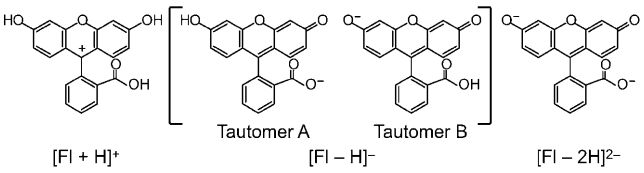
Spectroscopy

DOI: 10.1002/anie.201004366

On the Intrinsic Photophysics of Fluorescein**

*Peter D. McQueen, Sandeep Sagoo, Huihui Yao, and Rebecca A. Jockusch**

Fluorescein is used extensively for visualization and diagnostics in biological and medical applications. The popularity of fluorescein, which has been studied for over a century,[1] arises from its bright fluorescence and its ease of conjugation to biomolecules.[2] Fluorescein exists in up to seven different pH-dependent states:[3a] three neutral forms and four charged forms (Scheme 1). These forms each have different excitation



Scheme 1. Fluorescein cation (left), monoanion (center), and dianion (right).

and fluorescence emission properties, some of which are strongly solvent-dependent. To better understand the effect of the microenvironment on the spectroscopic properties of fluorescein, knowledge of its intrinsic (solvent-free) properties is crucial. Herein, we use the isolation capabilities of trapping mass spectrometry to individually probe the spectroscopy of the three fluorescein charge states. An unexpected result is that the brightest form of fluorescein in solution, the dianion, does not fluoresce significantly in the gas phase.

The absorbance and the quantum yield of fluorescein in solution vary significantly with the protonation state. The fluorescein dianion ($[Fl-2H]^{2-}$; Scheme 1) has the highest molar absorptivity (ca. $10^5\,M^{-1}cm^{-1}$ at $\lambda_{max}^{ab} = 490$ nm in water) and fluorescence quantum yield (0.92).[3] The monoanion ($[Fl-H]^-$) is also fluorescent, but has a lower absorptivity (two maxima of ca. $30\,000\,M^{-1}cm^{-1}$ at $\lambda_{max}^{ab} = 450$ and 470 nm in water) and fluorescence quantum yield (0.37).[3] Fluorescence upon excitation of cationic (and neutral) fluorescein is observed; however, this fluorescence is believed to occur through deprotonation in the excited state, thus forming the fluorescent excited monoanionic species. The effective fluorescence quantum yield for the fluorescein cation is 0.18, which reflects both the efficiency of the excited state proton transfer reactions and the quantum yield of the monoanion.[3a]

The fluorescein dianion exhibits significant solvatochromism.[4] This observation was first reported by Martin,[4a] who showed that as the solvent was changed from H_2O to dimethyl sulfoxide (DMSO), the absorption maximum for the dianion shifted from 490 nm to 520 nm. The observed solvatochromism was attributed to the hydrogen bonds between the fluorescein dianion and the solvent being stronger in the ground state than in the excited state, thus increasing the gap between the S_0 and S_1 electronic energy levels as the hydrogen-bonding ability of the solvent increases.

Whereas there is a breadth of information on the behavior of fluorescein in solution, studies of the properties of fluorescein in the gas phase have been limited to computational work.[5] Jang et al.[5b] have performed electronic structure theory calculations for nine different fluorescein tautomers in vacuo and in DMSO and water. Computations at the B3LYP/6-31++G** level of theory with a Poisson–Boltzmann continuous solvation approach showed that the most stable conformers of cationic and dianionic fluorescein in solution are similar to the most stable gas-phase forms. However, depending on the environment of the fluorophore, different forms of the monoanion are stabilized. The most stable form in the gas phase was found to be tautomer B (Scheme 1), which is deprotonated on the xanthene moiety. Deprotonation at the carboxylic acid group (Scheme 1, tautomer A) was 21 kJ mol^{-1} less favorable. Tautomers A and B are isoenergetic in DMSO, while in water tautomer A was favored over tautomer B by 1 kJ mol^{-1}. Raman and FTIR experiments are indicative of the predominance of tautomer A in aqueous solution.[6] Very recently, evidence has also been found for the presence of a small amount of tautomer B in non-hydrogen-bonding solvents such as DMSO.[7] Characterization of the monoanion in solution is challenging because of the presence of multiple groups with similar pK_a values, which makes it impossible to isolate the monoanionic form alone in solution.

The optical properties of the monoanionic, dianionic, and cationic forms of gas-phase fluorescein, which are formed by electrospray ionization, have been individually probed by using a quadrupole ion trap (QIT) mass spectrometer that has been modified to enable gas-phase spectroscopic studies.[8] Figure 1 shows the photodissociation (PD) mass spectra for the fluorescein cation ($[Fl+H]^+$), monoanion ($[Fl-H]^-$), and dianion ($[Fl-2H]^{2-}$). The PD mass spectrum of the fluorescein cation (m/z 333, Figure 1a) is the most complex as the spectrum shows numerous product ions. The most abundant product ion, m/z 287, corresponds to a loss of 46 Da from the precursor ion. The product ion is likely formed by the loss of

[*] P. D. McQueen, S. Sagoo, H. Yao, Prof. R. A. Jockusch
Department of Chemistry, University of Toronto
80 St. George Street, Toronto, ON M5S 3H6 (Canada)
Fax: (+1) 416-978-8775
E-mail: Rebecca.jockusch@utoronto.ca
Homepage: http://www.chem.utoronto.ca/staff/JOCKUSCH

[**] Funding for this project was provided by the Natural Sciences and Engineering Research Council of Canada (NSERC), the Canada Research Chairs program, Canada Foundation for Innovation (CFI), and the Government of Ontario.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201004366.

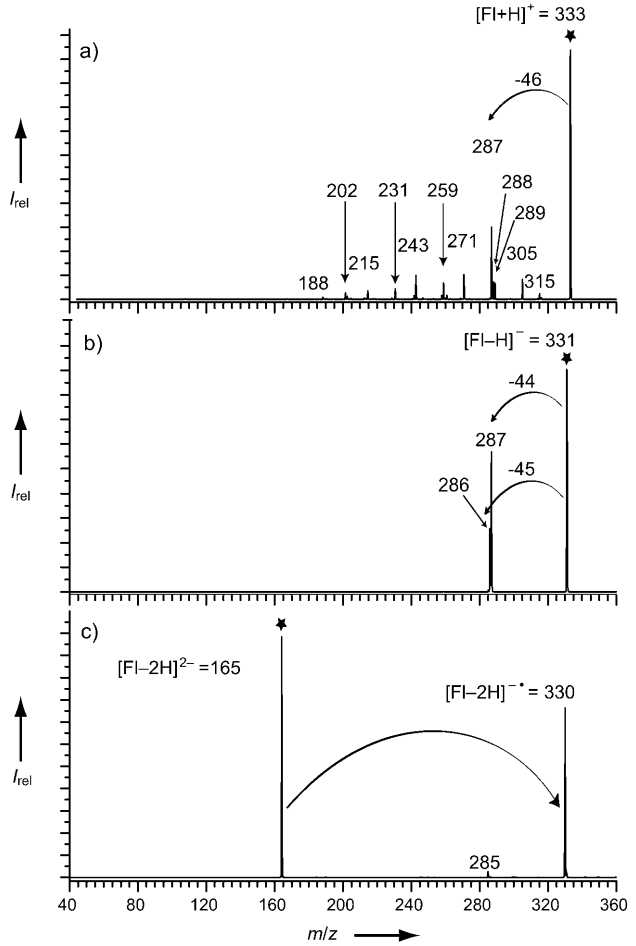


Figure 1. Photodissociation mass spectra of monoisotopically isolated fluorescein a) cation, b) monoanion, and c) dianion. Asterisks mark the parent ion. I_{rel} stands for relative intensity. Excitation wavelength (λ_{ex}), power (P), and irradiation times (t_{ex}) used were: a) $\lambda_{ex}=430$ nm, $P=20$ mW, $t_{ex}=700$ ms; b) $\lambda_{ex}=520$ nm, $P=2$ mW, 500 ms; c) $\lambda_{ex}=500$ nm, $P=0.3$ mW, $t_{ex}=100$ ms.

formic acid from the pendant benzoic acid moiety. Since the xanthene ring system is the chromophore that is excited upon irradiation by visible light, the loss of formic acid indicates that the excitation energy has been redistributed through the molecule prior to dissociation. The PD mass spectrum for the fluorescein monoanion (*m*/*z* 331, Figure 1b) is much simpler than that of the cation. The major fragment is at *m*/*z* 287, which corresponds to a loss of 44 Da. This fragment is likely to arise from loss of carbon dioxide from the benzoic acid moiety. Similar tandem mass spectra that show the same product ions from the fluorescein cation and the monoanion, were generated by using multiple collisionally activated dissociation (CAD) in the QIT (see the Supporting Information) and infrared multiple photon dissociation (IRMPD) using a CO_2 laser (data not shown).

The primary photodissociation product observed for the dianion (*m*/*z* 165) is a singly-charged ion at *m*/*z* 330 (Figure 1c). This product corresponds to a radical monoanion formed by the loss of an electron from the dianion [Equation (1)]:

$$[\mathrm{Fl}-2\,\mathrm{H}]^{2-} + h\nu \rightarrow [\mathrm{Fl}-2\,\mathrm{H}]^{-} + \mathrm{e}^{-} \quad (1)$$

The facile removal of an electron from the fluorescein dianion is not surprising because the small size of the dianion makes it less likely to retain two negative charges. However, this electron-detachment pathway is unique to the photodissociation by visible light; irradiation with a CO_2 laser and CAD do not result in the product at *m*/*z* 330. Instead, a singly charged product is observed at *m*/*z* 285 (see the Supporting Information); this fragment is a minor product ion at all wavelengths of the visible PD mass spectrum (Figure 1c).

Visible action spectra for the fluorescein cation, monoanion, and dianion in the gas phase are shown as solid squares in Figure 2. The action spectra for the cation and the monoanion were obtained by monitoring the yield of all the fragment ions as a function of the irradiation wavelength. Similarly, the action spectrum for the dianion corresponds to the formation of the *m*/*z* 330 product ion, that is, to the

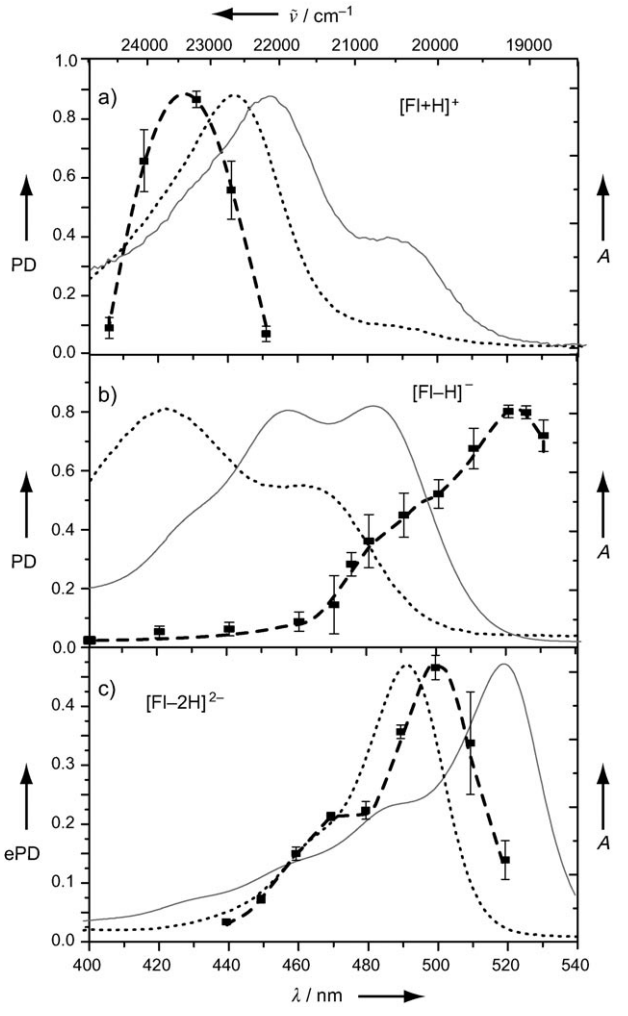


Figure 2. Visible action spectra(■---photodissociation (PD) or electron photodetachment (ePD) yield as a function of excitation wavelength) of gaseous fluorescein a) cation, b) monoanion, and c) dianion. The thick dashed lines are a guide to the eye. Also shown are absorption spectra of 1.5 μM fluorescein measured in water (-----) and DMSO (——) at pH a) 1.95, b) 5.67, and c) 11.65. The pH value of the solution was adjusted by using acetic acid or NaOH.

detachment of an electron. The absorption spectra of fluorescein in water and in solutions in DMSO at different pH values are also shown in Figure 2.

The action spectra for the fluorescein cation and dianion in the gas phase are similar to the absorption spectra obtained in solution (Figure 2). The absorption maximum of the cation in DMSO is located at 450 nm, while the absorption maximum in the gas phase is slightly higher in energy (420–430 nm). The absorption maximum of the dianion in the gas phase also lies at higher energy (500 nm) compared to DMSO (520 nm). The action spectrum of the dianion shows a shoulder at 1300 cm^{-1}, which is comparable to that observed in the solution absorption spectra.

The action spectrum of the monoanion in the gas phase (Figure 2b) is quite different from its spectrum in solution. In the latter case, multiple absorption maxima are observed at approximately 425 nm, 460 nm, and 485 nm in DMSO. The action spectrum of the monoanion in the gas phase shows a peak at 520 nm, which is significantly lower in energy than the maxima in DMSO. This result is contrary to the shifts observed for the cation and the dianion, and strongly suggests the presence of different forms of the monoanion in solution and in the gas phase. Unfortunately, the gas-phase action spectrum does not extend beyond 530 nm because of a significant drop-off in laser power at longer wavelengths.

Several pieces of evidence led us to conclude that the fluorescein monoanion exists as tautomer B in the gas phase. Mchedlov-Petrossyan and co-workers have recently reported an absorption spectrum of tautomer B in DMSO. This spectrum was obtained by subtracting contributions from other species present in solution. The resulting absorption spectrum looks remarkably similar to the action spectrum of the monoanion in the gas phase; however, the extracted spectrum is shifted towards lower energy with its main feature at 525 nm and a shoulder at approximately 490 nm.[7] The presence of a small peak at 520 nm in the extracted absorption spectrum in DMSO recorded previously by Klonis and Sawyer should also be noted.[4b] Comparison with fluorescein derivatives such as Rose Bengal A, for which the analogue of tautomer B predominates, also suggests that tautomer B will absorb at higher wavelengths than the dianion;[9] this is consistent with the gas-phase action spectra shown here. Further support for the presence of tautomer B in the gas phase comes from electronic structure theory calculations, which predict substantial stabilization of tautomer B upon removal from a hydrogen-bonding environment.[5b]

Significantly less energy is required to remove an electron from the fluorescein dianion than to fragment the monoanion or the cation. The rate of photoinduced electron detachment from $[Fl-2H]^{2-}$ varies linearly with laser power with zero intercept (see the Supporting Information), thus indicating that the removal of an electron results from the absorption of a single photon. In contrast, power dependence and kinetics measurements for the fluorescein monoanion[10] and cation photodissociation (see the Supporting Information) are more complex, hence indicating that these charge states do not undergo simple, single-photon dissociation. The vertical electron-detachment energy for the dianion in the gas phase, calculated at the B3LYP/6-311++G(2d,2p) level of theory, is 72 kJ mol^{-1}, which is significantly lower than the energy available from a 520 nm photon (230 kJ mol^{-1}). The cross-section for electron detachment from the fluorescein dianion is found to be 6×10^{-18} cm^2 at 500 nm; this value is similar to that found for a model chromophore of green fluorescent protein,[11] but about 50 times less than the absorption cross-section in solution.

No fluorescence was detected from the gas-phase fluorescein dianion ($\lambda_{ex}=500$ nm) or the cation ($\lambda_{ex}=430$ nm). For the monoanion, weak fluorescence is observed.[10] Dispersed fluorescence spectra with a signal-to-noise ratio of approximately 25 at the absorption maximum are obtained for gaseous rhodamine dyes under similar experimental conditions.[8] Thus, the dominant pathway for energy loss in gas-phase fluorescein is not fluorescence. This result is not surprising for the cation, which is believed to undergo proton transfer in the excited state. The weak fluorescence observed for the monoanion is the subject of another report.[10] The lack of observed fluorescence from the dianion is noteworthy because of its high (ca. 0.9) quantum yield in solution. It is evident that electron photodetachment out-competes fluorescence from the dianionic species in the gas phase. This observation suggests that the time frame for electron detachment is much shorter than the solution fluorescence lifetime, that is, the electron detachment probably occurs in less than a nanosecond.

In summary, we have measured action spectra for gaseous fluorescein in its cationic, anionic, and dianionic forms. The spectra obtained for the cation and the dianion are similar to those of the solution-phase ions. In contrast, the monoanionic form of fluorescein revealed a large shift in its absorption maximum, thus suggesting the presence of different forms of fluorescein in solution and in the gas phase. The fluorescein dianion, which is favored as a quantum yield standard in solution, does not fluoresce significantly in the gas phase. Instead, the dominant deactivation pathway for the dianion in the gas phase is the photodetachment of an electron.

Experimental Section

Fluorescein (Sigma–Aldrich, Oakville, Ontario, Canada) was dissolved in methanol/water (70:30 for cation and monoanion, 30:70 for dianion) to a concentration of 1.5 μM for electrospray ionization (ESI) measurements. The desired charge state of fluorescein was mass selected and stored in a quadrupole ion trap mass spectrometer (Bruker Esquire 3000+, Bruker Daltonik, Germany), which is modified for spectroscopy.[8] Gas-phase fluorescein ions were excited by using the frequency-doubled output of a Titanium-Sapphire laser (Tsunami, Spectra Physics, California, USA), which operates at 80 MHz, with a 130 fs pulse duration. The power of the excitation irradiation was adjusted to the desired value by using a variable neutral density filter. The passage of the excitation irradiation into the ion trap was controlled by a shutter, which was triggered from the Esquire Control software (Bruker Daltonik) after ion isolation. During irradiation, fluorescence was collected through a hole in the ring electrode and sent to a spectrograph and electron-multiplying charge-coupled device for detection. After the irradiation period, product ions and remaining parent ions were scanned out of the trapping region to measure a mass spectrum. Photodissociation yields for the action spectra were calculated as the sum of fragment-ion

intensities, and were normalized to the total (fragments + precursor) ion intensity. For kinetics and power-dependence measurements, photodissociation yields were computed by monitoring the disappearance of the precursor ion by bracketing photodissociation experiments (laser on) with control experiments in which the shutter remains closed (laser off), as described previously.[11a]

Received: July 16, 2010
Published online: October 22, 2010

Keywords: fluorescence · laser spectroscopy · mass spectrometry · photophysics · solvent effects

[1] A. Baeyer, *Ber. Dtsch. Chem. Ges.* **1871**, *4*, 555 – 558.
[2] R. P. Haugland, *The Handbook—A Guide to Fluorescent Probes and Labeling Technologies*, 10th ed., Invitrogen, San Diego, **2005**.
[3] a) R. Sjöback, J. Nygren, M. Kubista, *Spectrochim. Acta Part A* **1995**, *51*, L7 – L21; b) N. Klonis, W. H. Sawyer, *J. Fluoresc.* **1996**, *6*, 147 – 157; c) D. Magde, R. Wong, P. G. Seybold, *Photochem. Photobiol.* **2002**, *75*, 327 – 334.
[4] a) M. M. Martin, *Chem. Phys. Lett.* **1975**, *35*, 105 – 111; b) N. Klonis, W. H. Sawyer, *Photochem. Photobiol.* **2000**, *72*, 179 – 185; c) N. Klonis, A. H. A. Clayton, E. W. Voss, W. H. Sawyer, *Photochem. Photobiol.* **1998**, *67*, 500 – 510.
[5] a) A. Tamulis, J. Tamuliene, M. L. Balevicius, Z. Rinkevicius, V. Tamulis, *Struct. Chem.* **2003**, *14*, 643 – 648; b) Y. H. Jang, S. G. Hwang, D. S. Chung, *Chem. Lett.* **2001**, 1316 – 1317; c) B. Acemioglu, M. Arik, H. Efeoglu, Y. Onganer, *J. Molec. Struct. (Theochem)* **2001**, *548*, 165 – 171.
[6] L. L. Wang, A. Roitberg, C. Meuse, A. K. Gaigalas, *Spectrochim. Acta Part A* **2001**, *57*, 1781 – 1791.
[7] N. O. Mchedlov-Petrossyan, N. A. Vodolazkaya, N. V. Salamanova, A. D. Roshal, D. Y. Filatov, *Chem. Lett.* **2010**, *39*, 30 – 31.
[8] Q. Bian, M. W. Forbes, F. O. Talbot, R. A. Jockusch, *Phys. Chem. Chem. Phys.* **2010**, *12*, 2590 – 2598.
[9] a) N. O. Mchedlov-Petrossyan, N. A. Vodolazkaya, Y. N. Surov, D. V. Samoylov, *Spectrochim. Acta Part A* **2005**, *61*, 2747 – 2760; b) N. O. Mchedlov-Petrossyan, V. I. Kukhtik, V. D. Bezugliy, *J. Phys. Org. Chem.* **2003**, *16*, 380 – 397.
[10] H. Yao, S. Sagoo, R. A. Jockusch, **2010**, unpublished results.
[11] a) M. W. Forbes, R. A. Jockusch, *J. Am. Chem. Soc.* **2009**, *131*, 17038 – 17039; b) M. W. Forbes, A. M. Nagy, R. A. Jockusch, unpublished results.

