Techniques for Neutral Gas Phase Spectroscopy
Exemplar: The Green Fluorescent Protein Chromophore

Contact *j.greenwood@qub.ac.uk*

Jason B. Greenwood, Jordan Miles, Simone De Camillis

Centre for Plasma Physics, School of Maths and Physics
Queen's University Belfast, Belfast, BT7 1NN, U.K.

Helen H. Fielding, Helen C. Hailes, Lijian Zhang

Department of Chemistry, University College London
20 Gordon Street, London WC1H 0AJ, U.K

Introduction: Production of Molecules in the Gas Phase

The study of biological building blocks in the gas phase provides essential understanding of the intrinsic molecular properties which is valuable for complementary studies in solution or *in vitro*. By studying isolated molecules the influence of the environment can also be removed making theoretical studies more tractable. However, liberation into the gas phase necessarily involves input of energy to the molecules which may result in decomposition before they can be studied. Of the gas-phase techniques which have been developed, the most widely used are those of electrospray and matrix assisted laser desorption and ionization which can be directly coupled to mass spectrometers since some of the molecules become charged through protonation or de-protonation[1]. Electrospray has also been used by a number of groups to confine charged molecules in traps or storage rings for "action spectroscopy" experiments where energy deposition via photon absorption or collisions is studied from the resulting fragmentation[2]. However, to study neutral molecules different approaches are needed such as thermal evaporation or laser desorption using a nanosecond laser to rapidly heat the underlying surface[3,4].

An alternative technique is laser induced acoustic desorption (LIAD) in which a sample is deposited on a thin foil but in this case the desorption laser irradiates the foil on the opposite side to that on which the sample resides[5,6]. There is some debate about the desorption mechanism which could be due to a combination of an intense acoustic wave generated by the rapid heating of the foil, thermal expansion, and the transient temperature increase, but the result is a clean plume of neutral molecules[7]. To date this technique has been employed with low repetition rate ns lasers but development of a kHz LIAD source would be necessary to fully exploit experiments using modern femtosecond lasers. In this report an investigation of the LIAD mechanism is undertaken using a range of laser parameters for desorption of the sample and ionisation of the plume, followed by mass spectrometry of the products. This set-up was then used to generate a neutral gas phase molecular target of the green fluorescent protein chromophore to measure its absorption spectrum.

Absorption Spectrum of the Green Fluorescent Protein

Many natural systems have evolved so that chemical chromophores within protein structures are exploited for pigmentation and in a range of photochemical processes. Of particular significance for *in-vitro* cell biology is the green fluorescent protein (GFP) discovered in the jellyfish Aequorea Victoria[8], which has been extensively used as a genetically encoded fluorescent marker[9]. The GFP chromophore's intrinsic properties have been extensively investigated using p-hydroxybenzilidene-imidazolinone (p-HBDI) as a model system. However, after nearly 15 years of study a complete understanding of this molecule's photochemical properties remains elusive.

The intrinsic absorption properties of the anion have been studied in ion storage devices through by action spectroscopy[10, 11] which suggests that the protein environment is closer to that of an isolated molecule rather than in solution. However, there is an ongoing debate about these results since the observed action (dissociation) often requires absorption of more than one photon and there is a competing electron detachment channel[12]. Surprisingly there are no previous direct experimental results for the neutral chromophore, only action spectroscopy for model systems with substituted charged groups[13]. Here we report the first direct measurement of the absorption spectrum of the neutral.

Experimental Setup

The experiment was performed using the SFTC Central Laser Facility UFL1 Loan Laser. This consisted of a Light Conversion Pharos femtosecond laser operating at 1028 nm, 250 fs, 200 μJ and 10 kHz, which was used to pump an Orpheus/Lyra optical parametric amplifier (tuneable from 310-2500 nm. These outputs were used for desorption of neutral molecules and/or ionisation of the resulting plume. An Opotek laser producing 355 nm, 4 ns, 300μJ, pulses and a CW, 532 nm, 100 mW laser were also used for molecular desorption. The experimental set up is shown in Figure 1 and has been described in detail elsewhere[14].

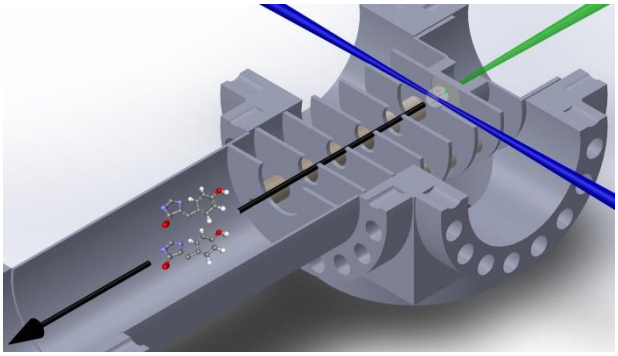

Figure 1: The experimental interaction region. The solid sample is placed on a thin foil (10 μm) which is irradiated with a desorption laser on the reverse side. The resulting plume is ionised by a femtosecond laser and the ions extracted into a time of flight mass spectrometer.

Investigation of the Laser Desorption Process

Using the amino acid phenylalanine as a test sample, signal was readily obtained when the Opotek ns pulses (300μJ) were focused to an intensity of 10^8 Wcm^{-2} on the foil with the plume ionised by the 1028 nm fundamental output of the Pharos focused to an intensity of 10^{13} Wcm^{-2}. Using a beam splitter, part of the femtosecond beam was then used as the desorption laser (replacing the ns pulses), giving a higher intensity (up to 10^{11} Wcm^{-2}) but lower pulse energy (100 μJ). Despite investigating several different foil materials (titanium, stainless steel, tantalum and tungsten), no signal could be obtained when using the femtosecond pulses, except when the repetition rate of the laser was increased from 10s of Hz to kHz when signal was obtained from the titanium and stainless steel foils (which both have low thermal conductivities). This strongly suggests that the LIAD process is actually driven by thermal processes rather than the intense acoustic wave generated by the laser-foil interaction.

Absorption Spectrum of the GFP Chromophore

The GFP chromophore *p*-HBDI was studied along with 3,5-dimethoxy-4-hydroxybenzylidene-1,2-dimethylimidazolinone (DMHBDI) and 3,5-difluoro-4-hydroxybenzylidene-1,2-dimethylimidazolinone (DFHBDI), for which electron donating and withdrawing groups are added respectively to the chromophore (Figure 2). To generate gas phase targets of these solids they were deposited on a stainless steel foil and gently heated by a 100 mW, 532nm, CW laser to a temperature of about 50 °C. The molecules were exposed to femtosecond pulses from the Orpheus/Lyra focused to an intensity of 1.5×10^{10} Wcm^{-2} in the wavelength range 315-420 nm.

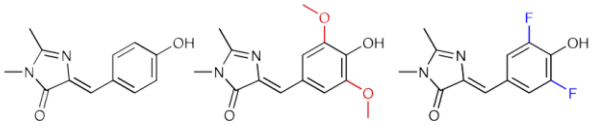

Figure 2: *p*-HBDI, DMHBDI, and DFHBDI

As p-HBDI has an ionization energy of 7.4 eV [15], for wavelengths greater than 335 nm three photons are required for ionisation which can be resonantly enhanced via the $S_1 - S_0$ excitation of a $\pi \rightarrow \pi^*$ transition on the phenol ring. The total ion yields from resonantly enhanced multi-photon ionisation (REMPI) as a function of wavelength are shown in Figure 3. For p-HBDI and DFHBDI, as the wavelength moves deeper into the UV, the ion yield increases to a maximum at 330-340 nm due the $S_1 - S_0$ transition. The fact that the yield does not reduce at shorter wavelengths can be attributed to the transition from 3 to 2 photons being required for ionisation. For DMHBDI the absorption band is clearer as it is red-shifted to a peak at 360 nm. This shift can be readily understood since the electron donating groups increase the electron de-localisation of the molecule bringing the π and π^* levels closer together.

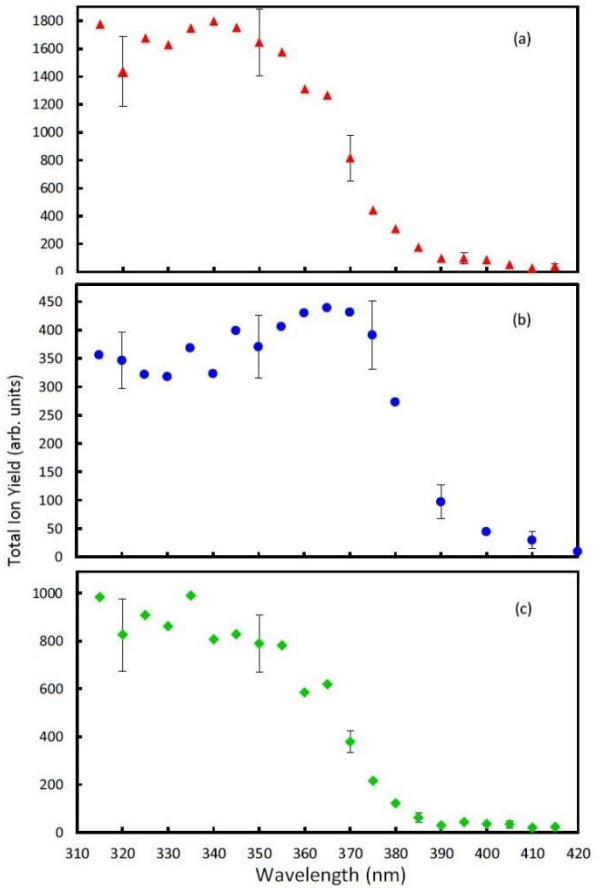

Figure 3: REMPI spectra for (a) *p*-HBDI, (b) DMHBDI, (c) DFHBDI

Conclusions

We have investigated the LIAD process for generating clean, neutral plumes of complex gas phase molecules using a variety of laser wavelengths and pulse parameters. From our experiments it is evident that the desorption process is not dependent on the intensity of the laser-foil interaction but rather on the pulse energy. This suggests that it is primarily a thermal rather than an acoustic wave which is driving detachment of molecules from the surface of the foil. For high repetition rate conditions and low thermal conductivity foils it was also evident that the average power of the laser was high enough to raise the average foil temperature sufficiently for thermal evaporation. This heating effect may well be the main desorption mechanism in a number of studies in the literature. However, some studies in which thin single crystal layers of the sample were deposited on the foil, intact desorption of large, labile molecules was possible[16]. In these cases desorption may be due to the mechanical mismatch between the foil and analyte resulting in fracturing of the crystalline layer[7].

Therefore the sample preparation appears critical for generating gas phase plumes of complex labile molecules using "LIAD". However, it may not be trivial to form a thin crystalline layer reliably for each sample under investigation. Pulsed laser desorption techniques have shown that intact, labile molecules can be produced if they are immediately cooled by entrainment into a gas jet[3,4]. The disadvantage is that the laser directly irradiates the surface on which the sample is deposited which can inevitably lead to some molecular breakup and contamination with surface material or impurities. If the same rapid heating can be achieved by irradiating the reverse side of a foil followed by gas jet cooling, the outcomes could be much improved, particularly if a clean target is required, as for instance in an electron spectroscopy experiment.

For the GFP chromophore p-HBDI studied here, gentle continuous heating of the foil with a CW laser was sufficient to create a gas phase target. The apparatus makes efficient use of the sample as it is very close to the interaction region unlike a typical evaporation source. Our REMPI absorption spectra show that the $S_1 - S_0$ transition has a peak which is shifted by about 55 nm (0.5 eV) when the chromophore is removed from the protein into vacuum. This contradicts earlier claims that the environment experienced by the chromophore inside the protein is similar to the vacuum[10,11]. It is perhaps better to say that the chromophore is very sensitive to the chemical modifications and the local environment in which the presence of H-bonds control the molecular conformation. This flexibility may be the reason that this chromophore has been exploited in nature. These results provide further understanding of this unique system but also provide insight which may be valuable for the design of synthetic chromophores used in future cell studies.

Acknowledgements

This work was supported by the CLF Laser Loan Scheme, EPSRC (EP/L005646/1), Leverhulme Trust (RPG-2012-735) and Northern Ireland Department of Employment and Learning.

References

[1] A. El-Aneed, A. Cohen, J. Banou, *Appl. Spect. Rev.*, **44**, 210 (2009)
[2] J.A. Wyer, *Photophysics of Ionic Biochromophores*, Springer-Verlag ed. S.B. Nielsen and J.A. Wyer, pp21 (2013)
[3] G. Meijer et al. *Appl. Phys. B*, **51**, 395 (1990)
[4] U. Boesl et al. *Instrum. Sci. Tech.*, **16**, 151 (1987)
[5] A.V. Zinovev et al. *Anal. Chem.*, **79**, 8232 (2007)
[6] A.R. Dow et al. *Eur. J. Mass Spectrom.*, **18**, 77 (2012)
[7] A.V. Zinovev et al. *Acoustic Waves - From Microdevices to Helioseismology*, Ch. 16, Intechopen.com, ed. M.G. Beghi (2011)
[8] O. Shimomura et al. *Cell. Comp. Physiol.*, **59**, 223(1962)
[9] B.A. Griffin, S.R. Adams, R.Y. Tsien, *Science*, **281**, 269 (1998)
[10] S.B. Nielsen et al. *Phys. Rev. Lett.*, **87**, 228102 (2001)
[11] M.W. Forbes, R.A. Jockusch, *J. Am. Chem. Soc.*, **131**, 17038 (2009)
[12] C.R.S. Mooney et al., *J. Phys. Chem. A*, **116**, 7943 (2012)
[13] J. Rajput et al., *Phys. Chem. Chem. Phys.*, **11**, 9996 (2009)
[14] C.R. Calvert et al., *Phys. Chem. Chem. Phys.*, **14**, 6289 (2012)
[15] K.M. Solntsev et al. *J. Phys. Chem. Lett.*, **2**, 2593 (2011)
[16] R.C. Shea et al. *Anal. Chem.*, **79**, 1825 (2007)