td>1+</td>
<td>5-</td>
<td>1+</td>
</tr>
<tr>
<td>$P_{He,,trap}$ (mbar)</td>
<td>9.9×10^-4</td>
<td>9.9×10^-4</td>
<td>1.2×10^-3</td>
<td>1.2×10^-3</td>
</tr>
<tr>
<td>$t_{ex}$ (s)</td>
<td>5.20</td>
<td>5.20</td>
<td>1.05</td>
<td>1.05</td>
</tr>
<tr>
<td>$t$ (min)</td>
<td>40</td>
<td>20</td>
<td>60</td>
<td>30</td>
</tr>
<tr>
<td>$\lambda_{ex}$ (nm)</td>
<td>520</td>
<td>480</td>
<td>470</td>
<td>470</td>
</tr>
<tr>
<td>$P$ (mW)</td>
<td>20</td>
<td>3</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Filter</td>
<td>532LP</td>
<td>500LP</td>
<td>480LP</td>
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</table>

<table>
<thead>
<tr>
<th></th>
<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>ICC</td>
<td>3.0×10^4</td>
<td>3.0×10^4</td>
<td>2.5×10^4</td>
<td>2.5×10^4</td>
</tr>
<tr>
<td>Charge</td>
<td>6+</td>
<td>1+</td>
<td>5+</td>
<td>1+</td>
</tr>
<tr>
<td>$P_{He,,trap}$ (mbar)</td>
<td>9.9×10^-4</td>
<td>9.9×10^-4</td>
<td>6.0×10^-4</td>
<td>6.0×10^-4</td>
</tr>
<tr>
<td>$t_{ex}$ (s)</td>
<td>1.05</td>
<td>1.05</td>
<td>1.05</td>
<td>1.05</td>
</tr>
<tr>
<td>$t$ (min)</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>$\lambda_{ex}$ (nm)</td>
<td>450</td>
<td>450</td>
<td>450</td>
<td>450</td>
</tr>
<tr>
<td>$P$ (mW)</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Filter</td>
<td>460LP</td>
<td>460LP</td>
<td>460LP</td>
<td>460LP</td>
</tr>
</tbody>
</table>

**Mass spectrometry abbreviations**

- ICC – ion charge control;
- $P_{He,\,trap}$ – helium trap pressure;
- $t_{ex}$ (s) – laser excitation time;
- $t$ (min) – fluorescence collection time;
- $\lambda_{ex}$ – excitation wavelength;
- $P$ (mW) – laser power

**Molecule abbreviations**

- Et: ethidium;
- AOH^+: acridine orange;
- ds: double-stranded DNA (see scheme 2);
- ss1: GCGGGAATTGGGCG;
- Rh123: rhodamine 123;
- RhoGX: rhodamine green-x.
Figure C.1. Circular dichroism spectra for the (a) dsDNA solution and the (b) dsDNA-EB solution.
Figure C.2. Solution-phase fluorescence measured in 25 mM ammonium acetate for 0.1 μM AOH$^+$ (blue) and the ds-AOH$^+$ complex (light blue), made from 1.5 μM dsA and 0.1 μM AOH$^+$. The excitation wavelength was 490 nm, and the excitation and emission slits bandwidths were 5.0 nm.
Figure C.3. Mass spectra of the bimolecular DNA G-quadruplex d(G₄T₄G₄)₂ under (a) harsh (capillary exit = -325 V, Oct 2 DC = -1 V, skimmer = -70 V) and (b) gentle (capillary exit = -275 V, Oct 2 DC = -6 V, skimmer = -50 V) mass spectrometry conditions. The number of coordinated ammonium ions is indicated in red.
Figure C.4. Structures of (a) Rh110 (b) Rh123 and (c) RhoGX.
Relative brightness of Rh123\(^+\), [ss1-\text{RhoGX}+3\text{H}]^{4+}\), and [ds-\text{RhoGX}+5\text{H}]^{6+}\) controls

A previous relative brightness measurement was “chained” together following analysis of the Rh123\(^+\) and [ss1-\text{RhoGX}+3\text{H}]^{4+}\) species on one day followed by [ss1-\text{RhoGX}+3\text{H}]^{4+}\) and [ds-\text{RhoGX}+5\text{H}]^{6+}\) measurements on a separate day. Under these conditions the intensity of Rh123\(^+\) to the [ds-\text{RhoGX}+5\text{H}]^{6+}\) complex was 7%. The results obtained from “chaining” versus performing the brightness measurement on the same day (Figure 2e-f) illustrate the difficulty of relative brightness measurements.

![Fluorescence emission spectra](image)

**Figure C.5.** Savitzky-Golay smoothed fluorescence emission spectra of Rh123\(^+\) (purple), [ss1-\text{RhoGX}+3\text{H}]^{4+}\) (red) and [ds-\text{RhoGX}+5\text{H}]^{6+}\) (blue) under identical mass spectrometry and fluorescence excitation conditions. Equivalent mass spectrometry conditions ensured identical ICC values, helium pressure, ion accumulation time, irradiation time, and \(q\text{z}\) trapping parameter. Equivalent fluorescence excitation conditions used identical excitation wavelength and fluorescence accumulation times. The laser power was 0.5 mW for Rh123 and 2.0 mW for ss- and ds-\text{RhoGX}. \(m/z\) values, charge state, and mass are included in parentheses next to the corresponding complex.
Figure C.6. Normalized fluorescence emission spectra for Rh123 (red), [ds-RhoGX+5H]^{6+} (green), and [ds-RhoGX+4H]^{5+} (blue) complexes. A 460 nm long-pass filter was used for these measurements, clipping part of Rh123’s fluorescence emission spectrum.
Figure C.7. Photodissociation of (a) H33342, (b) H34580, (c) H33258, (d) DAPI, (e) ethidium, (f) AOH, and (g) SG in complex with dsDNA as a function of laser power. Excitation wavelengths for complexes of dsDNA with Hoechst dyes/DAPI were 385 nm and 480 nm for complexes of dsDNA with acridine orange, SYBR Green I, and ethidium. The inset of Fig. C.6d displays ln(PD Yield) versus power to illustrate linearity. Details about fitting equations, fitted parameters, and observed fragment ions are listed for each complex.
Figure C.8. Photodissociation MS/MS for complexes of dsDNA with (a) H33342, (b) H34580, (c) H33258, (d) DAPI, (e) ethidium, (f) AO, (g) SG. The star indicates the precursor ion.
Discussion of photodissociation power dependence (Figure C.7), MS/MS spectra (Figure C.8)

Figure C.7 shows the photodissociation power dependence for complexes of dsDNA with Hoechst dyes, DAPI, ethidium, acridine orange, and SG. Details of fitting and fitted parameters can be found below. The inset of Figure C.6d indicates that the \([\text{ds-7H}+\text{DAPI}]^6\) complex proceeds via single photon dissociation. In contrast, the \([\text{ds-10H}+4\text{SG}]^6\) (Fig. C.7g) and \([\text{ds-9H}+3\text{Et}]^6\) (Fig. C.7e) complexes require fitting of higher-order parameters, consistent with multiple photon dissociation that may accompany the dissociation of these complexes via strand separation. We note that at certain laser powers, \([\text{ds-10H}+4\text{SG}]^6\) and \([\text{ds-9H}+3\text{Et}]^6\) complexes show an increase in precursor ion intensity, suggesting an increase in complex abundance upon irradiation. This phenomenon was unique only to these complexes, highly reproducible, and was not observed for other dsDNA-dye complexes or with isolated ethidium ions \((m/z 314)\) under similar conditions. Furthermore, we ruled out the possibility that these complexes were forming dimers in the gas phase that broke apart upon laser irradiation, by re-isolating these complexes at a subsequent MS\(^\text{n}\) step and monitoring the precursor ion intensity upon laser irradiation. Under these conditions, the precursor ion intensity for these two complexes continued to increase at low laser powers. For these reasons, we believe this behaviour may be associated with these dsDNA-dye complexes tendency to dissociate via strand separation. The photodissociation power dependence of \([\text{ds-10H}+4\text{SG}]^6\) and \([\text{ds-9H}+3\text{Et}]^6\) complexes highlights that dissociation of the \([\text{ds-10H}+4\text{SG}]^6\) complex occurs much easier than the \([\text{ds-9H}+3\text{Et}]^6\) complex. This may highlight why fluorescence was detected for the \([\text{ds-9H}+3\text{Et}]^6\) complex but not for the \([\text{ds-10H}+4\text{SG}]^6\) complex – dissociation competes with fluorescence for the \([\text{ds-10H}+4\text{SG}]^6\) complex at laser powers typically used for fluorescence.

The fits to the data for complexes of the Hoechst dyes and acridine orange with dsDNA (Figs. C.7a-c, f) require the inclusion of two components with different power dependencies.
This is particularly interesting as the only requirement for photodissociation is that a complex possesses sufficient energy to surpass a dissociation threshold. As such, photodissociation tends not to be highly sensitive to conformation. However, the photodissociation power dependence plots of the [ds-6H+AOH+]\(^5\) complex provides evidence of two populations, which may indicate AOH\(^+\) binding to dsDNA via electrostatic attachment and intercalation. Power dependence plots for Hoechst dyes also support the presence of two dye populations when binding to dsDNA. However, preliminary work performed by our group on the intrinsic photophysics of isolated H33258\(^+\) ions (m/z 427) show that H33258\(^+\) has two components in its photodissociation power dependence (data not shown). Thus, the presence of two components in power dependence plots for complexes of Hoechst dyes with dsDNA may be due to the intrinsic photophysics of the Hoechst dyes, rather than two distinct binding modes.
Description of fitting equations for photodissociation power dependence plots

This section provides a description of the fitting equations used to fit the data points in Figure C.5. In general, the photodissociation yield (PD Yield) depends on the precursor ion intensity during laser on and laser off periods:

\[
PD \text{ Yield} = 1 - \frac{I_{\text{laser on}}}{I_{\text{laser off}}} \]

The PD Yield is expressed as an \(n\)th order polynomial with respect to laser power (\(P\)):

\[
PD \text{ Yield} = y_1 e^{(-aP - bP^2 - cP^3)} \]

where \(a\), \(b\), and \(c\) are fitting parameters for a 1\(st\), 2\(nd\), and 3\(rd\) degree polynomial, respectively. For photodissociation power dependence curves where a single component is apparent, the natural logarithm can be applied to each side of Equation 2 to generate:

\[
\ln(PD \text{ Yield}) = -aP - bP^2 - cP^3
\]

Alternatively, for photodissociation power dependence plots where two exponential components are apparent, the PD Yield must be expressed as a sum of \(n\)th order polynomials with respect to power:

\[
PD \text{ Yield} = y_1 e^{(-aP - bP^2 - cP^3)} + y_2 e^{(-dP - eP^2 - fP^3)}
\]

<table>
<thead>
<tr>
<th>Complex</th>
<th>(y_1)</th>
<th>(a)</th>
<th>(b)</th>
<th>(c)</th>
<th>(y_2)</th>
<th>(d)</th>
<th>(e)</th>
<th>(f)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ds-H33342</td>
<td>0.46</td>
<td>6.6</td>
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Appendix D: Supplementary information for Chapter 5

Fürster Resonance Energy Transfer measurements probe the conformation adopted by double-stranded DNA ions in the gas phase – Extra Supplementary Information

Stephen V. Sciuto, Rebecca A. Jockusch

**Table D.1.** Experimental parameters employed for [DA-dsDNA]$^7$, [DA-dsDNA]$^8$, and [DA-dsDNA]$^9$ complexes

### Steady-state measurements

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<thead>
<tr>
<th></th>
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<th>[DA-dsDNA]$^9$</th>
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### Time-resolved measurements

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### Capillary exit measurements

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<td>t (min)</td>
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Figure D.1. Integrated fluorescence intensity as a function of wavelength for the complementary ssDNA sequences labeled with BODIPY Fl (blue squares) and BODIPY 576/589 (green circles) in the 4⁺ charge state. The ratio between BODIPY Fl and BODIPY 576/589 fluorescence is indicated in pink.
Figure D.2. The residuals from fitted donor lifetimes for double (dots with red line) and single (dots with blue line) exponential fits for (a) [DA-dsDNA]$^7$ and (b) [DA-dsDNA]$^8$ complexes. For the [DA-dsDNA]$^7$ complex, fitted lifetimes and amplitudes for double-exponential fits are $\tau_1 = 0.97 \pm 0.12 \text{ ns } (A_1 = 0.69 \pm 0.03)$ and $\tau_2 = 11.6 \pm 1.1 \ (A_2 = 0.31 \pm 0.01)$ compared to $\tau = 4.84 \pm 0.26 \text{ ns}$ for the single-exponential fit. For the [DA-dsDNA]$^8$ complex, fitted lifetimes and amplitudes for double-exponential fits are $\tau_1 = 1.5 \pm 0.35 \text{ ns } (A_1 = 0.46 \pm 0.03)$ and $\tau_2 = 10.2 \pm 0.8 \ (A_2 = 0.54 \pm 0.03)$ compared to $\tau = 7.01 \pm 0.28 \text{ ns}$ for the single-exponential fit.
**Figure D.3.** Time-resolved fluorescence for (a) [D-dsDNA]$^7$ and (b) [D-dsDNA]$^8$ complexes. The residuals from double (dots with red line) and single (dots with blue line) exponential fits are shown below the time-resolved curve. For the [D-dsDNA]$^7$ complex, fitted lifetimes and amplitudes for double-exponential fits are $\tau_1 = 2.73 \pm 1.05 \text{ ns} \ (A_1 = 0.15 \pm 0.01)$ and $\tau_2 = 12.6 \pm 0.44 \ (A_2 = 0.85 \pm 0.01)$ compared to $\tau = 11.5 \pm 0.15 \text{ ns}$ for the single-exponential fit. For the [D-dsDNA]$^8$ complex, fitted lifetimes and amplitudes for double-exponential fits are $\tau_1 = 3.9 \pm 3 \text{ ns} \ (A_1 = 0.14 \pm 0.07)$ and $\tau_2 = 11.9 \pm 1.3 \ (A_2 = 0.86 \pm 0.01)$ compared to $\tau = 10.9 \pm 0.27 \text{ ns}$ for the single-exponential fit.
Estimation of $R_{0,\text{gas}}$ for the FRET dye pair (BODIPY Fl and BODIPY 576/589)

The Förster distance ($R_0$) of a general dye pair is the critical parameter that dictates the donor-acceptor distance, and depends on the spectroscopic properties of the two dyes and their chemical environment. $R_0$ can be expressed in Å through equation 1:

$$R_0 = 0.211 \left( \frac{\kappa^2 \Phi_D J}{n^4} \right)^{1/6}$$

where $\kappa^2$ describes the orientation of donor and acceptor dipoles (assumed to be 2/3, see below), $\Phi_D$ is the quantum yield of the donor, $J$ is the spectral overlap integral between donor emission and acceptor absorption, and $n$ is the index of refraction of the dye environment. Unfortunately, several of these parameters ($\kappa^2$, $\Phi_D$, and $J$) are not directly measurable in the gas phase. Thus, the following section will outline the assumptions that we have made to estimate $R_0$.

While $\kappa^2$, $\Phi_D$, and $J$ are not directly measurable in the gas phase, based on the spectroscopic properties of BODIPY Fl and BODIPY 576/589 we can assume that $\Phi_D$ and $J$ are roughly similar in the gas phase. For example, the fluorescence lifetime of BODIPY Fl in MeOH has been reported as ~5.9 ns.\textsuperscript{1} The relationship between the radiative rate ($k_r$) and the square of the index of refraction of the solvent ($n^2$) can be expressed as $k_r \propto n^2$. Thus, upon transfer of BODIPY Fl from MeOH ($n = 1.33$) to the gas phase ($n = 1.0$), if the non-radiative deactivation rate constant ($k_{nr}$) does not change significantly we should observe a doubling of BODIPY Fl’s lifetime. Indeed, time-resolved fluorescence of [D-dsDNA] controls fall within this range (~10.9–12.6 ns). Thus, since it appears that the radiative and non-radiative rates of BODIPY Fl are not dramatically different in the gas phase, $\Phi_D$ is likely similar in the gas phase. Likewise, the small shifts in gaseous emission maxima for BODIPY Fl and BODIPY 576/589 relative to H$_2$O (200 cm$^{-1}$ and 300 cm$^{-1}$, respectively) suggest that these fluorophores’
fluorescence properties show minimal sensitivity to their environment. This suggests that $J$ for this donor-acceptor pair is similar in the gas phase.

The largest uncertainty in our estimate of $R_0$ arises from the dipole orientation factor, $\kappa^2$, which is typically assumed to be $\sim 2/3$ when both donor and acceptor dipoles are freely rotating. We believe this is a reasonable assumption for our gas-phase studies as both BODIPY fluorophores are electronically neutral and are separated from the oligonucleotide via $C_3$ spacers, minimizing the opportunity for the BODIPY fluorophores to interact with their respective DNA sequences. However, it is possible that the BODIPY dyes interact with DNA, resulting in a $\kappa^2$ value that is different than $2/3$ (possible range of $\kappa^2$ lies between 0–4).

As there is no reported $R_{0,\text{soln}}$ between BODIPY Fl and BODIPY 576/589, we chose to use the reported $R_{0,\text{soln}}$ between BODIPY Fl and BODIPY 581/591 ($R_{0,\text{soln}} = 60 \, \text{Å}$) in Tris buffer ($n = 1.35$). Using the assumptions described above for $\kappa^2$, $\Phi_D$, and $J$, we assume that the gaseous Förster distance can be explained predominantly through the change in refractive index. Thus, as $R_0 \propto n^{2/3}$, we estimate that the Förster distance is $\sim 22\%$ higher in the gas phase compared to solution, or $\sim 73 \, \text{Å}$.

References