



Condensed-Phase Applications of Cavity-Based Spectroscopic Techniques

by

Simon Neil

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Doctor of Philosophy

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*University of Oxford
Physical and Theoretical Chemistry Laboratory
and
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ABSTRACT

This thesis describes the development and application of condensed-phase cavity-based spectroscopic techniques—namely cavity ring-down spectroscopy (CRDS); cavity enhanced absorption spectroscopy (CEAS); broadband cavity enhanced absorption spectroscopy (BBCEAS) and evanescent wave (EW) variants of all three.

The recently-developed cavity technique of EW-broadband cavity enhanced absorption spectroscopy (EW-BBCEAS) has been used—in combination with a supercontinuum source (SC) and a sensitive, fast readout CCD detector—to record of the full visible spectrum (400–700 nm) of a silica-liquid interfacial layer (with an effective thickness *ca.* 1 μm), at rapid acquisition rates (> 600 Hz) that are sufficient to follow fast kinetics in the condensed phase, in real time. The sensitivity achieved ($A_{\text{min}} = 3.9 \times 10^{-5}$) is comparable with previous EW-CRDS and EW-CEAS studies, but the spectral region accessed in this broadband variant is much larger. The study of liquid|air interfaces using EW cavity-based techniques is also illustrated for the first time.

The first application of BBCEAS to the analysis of microfluidic samples, flowing through a microfluidic chip, is illustrated. Proof-of-principle experiments are presented, demonstrating the technique's ability to provide full visible broadband spectral measurements of flowing microfluidic droplets, with both high detection sensitivity ($\alpha_{\text{min}} < 10^{-2} \text{ cm}^{-1}$) and excellent spatial and temporal resolution: an SC light source and sensitive, fast readout CCD allowed measurement repetition rates of 273 Hz, whilst probing a very small sample volume (*ca.* 90 nL).

A significant portion of this thesis is devoted to demonstrating the powerful capabilities of CEAS, CRDS and BBCEAS in monitoring radical recombination reactions and associated magnetic field effects (MFEs) in solution. The efficacy of CEAS as a high-sensitivity MFE detection method has been established in a proof-of-principle study, using narrow band CEAS in combination with phase-sensitive detection: MFE-induced absorbance changes of *ca.* 10^{-6} could be detected using the modulated CEAS technique and the data are shown to be superior to those obtained using conventional transient absorption (TA) methods typically employed for MFE measurements. The powerful capabilities of CRDS in monitoring radical recombination reactions and associated MFEs are also demonstrated. In particular, a pump-probe CRDS variant allows not only high sensitivity (A_{min} on the order 10^{-6}), but also sub-microsecond time-resolution. Combined, these features represent significant advantages over TA. Finally, SC-BBCEAS is used to measure full visible spectra of photoinduced reactions and their MFEs. The applicability of this approach to *in vitro* MFE studies of *Drosophila* cryptochrome is demonstrated—the results mark the first *in vitro* observation of a magnetic field response in an animal cryptochrome, a key result supporting the hypothesis that cryptochromes are involved in the magnetic sense in animals.

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Publications arising from this work

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2. Schnippering, M., Neil, S. R. T., Mackenzie, S. R. & Unwin, P. R., Evanescent wave cavity-based spectroscopic techniques as probes of interfacial processes, *Chem. Soc. Rev.* **40**, 207-220, (2011).
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5. Van der Sneppen, L., Hancock, G., Kaminski, C. F., Laurila, T., Mackenzie Stuart, R., Neil, S., Peverall, R., Ritchie, G. A. D., Schnippering, M. & Unwin, P. R., Following interfacial kinetics in real time using broadband evanescent wave cavity-enhanced absorption spectroscopy: a comparison of light-emitting diodes and supercontinuum sources, *Analyst* **135**, 133-139, (2010).

Acronyms

AOM	acousto-optic modulator
AQDS	anthraquinone-2,6-disulphonate
AtCry	<i>arabidopsis thaliana</i> cryptochrome
ATR	attenuated total internal reflection
BB	broadband
CCD	charge coupled device
CEAS	cavity enhanced absorption spectroscopy
CEF	cavity enhancement factor
CRDS	cavity ring-down spectroscopy
CW	continuous wave
DABCO	1,4-diazabicyclo[2.2.2]octane
DmCry	<i>Drosophila melanogaster</i> cryptochrome
DAQ	digital acquisition
DC	direct current
EcPL	<i>Escherichia coli</i> photolyase
EP	escape product
ESR	electron spin resonance
EW	evanescent wave
FAD	flavin adenine dinucleotide
FBG	fiber Bragg grating
FDS	fiber delivery system
F-pair	freely-diffusing pair
(F)FT	(fast) Fourier transform
FMN	flavin mononucleotide
FWHM	full width half maximum
GP	geminate product
G-pair	geminate pair
HEWL	hen egg white lysozyme
HPLC	high-performance liquid chromatography
IR	infrared
ISC	intersystem crossing
LED	light emitting diode
LC	liquid chromatography
LCW	liquid core waveguide
LFE	low field effect
LIA	lock-in amplifier
MARY	magnetically altered reaction yield
MFE	magnetic field effect
MTHF	methenyltetrahydrofolate
NICE-OHMS	noise immune cavity enhanced optical heterodyne molecular spectroscopy
NMR	nuclear magnetic resonance

PCF	photonic crystal fiber
PMT	photomultiplier tube
QE	quantum efficiency
RP	radical pair
RPM	radical pair mechanism
SC	supercontinuum
SDS	sodium dodecyl sulfate
SNR	signal to noise ratio
S-T	singlet-triplet
TA	transient absorption
TH	thionine
TIR	total internal reflection
Trp	tryptophan
TTL	transistor-transistor logic
UV	ultra-violet
Vis	visible
Xa	xanthone

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Chapter 1

Introduction

Numerous well-developed spectroscopic techniques exist for probing various properties of matter in the liquid-phase, including: ultra-violet/visible (UV/Vis) spectroscopy and fluorescence spectroscopy, giving information on electronic structure; Fourier transform infrared (FT-IR) spectroscopy and Raman spectroscopy, which allow functional group identification *via* vibrational structure; and spectroscopic techniques used to probe the local chemical environments of molecules *via* nuclear magnetic resonance (NMR) and electron spin resonance (ESR). Of these, those techniques based on detection of optical absorption are very widely used, but relatively insensitive. Work reported in this thesis concerns the development and application of numerous condensed-phase optical cavity-enhanced techniques (namely: cavity ring-down spectroscopy (CRDS), cavity enhanced absorption spectroscopy (CEAS), broadband cavity enhanced absorption spectroscopy (BBCEAS) and evanescent wave (EW) variants of all three), which allow dramatic improvements in optical absorption detection sensitivity. The background of these techniques (based on direct detection of optical absorption) and their basic principles are discussed in the present chapter.

This chapter begins with a general discussion of direct absorption spectroscopy. Section 1.2 deals with the fundamental principles of three popular optical cavity-enhanced techniques (CRDS, CEAS, BBCEAS). These principles are developed further in Section 1.3, whilst Sections 1.4 and 1.5 deal with important instrumental requirements (light sources and cavity design) that must be considered. Section 1.6 provides a review of previous implementations of CRDS, CEAS and BBCEAS in the condensed phase. Finally, the remaining Chapters of this thesis are summarized in Section 1.7. Parts of this Chapter have been published in a review article: [Schnippering, M., Neil, S. R. T., Mackenzie, S. R. & Unwin, P. R., Evanescent wave cavity-based spectroscopic techniques as probes of interfacial processes, *Chem. Soc. Rev.* **40**, 207-220, \(2011\)](#) - Reproduced by permission of The Royal Society of Chemistry.

1.1 The Beer-Lambert law and conventional direct absorption spectroscopy

Single-pass absorption spectroscopy is nearly universally applicable and allows non-invasive, *in situ*, quantitative sample analysis *via* use of the Beer-Lambert law.

Pierre Bouguer was the first to recognize that the attenuation of a light beam passing through a particular material is directly proportional to the material's thickness.¹ This law was re-discovered by Johann Lambert in 1760,² and later developed by August Beer, in 1852,³ to include the effect of sample concentration on the fraction of light absorbed. The resulting Beer-Lambert law relates sample absorbance (A) to the attenuation of a light beam as it makes a single pass through a sample:

$$A = -\log_{10} \frac{I}{I_{in}} \quad (1.1)$$

where I_{in} is the light intensity incident upon the sample and I is the intensity transmitted through the sample.

A can be further described by:

$$A = \varepsilon Cl \quad (1.2)$$

where ε is the molar decadic absorption coefficient, C the concentration and l the optical path length through the sample.

By convention, in analytical chemistry Equation 1.1 is formulated using a decadic logarithmic scale (*i.e.*, $\log_{10}$). By contrast, the gas-phase community typically quantifies absorption (and scattering) losses in terms of natural logarithms, *i.e.*:

$$A' = \alpha l = -\ln \frac{I}{I_{in}} \quad (1.3)$$

where α is the absorption coefficient of the sample.

Equations 1.2 and 1.3 can, therefore, be related using a conversion factor, $\ln 10$ (= 2.3026)

$$A = \varepsilon Cl = \frac{\alpha l}{2.3026} \quad (1.4)$$

In conventional (single-pass) direct absorption spectroscopy, l is typically well-known and hence α (and thus C or ε) can be determined conveniently without the need for any independent calibration. However, for reasons discussed below, single-pass direct absorption spectroscopy is limited in its dynamic range, especially when studying samples with low concentrations or molar absorption coefficients.

The minimum detectable absorbance in a conventional single-pass absorption experiment can be defined as:

$$2.3026A_{\min} = \alpha_{\min} l = \frac{\Delta I_{\min}}{I_{\text{in}}} \quad (1.5)$$

where $\Delta I_{\min}$ is the minimum detectable change in the light intensity resulting from sample absorption. Therefore, in order to achieve a high sensitivity (low $A_{\min}$), very small changes in light attenuation must be distinguished from a relatively large background. For example, in order to achieve $A_{\min} = 1 \times 10^{-5}$, $\Delta I_{\min}/I_{\text{in}}$ must be determined to within 2 parts in 10^5 . This is challenging, even for modern commercial instruments using stabilized light sources.

1.2 High-sensitivity absorption detection in the condensed-phase

Highly sensitive direct absorption methods are desired for a variety of condensed-phase applications. Thin films (with concomitantly short path lengths), biological samples (typically low concentrations) and liquid chromatography (LC) analytes (small α and/or l) all exhibit inherently small absorbances. Similarly the microfluidics community is driving instrument miniaturization resulting in very small path lengths (and, therefore absorbances).⁴ Sensitive

detectors can also be used to achieve high temporal resolution (*via* reductions in signal averaging time) permitting the study of reaction kinetics in real time.

While a number of highly sensitive indirect approaches to condensed-phase absorption detection exist (for example, thermal lens spectroscopy, photo-acoustic spectroscopy and non-linear optical techniques such as sum frequency mixing and degenerate four wave mixing), these techniques typically lack the main benefits of direct absorption spectroscopy: simplicity, self-calibration, and near-universal applicability. Therefore, numerous attempts have been made to enhance the sensitivity of direct absorption spectroscopy.

It is clear from Equations 1.2 and 1.3, that, for a sample of fixed α (or $C\epsilon$), an increase in l generates a larger measurable absorbance. Therefore, in principle the sensitivity of direct absorption spectroscopy is linearly dependent on l and one obvious approach to improving $A_{\min}$ is to increase l by changing the dimensions of the sample itself. An elegant solution for liquid-phase samples is to use a liquid core waveguide (LCW) to increase l , whilst maintaining a low sample volume.^{5,6} LCWs rely upon total internal reflection (see Section 1.6.2) to constrain probe light as it passes through a hollow core fiber, filled with a liquid sample. Such fibers have relatively small diameters and can allow l on the order of meters, with microliter-sized samples. However, the poor spatial resolution inherent to LCW-based techniques makes them inappropriate for certain liquid-phase applications.

Herriot- and White-type cells seek to increase l by making multiple passes of light through a sample, *via* multiple reflections between two highly reflective mirrors. Light is injected into the cell through an aperture in (or adjacent to) one of the mirrors. The injection angle leads to migration and spatial separation of the beam during its multiple passes, thereby avoiding interference effects. Whilst these long pass cells can increase l by one or even two orders of magnitude, the beam's migration reduces spatial resolution and places significant limitations

on the cell geometry. In practice this means larger cell sizes and, therefore, larger sample volumes are required, which restricts their use in many liquid-phase applications.

The practical drawbacks—spatial resolution degradation and sample volume increase—typically associated with increasing the optical path length, are circumvented by optical cavity-based spectroscopic techniques: by spatially overlapping the multiple passes of light between two or more mirrors, the same sample region is probed after each reflection or round trip between the mirrors, effectively limiting the minimum sample volume to the beam diameter and per-pass optical path length through the sample. Spatial overlap of the probe beam between the mirrors results in the formation of an optical resonator (cavity), with interference fringes that lead to standing wave patterns (modes), only allowing transmission (and storage) of light for certain resonant frequencies. This light can be used to sensitively monitor sample absorption *via* a number of different experimental approaches. Three of the most popular optical cavity-based techniques, CRDS, CEAS and BBCEAS, are described in the following sections.

1.2.1 Cavity ring-down spectroscopy

Since its introduction in 1988 by O’Keefe and Deacon,⁷ CRDS has proved a powerful technique for spectroscopic analysis of molecules, clusters and trace gas species. Numerous variants of CRDS have been developed and these have been reviewed in detail elsewhere.⁸⁻¹² In this section, the basic principles of CRDS are described in terms of a conceptually simple variant, pulsed CRDS using a two-mirror cavity. It should also be noted that although CRDS was conceived and largely developed in the gas phase, the basic principles of the technique are applicable, with only minor modifications, to the condensed-phase.

In the most conceptually simple implementation of CRDS, a pulse of light (typically from a laser) is injected into the cavity, through the rear of one of the mirrors (see Figure 1.1).

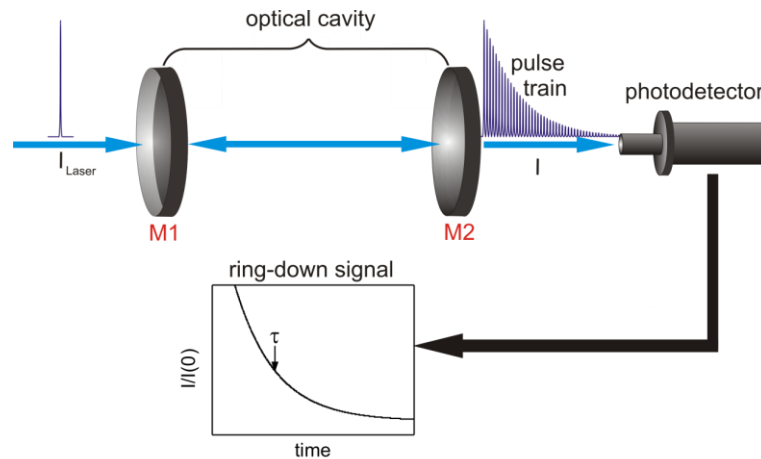


Figure 1.1 Schematic of a (linear) Fabry–Perot cavity. A light pulse, intensity I_{Laser} , enters the cavity from the left through mirror M1. The light intensity transmitted through the output mirror (M2) is measured with a photodetector. Following an initial rise (during the pulse), the light intensity decays exponentially from an initial value, $I(0)$, and its temporal profile ('ring-down', characterized by a decay constant, τ) is usually observed as a smoothed single exponential curve that envelops an unresolved pulse train.

Following the pulse the intracavity light intensity decays exponentially as a result of absorption/scattering losses within the cavity and transmission through the cavity mirrors. The decay is monitored as a function of time (using the small amount of light exiting the cavity *via* transmission through the rear mirror) and fitted to yield a decay time constant (the 'ring-down time', τ), which may be used to determine the total per-pass absorbance as described below.

If the duration of the laser pulse is shorter than the pulse's round-trip time within the cavity, the output signal is a train of pulses with decreasing intensity. For the simple two-mirror cavity shown in Figure 1.1, comprising mirrors with identical reflectivity, R , the light intensity at the detector after the first pass through the cavity, $I(0)$, can be described by:

$$I(0) = I_{\text{laser}} T^2 e^{-\alpha l} \quad (1.6)$$

where I_{laser} is the intensity of the injected laser pulse, T is the mirror intensity transmittivity (for ideal mirrors, $T = 1 - R$, although in practice T is typically limited by

absorption/scattering losses to 0.1–0.5 times $1 - R$) and $e^{-\alpha l}$ is the one-pass light intensity attenuation described by the Beer-Lambert law (see Equation 1.3). For each subsequent round trip through the cavity, the intensity reduces by an additional factor of $R^2 e^{-2\alpha l}$. Therefore, after n round trips through the cavity, the pulse intensity at the detector can be described by:

$$I(n) = I(0)[Re^{-\alpha l}]^{2n} = I(0)e^{-2n(-\ln R + \alpha l)} \quad (1.7)$$

The detector is typically unable to resolve the pulse train, and instead the cavity's output signal is usually observed as a smoothed single exponential curve (that envelops the unresolved pulse train). Therefore the discrete variable n can be exchanged for a continuous time variable, t :

$$t = \frac{2L}{c} n \quad (1.8)$$

where $\frac{2L}{c}$ is the time taken for light to make one round-trip within the cavity.

Therefore the time-dependent light intensity exiting the cavity, $I(t)$, can be described as:

$$I(t) = I(0)e^{-\frac{ct}{L}(-\ln R + \alpha l)} \quad (1.9)$$

From Equation 1.9, and defining τ to be the time taken for the initial light intensity to decrease to $I(0)/e$:

$$\tau = \frac{L}{c(-\ln R + \alpha l)} \quad (1.10)$$

which, for a high finesse cavity (with R close to 1 and, therefore, $-\ln R \approx 1 - R$), can be re-written as:

$$\tau = \frac{L}{c(1 - R + \alpha l)}. \quad (1.11)$$

For an empty cavity ($\alpha = 0$), the ring down time, τ_0 , can be described as:

$$\tau_0 = \frac{L}{c(1 - R)} \quad (1.12)$$

At this point it should be noted that, in practice, R actually represents an ‘effective’ mirror reflectivity, which not only describes the reflectivity of the mirror coating (R_{pure}), but also includes any additional background losses (a_b) within the cavity: $R = R_{\text{pure}}(1 - a_b)$. This is discussed further, in the context of condensed-phase cavity-based techniques, in Section 1.6.

With knowledge of both τ and τ_0 the per-pass absorbance resulting from an intracavity absorber may then be calculated by:

$$2.3026A = \alpha l = \left(\frac{1}{\tau} - \frac{1}{\tau_0} \right) \frac{L}{c} = \frac{\Delta\tau}{\tau_0 \tau} \frac{L}{c} \quad (1.13)$$

where $\Delta\tau = \tau_0 - \tau$

From Equation 1.13 the advantages of CRDS become clearer. Like conventional direct absorption spectroscopy, CRDS is self-calibrating and allows quantitative absorbance measurements. However, CRDS measurements are practically intensity-independent; the sensitivity of the technique does not rely on the ability to differentiate small differences between large signals, but rather on the ability to accurately determine small differences between exponential decays, as discussed below. This is an important factor, as pulse lasers typically suffer from significant pulse-to-pulse energy fluctuations.

1.2.1.1 CRDS sensitivity

The minimum detectable per-pass absorbance (A_{min}) that may be achieved by CRDS is ultimately dictated by the smallest measureable change in the ring-down time, $\Delta\tau_{\text{min}}$, and can be defined as:

$$2.3026A_{\text{min}} = \frac{\Delta\tau_{\text{min}}}{\tau_0^2} \frac{L}{c} = (1 - R) \times \left(\frac{\Delta\tau}{\tau_0} \right)_{\text{min}} \quad (1.14)$$

Using the example given in Section 1.1: for a conventional single pass absorbance measurement to achieve $A_{\text{min}} = 1 \times 10^{-5}$, $\frac{\Delta I_{\text{min}}}{I_{\text{in}}}$ must be determined to better than $2.3 \times 10^{-3} \%$.

According to Equation 1.14, to achieve the same level of sensitivity in CRDS using a 50 cm cavity with $\tau_0 = 1 \mu\text{s}$, $\frac{\Delta\tau}{\tau}$ need only be known to within 1.4 %. In the gas phase this is trivial (τ_0 values of 100 μs are not uncommon, and $\frac{\Delta\tau}{\tau} = 0.01$ readily achievable) and, as described in Section 1.6 and shown in later chapters, these conditions can also be achieved with relative ease in the condensed phase (despite the shorter ring-down times, due to scattering and background absorption).

From Equation 1.14, it is also seen that, in principle, the sensitivity may be improved by increasing τ_0 (via R or L), or decreasing $\Delta\tau$ (by improving the accuracy with which the ring-down time is determined). However, the sensitivity of any CRDS experiment also relies on a number of practical aspects, which are discussed in terms of condensed-phase CRDS, in Section 1.6.1.1.

1.2.2 Cavity enhanced absorption spectroscopy

CEAS is closely related to CRDS. However, like conventional direct absorption spectroscopy (and unlike CRDS), CEAS relies on the measurement of fractional changes in light intensity to determine absorbance. CEAS has been described in great detail elsewhere (for example, Mazurenka *et al.*¹³ and references therein) and only a broad overview of the technique is given below.

In CEAS the sample is placed within a high finesse optical cavity, into which light is continuously injected. The light intensity within the cavity rapidly reaches a steady state at which point the light entering the cavity matches that lost from the cavity (to transmission through the cavity mirrors and to absorption/scattering). Measurements of the steady-state light intensity can then be used to determine intracavity absorbance, as described below.

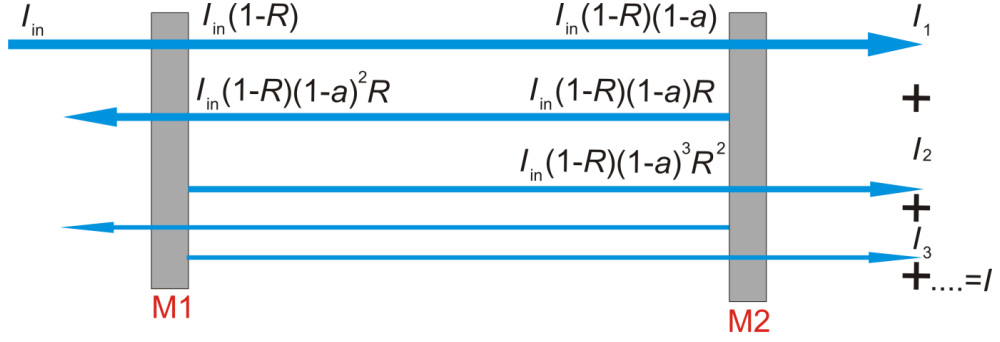


Figure 1.2 Schematic used to derive I from first principles (see text for details).

Figure 1.2 illustrates how the light intensity transmitted through the rear cavity mirror may be described. After the first pass of light through the cavity, the light intensity exiting the rear mirror (I_1) can be described as the product of the input light intensity and the transmission through the first (input) mirror ($T_1 = (1 - R)$, neglecting any scattering/absorptive losses within the mirror), the cavity medium (T_M) and finally through the second (output) mirror (T_2). Thus, assuming $T_1 = T_2 = 1 - R$ and $T_M = 1 - a$, where a is the per-pass light attenuation:

$$I_1 = I_{\text{in}}(1 - R)(1 - a)(1 - R) \quad (1.15)$$

and for an infinite number (n) of passes:

$$I_{\text{out}} = I_{\text{in}}(1 - R)^2(1 - a) \sum_{n=0}^{\infty} R^{2n}(1 - a)^{2n} \quad (1.16)$$

For $R < 1$ and $a < 1$, the summation term is an infinite geometric series, which converges and may be rewritten as:

$$I = I_{\text{in}} \frac{(1-R)^2(1-a)}{1-R^2(1-a)^2} \quad (1.17)$$

In the absence of any per pass losses, the light intensity, I_0 , may then be described as:

$$I_0 = \frac{I_{\text{in}}(1-R)^2}{1-R^2} \quad (1.18)$$

The ratio of Equations 1.17 and 1.18 gives:

$$\frac{I_0}{I} = \frac{1 - (R(1-a))^2}{(1-R^2)(1-a)} \quad (1.19)$$

Assuming Beer-Lambert absorption, $1 - a = e^{-\alpha l}$, Equation 1.19 may be recast, to yield the intracavity absorbance, given by Fiedler *et al.*:¹⁴

$$2.3026A = \alpha l = -\ln \left[\frac{1}{2R^2} \left(\sqrt{4R^2 + \left(\frac{I_0}{I} (R^2 - 1) \right)^2} + \frac{I_0}{I} (R^2 - 1) \right) \right] \quad (1.20)$$

In lim $R \rightarrow 1$ and $a \rightarrow 0$ (*i.e.*, high reflectivity mirrors and low intracavity losses), Equation 1.20 simplifies to:

$$2.3026A = \alpha l = \left(\frac{I_0}{I} - 1 \right) (1 - R) \quad (1.21)$$

$\left(\frac{I_0}{I} - 1 \right)$ thus represents the experimental observable which is directly proportional to the absorbance. The constant of proportionality, $(1 - R)^{-1}$, is known as the cavity enhancement factor (CEF).

As discussed previously in Section 1.2.1, it is important to remember that additional background losses (a_b) are also folded into R .

From Equations 1.20 and 1.21, a major drawback of CEAS is apparent: unlike CRDS, CEAS is unable to provide an absolute measure of absorbance without a prior calibration to determine R (and the CEF). This calibration can be performed using a CRDS measurement (of τ_0 , see Equation 1.12), or using a calibrant species whose absorption cross section is well known. However, any error in determining R results in a decrease in the sensitivity of CEAS, as touched upon in later sections of this thesis.

For most CEAS applications, the approximations leading to equation 1.21 are valid.^{15,16} However, it is important, for any given CEAS experiment, to predict the conditions under which

these approximations break down, particularly in condensed-phase applications where intracavity absorption and/or scattering losses may be large.

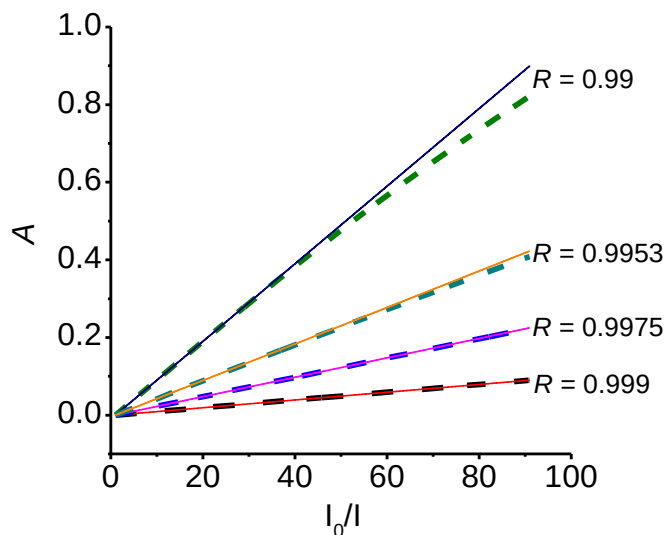


Figure 1.3 The relationship between CEAS-measured fractional light intensity change and absorbance, calculated using equations 1.20 (dashed lines) and 1.21 (solid lines). Results are shown for various cavities, with different R .

This is exemplified by Figure 1.3, which shows a comparison of A , calculated as a function of $\frac{I_0}{I}$ from Equations 1.20 and 1.21. At low R and high light-attenuation within the cavity, *e.g.* $R = 0.99$ and $\frac{I_0}{I} > 50$, there is a significant difference between the results of Equations 1.20 and 1.21, due to breakdown in the approximations leading to Equation 1.21.

1.2.2.1 CEAS sensitivity

In CEAS, the minimum detectable absorbance may be described by:

$$2.3026A_{\min} = \alpha_{\min}l = (1 - R) \left(\frac{\Delta I}{I_0} \right)_{\min} \quad (1.22)$$

where $\Delta I_{\min}$ is the smallest measureable change in the transmitted light intensity. Note the similarity to Equation 1.14, for CRDS. Unlike CRDS, however, the sensitivity is now genuinely intensity dependent and a comparison with Equation 1.5 demonstrates that, in principle, the sensitivity in CEAS is enhanced by a factor of $(1 - R)^{-1}$ (the CEF, see Section 1.2.2) with respect to a single pass experiment, and may be improved by increasing the mirror reflectivity

and by minimising $\frac{\Delta I_{\min}}{I_0}$. However, in practice, as R is increased the transmitted light intensity decreases and the signal to noise ratio (SNR) of this transmitted intensity can ultimately limit the sensitivity.¹⁵ Additionally, in the condensed phase, absorptive and scattering losses to intracavity elements and solvents can limit the CEF and sensitivity (discussed further in Section 1.6).

1.2.3 Broadband CEAS

CEAS was initially developed using spectrally narrow linewidth light sources. In such techniques, spectral information is typically produced by scanning the laser frequency. The scanning process prohibits rapid spectral acquisition. However, a straightforward extension to CEAS involves the use of a spectrally broadband light source, giving a broadband (BB)CEAS variant; BBCEAS was conceived (in the gas phase) by Fiedler *et al.*¹⁴ For the purpose of this thesis, the term BBCEAS is applied to any CEAS technique that has the potential to measure absorbance over multiple wavelengths simultaneously without the need for laser scanning. The multiplexing capabilities of BBCEAS are typically achieved by measuring the light intensity exiting the cavity as a function of wavelength, across all wavelengths, λ , simultaneously and determining $A(\lambda)$ *via* Equation 1.23 (which is simply a wavelength-specific version of Equation 1.21)

$$2.3026A(\lambda) = \alpha(\lambda)l = \left(\frac{I_0(\lambda)}{I(\lambda)} - 1\right)(1 - R(\lambda)) \quad (1.23)$$

BBCEAS is discussed further in this Chapter, in Sections 1.4.2 and 1.6.1.2, with a focus on the practical aspects of the technique. However, in order to design and build a (BB)CEAS- or, indeed, any cavity-based instrument, the behaviour of light within the optical cavity, and the practical implications of this behaviour, must be considered in more detail. This is done in the next section.

1.3 Cavity mode structure

As mentioned in Section 1.2, cavity-based techniques rely upon the interference effects that occur within an optical resonator. These effects have been ignored in the description of CRDS and (BB)CEAS above. However, as discussed below, the interference effects have important practical implications and must be understood in more detail.

Interference effects are caused by the multiple spatially overlapping reflections that generate standing wave patterns ('modes') within the cavity. The resonator modes can be further defined in terms of longitudinal and transverse modes. Each longitudinal mode occurs at a different frequency (with all longitudinal modes being equally spaced). The frequency spacing between longitudinal modes is known as the free spectral range, $\Delta\nu_{\text{FSR}} \left(= \frac{c}{2nL} \right)$, where n is the refractive index of the medium inside the cavity. The full width half maximum (FWHM) of the cavity modes is defined as:

$$\text{FWHM} = \frac{\Delta\nu_{\text{FSR}}}{\pi} \left(\frac{1-R}{\sqrt{R}} \right) = \frac{\Delta\nu_{\text{FSR}}}{F} \quad (1.24)$$

where F is the finesse (see Figure 1.4).

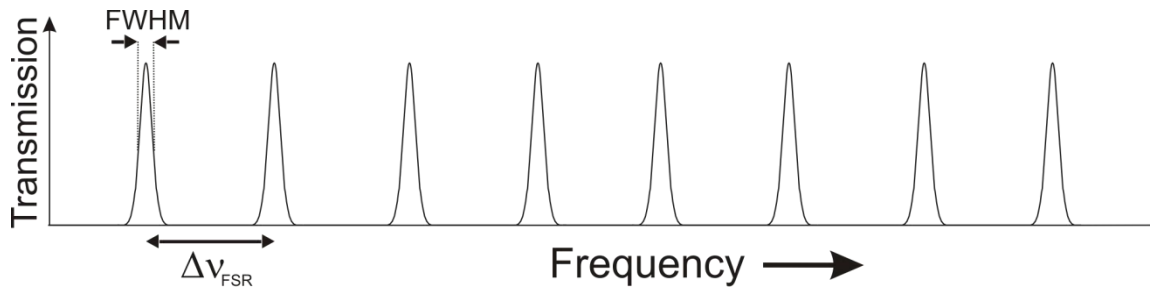


Figure 1.4 Schematic of the frequency dependent transmission of an optical cavity, ignoring contributions from higher order transverse modes. The larger the cavity finesse, the narrower the peak FWHM relative to $\Delta\nu_{\text{FSR}}$.

Each longitudinal mode is associated with a transverse mode describing the cross sectional intensity pattern of the beam. The fundamental transverse mode (often called the TEM_{00}

mode) is typically Gaussian in shape. Higher order transverse modes are separated in frequency from one another and from the TEM_{00} mode.

An important implication of the cavity's mode structure is that light can be efficiently coupled into the cavity only if it is resonant with one or more longitudinal modes and, assuming this condition is fulfilled, absorption measurements may only be made when the frequencies of the cavity resonance and sample absorption are coincident. For a typical cavity applied to the condensed phase, FWHM is of the order 10^5 Hz and $\Delta \nu_{FSR}$ is of the order 10^8 Hz. Therefore, the mode structure is a series of very sharp features, which should be compared to the linewidths of the sample absorption and spectral bandwidth of the probe light ($\Delta \nu_{source}$) when designing a cavity-based experiment.

In the condensed-phase, strong inhomogeneous broadening typically results in very broad absorption features (on the order of tens to hundreds of nanometers, spanning thousands of cavity modes). Hence, coincidence between the sample absorption and cavity resonance is easily achieved. However, achieving coincidence between the probe light and cavity mode frequencies can be more challenging, depending on the light source used. Light sources suitable for cavity based spectroscopy can be characterised by their spectral bandwidth, by comparison with the cavity's free spectral range. Some of the more common cavity light sources and their applicability to CRDS and (BB)CEAS are discussed in the next section.

1.4 Light sources

In a similar trend to developments in the gas-phase, initial condensed-phase cavity-based studies were variants of CRDS employing high powered tuneable pulsed lasers,^{17,18} whereas more recently the use of continuous wave (CW) diode lasers, in both CRDS- and CEAS-based approaches, has become popular. The implementation and relative merits of these light

sources in gas-phase CRDS and CEAS are discussed in detail elsewhere and much of this information is directly applicable to the condensed phase.^{9,13} However, it is appropriate to discuss briefly, the essential features of light sources suitable for condensed-phase CRDS, CEAS and BBCEAS.

1.4.1 CRDS and CEAS light sources

1.4.1.1 Pulsed sources

One of the most common CRDS light sources, tuneable pulsed lasers, typically provide emission with bandwidths on the order of GHz—sufficiently broad as to excite multiple cavity modes simultaneously (illustrated in Figure 1.5). The high pulse energy of these lasers are unmatched by other light sources and transmitted signals are usually well beyond noise levels of modern detectors (*e.g.*, photomultiplier tubes (PMTs), or photodiodes). Pulsed light source variants of CEAS also exist (in the gas phase), although these are less common and have not been implemented in the condensed phase.¹⁹

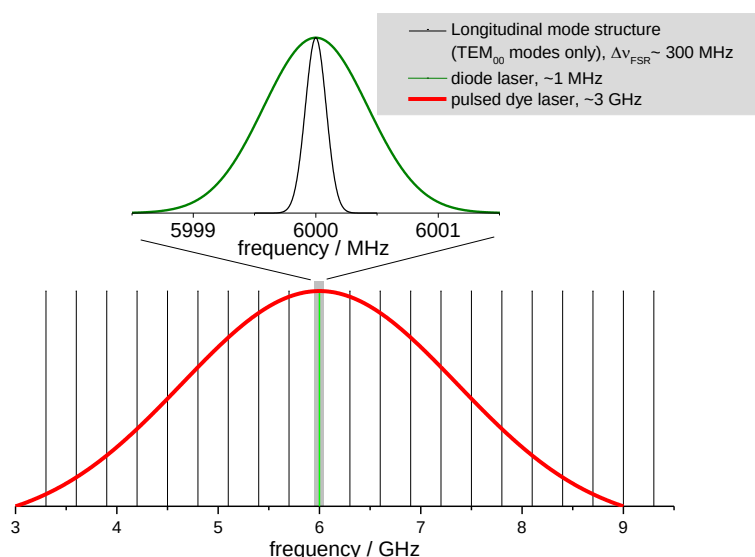


Figure 1.5 The spectral bandwidth of emission from a typical pulsed dye laser (red line) and diode laser (green line) in comparison with the longitudinal mode structure of a cavity (black line) with $L = 50$ cm and $F = 1500$.

In principle, the tunability of dye lasers allows acquisition of full spectral information, *i.e.* absorbance as a function of wavelength. However, in practice tuneable light sources are

unsuitable for monitoring rapid spectral changes in condensed-phase CRDS; tuneable lasers are typically unable to scan with sufficient speed across the wide wavelength ranges spanned by condensed-phase absorption spectra. Hence, examples of CRDS studies employing pulsed tuneable light sources to acquire absorption spectra in the condensed phase are comparatively scarce,^{17,20-22} and in this regard the technique may be considered somewhat one-dimensional.

Additionally, the low repetition rates (*ca.* 10 Hz) at which pulsed sources operate can significantly limit their application to kinetic measurements.

1.4.1.2 CW sources

Continuous wave light sources have become increasingly inexpensive over the last decade. As a result, their use in CRDS and CEAS has become commonplace. The types of CW sources employed for condensed-phase CRDS or CEAS are described below.

Narrow linewidth CW diode lasers (*i.e.*, highly monochromatic, typically external cavity diode lasers) typically have bandwidths (*ca.* 1 MHz) significantly smaller than the free spectral range of the cavity (as illustrated in Figure 1.5), complicating the coupling of light into the cavity; the laser either needs to be locked to a cavity mode (or vice versa), or one of either the cavity or laser needs to be scanned across several modes.²³ However, when coupling is achieved, narrow linewidth lasers provide the best way to make use of the full finesse of a cavity. Essentially when resonant with a cavity mode all (or a large fraction of) light can enter the cavity, producing maximum circulating light levels determined by the product of the input intensity, I_{in} , and the cavity finesse.

In CRDS, the simplest implementation of narrow linewidth lasers involves scanning the cavity or laser across a mode, allowing the intra-cavity light levels to build up to a predetermined threshold. Once this threshold has been reached, the light entering the cavity is rapidly

extinguished (for example using an acousto-optic modulator, AOM,) and the resulting ring-down decay is monitored.

The use of narrow linewidth lasers for CEAS introduces significant experimental complexity. This complexity stems from the fact that even small instabilities in laser frequency (*ca.* kHz), or cavity length (*ca.* nm) can result in loss of coincidence between the laser and cavity mode, and produce a large amount of noise in the transmitted light intensity. Certain CEAS variants (most notably Noise Immune Cavity Enhanced Optical Heterodyne Molecular Spectroscopy, NICE-OHMS) have been developed which mitigate for, or circumvent, this problem but their description is beyond the scope of this thesis (for an in-depth review of these techniques, the interested reader is directed to Mazurenka *et al.*¹³).

In CRDS, when the laser linewidth is wider than, or comparable with, the linewidth of a coincident absorption, the resulting ring-down decay is inherently multiexponential and must be treated with a complex fitting routine in order to accurately determine absorbance values.^{11,24} As a result, the pulsed and narrowband CW lasers described above are particularly popular in gas-phase CRDS applications, as they have linewidths that are spectrally narrow by comparison with gas-phase absorption linewidths (typically around 0.1 cm^{-1} or narrower). However, in the condensed-phase, where absorption linewidths are on the order of tens or hundreds of nanometers, broadband CW diode lasers with bandwidths on the order of a nanometer are suitable for use in CRDS.

In contrast to narrow linewidth lasers, broadband CW diode lasers may excite many thousands of cavity modes simultaneously, greatly simplifying the instrumentation required. Some broadband diodes may be 100% intensity-modulated at high repetition rates (typically kHz) using simple laboratory signal generators on a timescale useful for CRDS (*i.e.*, with rise/fall times of a few nanoseconds).²⁵⁻²⁸ If not, additional elements such as AOMs must be incorporated to provide a pulse train for CRDS.²⁹ In both cases the cavity is essentially filled

with light (typically to a steady state, determined by the quality factor of the cavity) and either the steady state light intensity exiting the cavity is monitored (in CEAS) or the continuous decay of circulating light intensity is monitored when the light the source is switched off (in CRDS). Alternatively, in the absence of an AOM, light from the cavity can reflect directly back into the laser, causing it to self-pulsate and making it suitable for use in CRDS.³⁰

1.4.2 Broadband light sources

Condensed-phase absorption spectra are rarely acquired using the light sources described in Section 1.4.1, which have comparatively narrow linewidths that prohibit rapid spectral acquisition (as discussed in Sections 1.2.3 and 1.4.1.1). To avoid laser tuning and allow rapid acquisition of spectral information, the light source used should have a bandwidth comparable to (or larger than) that of the absorption feature of interest. Broadband variants of CEAS are based on this principle (see Section 1.2.3) and—*via* use of either an incoherent source (*e.g.* light emitting diode, LED, or lamp, see section 1.4.2.1), or a coherent source (*e.g.* laser, see section 1.4.2.2, for example)—allow simultaneous acquisition of spectral information over wide wavelength ranges (tens or hundreds of nanometres), making them well-suited to use in the condensed phase.

1.4.2.1 Non-laser sources

The first BBCEAS experiment (in the gas phase) employed a (incoherent) Xe-arc lamp, emitting a continuous spectrum from the UV to the infrared, which was spectrally filtered to coincide only with the wavelength range of the high-reflectivity mirror coatings (*ca.* 40 nm).¹⁴ Gas-phase incoherent BBCEAS has also been performed using LEDs; the simple, hardy, compact and low-cost nature of LEDs has popularized their use in BBCEAS field instruments, for detection of atmospherically important trace gas species.^{31,32} Both Xe-arc lamp- and LED-based BBCEAS have also been applied to liquid-phase and interfacial studies.^{15,33-37} However, achieving efficient coupling of incoherent light sources into optical cavities is challenging and the control

of the beam size, focusing, or collimation within the cavity is limited. This can, in turn, limit the minimum volume of sample that can be probed. Furthermore, the low spectral irradiance of incoherent light sources can limit the time resolution of measurements, due to the length of time required to build up a sufficiently large signal at the instrument's detector. These problems can be overcome using intense, spatially coherent, broadband supercontinuum light sources (described in the next section).

1.4.2.2 Supercontinuum sources

Supercontinuum (SC) sources combine high irradiance (and spatial coherence) with extremely broad spectral coverage (typically > 1000 nm) and are, therefore, of great interest in many areas of applied spectroscopy including those based on optical cavities.

SC generation describes a range of non-linear effects that occur upon propagation of short (typically sub-picosecond) intense light pulses through a non-linear medium, resulting in spectral broadening. The effects involved are governed by dispersion in the non-linear medium and can include Raman scattering, solitons, self-phase modulation and four-wave mixing. SC generation was first identified (by Alfano and Shapiro, in 1970)³⁸ as spectral broadening of light from a high-power picosecond Nd:glass laser, when it was focussed into solid crystals: the light was spectrally broadened, to both the red and blue of the laser's operating wavelength (530 nm), resulting in white (*ca.* 400-700 nm) light generation. Modern (commercially available) SC sources use photonic crystal fibers (PCFs) as the non-linear medium. PCFs guide light *via* index differences in a cladding matrix, with a similar structure to that of certain crystals. Crucially, the dispersion of PCFs can be specifically tailored for use at a variety of pumping wavelengths, durations and powers, to produce emission with extremely wide and well-controlled spectral coverage. A detailed description of the numerous types of pumping sources and PCFs available and their relative merits is beyond the scope of this thesis. For the interested reader, good overviews of SC generation methods and mechanisms in PCFs are given by Kaminski *et al.*³⁹ and

NKT Photonics (a commercial SC source manufacturer).⁴⁰ Recently, a number of SC-based BBCEAS studies (which include work in this project),^{12,37,41-43} and a BBCRDS study,⁴⁴ have been performed in the condensed phase. The SC sources used in this project are characterized in Chapter 2 and results of individual experiments are described in Chapters 3, 4 and 8.

1.5 Cavity design

In addition to the light-source considerations described in Section 1.4, the cavity stability (described below) must also be considered when designing any basic condensed-phase CRDS or (BB)CEAS setup.

There are several varieties of the simple two mirror linear cavity arrangement, the properties of which depend on the radii of curvature of the individual cavity mirrors (r_1 and r_2) and the cavity length (L). Cavities used for CRDS and CEAS must form stable optical resonators to avoid unnecessary light leakage. A stable cavity is one in which light is refocused after each pass/round trip between the mirrors. To achieve this, practical cavities incorporate at least one concave element and satisfy the optical stability condition:

$$0 < \left(1 - \frac{L}{r_1}\right) \left(1 - \frac{L}{r_2}\right) < 1 \quad (1.25)$$

A variety of common stable cavity geometries and their relative merits have been discussed in detail elsewhere.¹³

When designing extremely high-finesse cavities for excitation by narrowband laser sources (where $\Delta \nu_{\text{source}} < \Delta \nu_{\text{FSR}}$) the specific stable cavity geometry can be of vital importance. For example, in a so called ‘confocal’ geometry, where ($L = r_1 = r_2$), transverse modes are suppressed and only longitudinal modes of the cavity are excited, making light-coupling into the cavity experimentally challenging (see also Sections 1.3 and 1.4). However, choosing a cavity with geometry far from confocal can result in a so-called ‘white’ cavity, with an

extremely dense cavity mode structure (an extremely large number of transverse modes are excited, filling in the frequency space between adjacent longitudinal modes). A white cavity exhibits a frequency independent response and may, therefore, readily be excited using a narrowband laser. The choice between cavity geometries can also be important when minimising diffraction losses or the excitation of higher order transverse modes.

Whilst the above considerations of specific stable cavity geometries are important for many applications (particularly in the gas phase), the majority of condensed-phase CRDS and CEAS studies do not employ narrowband lasers as cavity light sources (see Sections 1.4 and 1.6). Additionally, in condensed-phase applications of CRDS/CEAS diffraction losses are typically minimal as losses are dominated by the mirrors, sample and any intracavity elements present. Therefore, provided they fulfill the stability condition, most condensed-phase cavity geometries are chosen to fit the specific experiment's requirements for sample constraint. A review of previous condensed-phase cavity-based techniques is given in the next section.

1.6 Implementation of CRDS and CEAS in condensed phases

Compared with the gas-phase, any implementation of CRDS or CEAS in the condensed phase must contend with higher solvent extinctions. As absorptive and scattering losses within any solvent also increase linearly with path length it is no longer clear that additional path length should lead to sensitivity gains. In fact, as already mentioned in Sections 1.2.1 and 1.2.2, significant background losses (a_b) in the condensed-phase must be folded into R in all of the aforementioned Equations, used to define A in CRDS and CEAS: $R = R_{\text{pure}}(1 - a_b)$. When a_b is large, $R \ll R_{\text{pure}}$ and increasing R_{pure} does little to increase the sensitivity of CRDS or CEAS.¹⁵

The caveats described above have led to a range of novel cavity designs, beyond those described in the previous sections. For example, optical cavities have been constructed

incorporating total internal reflection at a prism interface, to probe absorption within the evanescent field generated and study interfacial phenomena (see Section 1.6.2).^{29,45,46} Early applications of liquid-phase cavity-based techniques included the use of intra-cavity liquid cells placed at Brewster's angle to the cavity (optical) axis⁴⁷ or simply filling the cavity with liquid sample.^{48,49} More recently, various elegant approaches have been developed such as the use of liquid jets, or free-flowing soap films, aligned at Brewster's angle.^{50,51} However, the use of cuvettes remains the most common variant, both for the study of solution kinetics and, using precision optical cells, as an alternative to UV/visible spectroscopy in high-performance liquid chromatography (HPLC) detection.^{15,16,33-35,47,52-54}

The design of a condensed-phase CRDS/CEAS cavity often requires a compromise between a number of factors, including sensitivity, sampling volume and experimental simplicity. As a result, cavity designs tend to be tailored to a specific application. A more detailed review of previous condensed-phase CRDS and CEAS applications and associated cavity designs is presented in the next section.

1.6.1 Liquid-phase CRDS and CEAS

The development of cavity-based techniques in the liquid-phase has been driven largely by the demand for low-cost, highly sensitive detection methods in analytical chemistry. However, in future it is likely that optical cavities will find many other condensed-phase applications, along the lines of those reported in later chapters of this thesis. To place the developments achieved during this project in context, a broad review of previous liquid-phase CRDS and CEAS studies is given below.

1.6.1.1 Liquid-phase CRDS

Previous liquid-phase CRDS studies are summarized in Table 1.1 and described below.

Conceptually, the simplest liquid-phase implementation of CRDS is achieved by filling the cavity with the liquid analyte (Figure 1.6(a)). Pioneered by Zare and co-workers⁴⁸ this ‘wet-mirror’ approach has been applied to trace detection,^{30,55-57} solution reaction kinetics,⁴⁹ and eluent detection in LC.^{56,57}

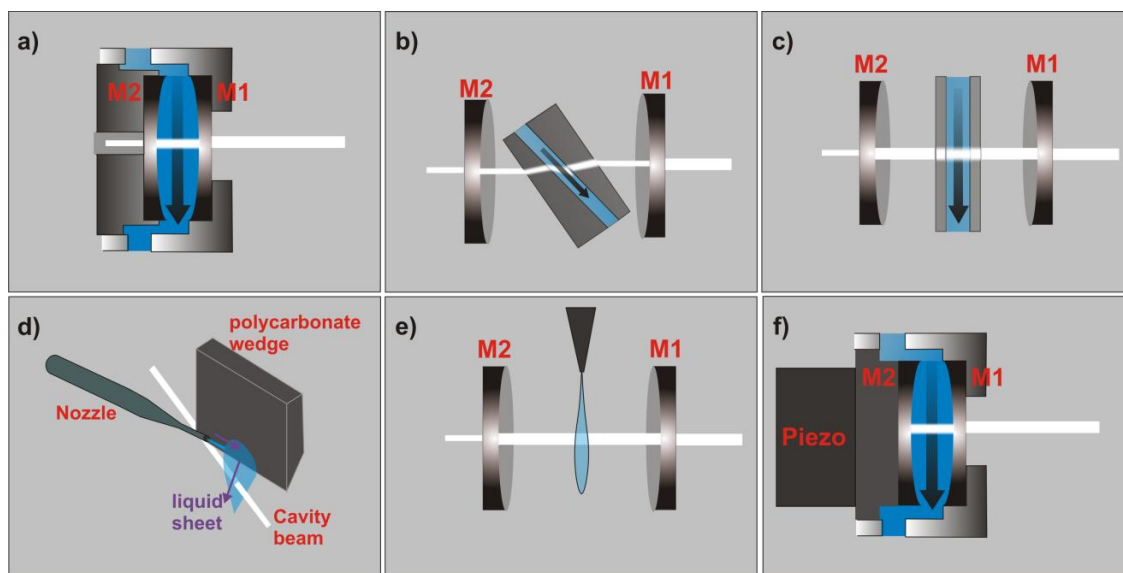


Figure 1.6 Various methods used to constrain liquid-phase samples within optical cavities for CRDS/CEAS studies (see text for details): a) wet-mirror cavity, b) Brewster's angle flow cell, c) normal incidence flow cell, d) liquid-sheet jet, e) soap film, f) high-finesse wet mirror cavity.

In the first wet-mirror cavity study (Hallock *et al.*⁴⁸ summarized in Table 1.1) the absorbance of various analytes, in a range of organic solvents, was measured with a high sensitivity ($\alpha_{\min} = 1 \times 10^{-6} \text{ cm}^{-1}$). In a follow-up study by the same group, a similar cavity design ($L = 23 \text{ cm}$) was used in conjunction with a broadband CW diode laser.³⁰ A very high measurement repetition rate (10 KHz) was achieved *via* acousto-optic modulation of the input beam, allowing extensive signal averaging and sensitivities comparable to those achieved in the analogous (pulsed laser) experiments described above,⁴⁸ whilst using a relatively small, inexpensive light source (see Section 1.4.1.2). The comparatively high temporal resolution available to this CW-CRDS variant was subsequently applied to study the reduction kinetics of methylene blue by ascorbic acid in acetonitrile.⁴⁹

In the studies described above, the presence of the solvent does not appear to interfere with the high-reflectivity mirrors needed for CRDS, or significantly alter their performance. However, for the cavity lengths used (*ca.* 20 cm), it is important to ensure that any solvent itself has negligible extinction in the wavelength region of interest, as this will scale linearly with the increased path length. At the wavelengths (620-670 nm) used by Zare and co-workers, spectrophotometric grade acetonitrile, hexane, toluene and cyclohexane showed enough optical transparency for use as solvents in the cavity, whereas methanol, ethanol, tetrahydrofuran, carbon tetrachloride and water exhibited too much background absorption (Figure 1.7 shows the absorption spectrum of water). Additionally, such large mirror separations necessitate large sample volumes, making this approach unsuitable for many analytical applications, such as eluent detection in HPLC.

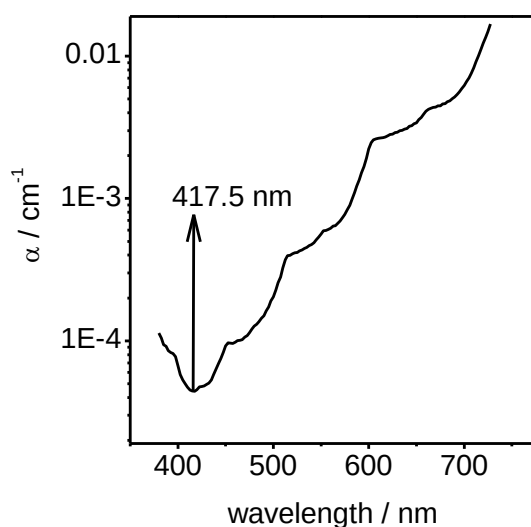


Figure 1.7 absorption spectrum of water, indicating a minimum at 417.5 nm. The graph was generated using data (obtained using an integrated cavity absorption meter) by Pope & Fry.⁵⁸

To minimize per-pass light losses and sample volumes in a wet-mirror arrangement, van der Sneppen *et al.*^{56,57} and Bahnev *et al.*⁵⁵ employed miniresonator wet-mirror cavities, with mirror spacings of 2 mm and internal volumes of 12 μL (see Table 1.1). Practically, this was achieved using a silicon rubber spacer, clamped leak-tight between the mirrors, forming the low-volume sample cell. The miniaturized CRDS technique was successfully implemented as a detection

method in conventional-sized liquid chromatography; 50 μL sample plugs flowed through the cell at a rate of 0.8 mL/min. Using $R = 0.99996$ mirrors at $\lambda = 532\text{ nm}$, the detection limit of the CRDS setup was found to be thirty times smaller when compared directly with a commercially available UV/Vis absorption detector.⁵⁶ The miniresonator-based CRDS approach was later extended to absorbance detection in the UV (at $\lambda = 355\text{ nm}$).⁵⁷ However, at these wavelengths, the sensitivity of CRDS is limited by the lack of suitably-coated commercially available high reflectivity mirrors (van der Sneppen *et al.*⁵⁷ were limited to mirrors with $R = 0.9995$ at 355 nm).

The main drawback of the miniresonator approach described above is the very short ring-down times (tens of nanoseconds—resulting from the use of small cavity lengths)—the utilization of which requires a fast instrument response (short laser pulses and fast sampling electronics), complicating the setup and potentially limiting the accuracy by which the ring-down time can be determined.

Where solvent absorption is a problem, or small sample volumes are required, the use of intracavity absorption cells offers a compromise solution. The first use of an intracavity cell (oriented at Brewster's angle with respect to the cavity axis) was by Xu *et al.* in their overtone study of liquid benzene.⁴⁷ Since then, custom-built cells have been developed in which every surface is presented at Brewster's angle to the cavity axis (Figure 1.6(b) and Table 1.1). These cells offer much improved sensitivity to UV/visible spectroscopy in the detection of components separated by HPLC, although calibration may be required when the solvent and solute (or multiple solvents, in a gradient separation) have different refractive indices.^{53,54} Whilst Brewster's angle arrangements seek to minimise reflections at the cell's interfaces, which would otherwise result in significant light losses from the cavity, this problem may be circumvented by using a standard cuvette, as an intracavity sample cell, in a normal incidence geometry, to trap back-reflections from the cell windows within the cavity (Figure 1.6(c)).⁵⁹ This

normal incidence geometry approach removes the need for expensive custom-designed flow cells and can be applied to study a wide range of solutions with different refractive indexes. However, in order to minimise baseline per-pass light losses, the cell must be well aligned within the cavity; intracavity cell alignment using a normal incidence geometry is more challenging than the more forgiving Brewster's angle arrangement¹⁵ and a high degree of parallelism and low surface roughness of the cell windows is also important.

It is also possible to perform liquid studies without any cell at all, as Alexander demonstrated with a novel "cell-free" liquid CRDS geometry making use of flowing liquid sheets (Figure 1.6(d)).⁵⁰ This approach is based upon liquid jet technology previously employed as the lasing medium in CW ring dye lasers and uses a jet of ethylene glycol, directed at a polycarbonate block, to form a liquid sheet oriented at Brewster's angle within an optical cavity. Whilst the sensitivity of this approach compares well with other liquid-phase CRDS studies (see Table 1.1), the high flow rate (*ca.* 200 ml min⁻¹) necessitates large total sample volumes and thus makes the technique unsuitable for certain applications. In an attempt to avoid this problem, Vogelsang *et al.* incorporated a slowly flowing (1.2 mL min⁻¹) soap film within an optical cavity.⁵¹ The soap film was generated by flowing a sodium dodecyl sulfate (SDS) solution over a simple oval-shaped wire loop, mounted vertically at Brewster's angle within the cavity (Figure 1.6(e)). This simple approach couples a small per-pass optical path length (9 μ m) and detection volume (*ca.* 28 nL) with an impressive absorbance sensitivity (4×10^{-6}).

Very recently, Kiwanuka *et al.* performed liquid-phase BBCRDS using an SC light source and a linear optical cavity containing a home-built intracavity cell (the windows of which could be independently adjusted to ensure an optimal alignment at normal incidence to the cavity axis).⁴⁴ The instrument employs a custom built avalanche photodiode array to simultaneously record ring-down signals (with nanosecond time resolution) in up to 64 different spectral channels and enables BBCRDS with a sensitivity comparable to that achieved using the

narrowband CRDS techniques described above and the (BB)CEAS techniques described in the next section.

In this project, time-resolved intracavity cuvette-based CRDS measurements, of photo-induced reactions and their magnetic field effects in solution, are demonstrated with an absorbance sensitivity on the order 10^{-6} (see Chapter 7).

1.6.1.2 Liquid-phase CEAS

Liquid-phase applications of CEAS (summarized in Table 1.1) are even scarcer than those of CRDS, despite the fact that larger dynamic ranges may be achieved. Most liquid-phase CEAS studies are variants of BBCEAS and seek to make use of the technique's multiplexing capabilities to record entire absorption spectra in a single measurement (see also Section 1.2.3). Fiedler *et al.* were the first to apply (BB)CEAS to the condensed phase, using a modified commercially-available double beam UV/Vis spectrometer, which had been retrofitted with an optical cavity to multiply reflect the instrument's sample beam through a cuvette oriented at normal incidence to the cavity axis.^{15,16} Additionally, by monitoring the reference beam of the spectrometer, background intensity fluctuations in the light source could be removed. Using these simple modifications, the sensitivity of the UV/Vis instrument was improved by a factor of *ca.* 10 (to give $\alpha_{\min} = 2 \times 10^{-5} \text{ cm}^{-1}$). In the same study, a BBCEA spectrometer was also constructed from scratch, using a Xe-arc lamp. The high-luminosity of this light source allowed further improvements in SNR and minimum detectable absorbance ($A_{\min} = 3 \times 10^{-6}$). In the present project, comparable sensitivities have been demonstrated using supercontinuum radiation light sources in BBCEAS variants (see Chapters 2-4 and 8).

Other liquid-phase BBCEAS studies have employed LED sources to study absorbance of analytes constrained within intracavity cuvettes, an HPLC cell and a liquid-filled cavity.³³⁻³⁵ LEDs provide a relatively low-cost and robust alternative to the commercial lamps used by Fiedler *et al.*¹⁵—although they are typically less powerful and have a narrower bandwidth than a lamp.

Importantly, as demonstrated by Seetohul *et al.* by coupling a broadband light source (in this case an LED) with the multiplex detection capability of a charge coupled device (CCD), BBCEAS allows the spectral separation of multiple liquid-phase analytes with overlapping absorption profiles.³⁴ It is envisaged that in future this powerful additional feature of BBCEAS will find more applications to the condensed phase.

Using a similar optical cavity setup to that for the SC-BBCRDS study described in Section 1.6.1.1, Kiwanuka *et al.* performed liquid-phase BBCEAS,⁴³ with SC light (ca. 480-700 nm) transmitted through the cavity and coupled to a grating spectrograph and CCD camera. This approach was used to make spectrally and temporally resolved measurements of oscillating absorbance during a Belousov–Zhabotinsky reaction.

In the studies described above, the CEF (see Section 1.2.2) and, in turn, the absolute absorbance spectrum, can in principle be calculated *via* a comparison of the cavity-enhanced absorption spectrum and a single-pass spectrum (appropriately scaled to the cavity concentration, C , and per-pass path length, l , see Equation 1.23). The single pass spectrum can be acquired easily using a UV/Vis spectrometer or simply by removing the cavity from the BBCEAS instrument.

Finally, it is important to note that the experimental difficulty in (BB)CEAS does not increase with decreasing cavity mirror separation (unlike CRDS, See Section 1.6.1.1). With this in mind McGarvey *et al.*⁶⁰ used a wet-mirror CEAS cavity with an extremely small mirror separation (equal to the per-pass path length through solution, 0.175 cm) and a microlitre-sized sample volume, in conjunction with a single mode probe laser locked to the cavity resonance (*via* piezo-control of the cavity's rear mirror, see Figure 1.6(f)), to achieve an extremely high finesse and sensitivity. However, this setup lacks the experimental simplicity and broadband capability associated with the BBCEAS studies described above, which are key to the studies described herein.

Study	Tech- nique	Approach	Cavity Light source	R	Baseline τ /ns, or CEF	λ /nm	$l \mid L$ /cm	Total Volume /dm ³	10^6 $\times A_{\min}$ /A.U	$10^6 \times$ $\alpha_{\min}$ /cm ⁻¹	LOD /M	$10^{-4} \times \epsilon$ /M ⁻¹ cm ⁻¹
Hallock ⁴⁸	CRDS	Wet mirror	Pulsed: Nd:YAG- pumped dye	0.9998	400	620-670	21 21	5×10^{-2}	9 (3 σ)	1 (3 σ)	<i>ca.</i> 1 $\times 10^{-11}$	10
Hallock ^{30,49}	CRDS	Wet mirror	CW: BB diode laser	0.9998	100	655	23 23	6×10^{-2}	3.3 (1 σ)	0.33 (1 σ)	---	---
van der Sneppen ⁵⁶	CRDS	Wet mirror	Pulsed: Nd:YAG	0.99996	65–75	532	0.2 0.2	1.2×10^{-5}	2.7	31	$1.5 - 2.0 \times 10^{-8}$	1-1.4
van der Sneppen ⁵⁷	CRDS	Wet mirror	Pulsed: OPO	0.99993	70–80	457	0.2 0.2	1.2×10^{-5}	2.7	31	1.7×10^{-8}	1.5
				0.9995	20–25	355	0.2 0.2	1.2×10^{-5}	13	150	7.5×10^{-8}	1.02
Bahnev ⁵⁵	CRDS	Wet mirror	Pulsed: Nd:YAG	0.9998	20–30	532	0.2 0.2	1.2×10^{-5}	27	310	2.5×10^{-9} (2 σ)	5.45
Xu ⁴⁷	CRDS	Cuvette, single or double, Brewster's	Pulsed: Excimer- pumped dye	0.9997	<i>ca.</i> 800	590 – 620	1 48	---	0.09	0.2	---	----
Alexander ⁵²	CRDS	Cuvette, Brewster's	Pulsed: Nd:YAG- pumped dye laser	---	---	635	---/---	---	---	---	---	---

Table 1.1 A comparison of previous liquid-phase absorbance measurements using optical cavity-based techniques. In some cases $A_{\min}$ has been calculated from $\alpha_{\min}$, or *vice-versa*.

Study	Technique	Approach	Cavity Light source	R	Baseline τ /ns, or CEF	λ /nm	$l \mid L$ /cm	Total Volume /dm ³	$10^6 \times A_{\min}$ /A.U	$10^6 \times \alpha_{\min}$ /cm ⁻¹	LOD /M	$10^{-4} \times \epsilon$ /M ⁻¹ cm ⁻¹
Snyder ⁵³	CRDS	Cuvette, True Brewster's	Pulsed: OPO	0.9993	2500	470	0.03 100	1×10^{-5}	3.2	246	3×10^{-8} (3 σ)	0.9
Bechtel ⁵⁴	CRDS	Cuvette, True Brewster's	CW: narrowband diode laser	---	5800	488	0.03 100	1×10^{-5}	0.2	15	---	---
van der Sneppen ⁵⁹	CRDS	Cuvette, normal incidence	Pulsed: Nd:YAG or Nd:YAG-pumped dye laser	0.9998	60–70	355	1 4	8×10^{-5}	13	30	4.8×10^{-8}	0.73–1.02
				0.9991	12–18	273	1 4	8×10^{-5}	420	970	$0.5 - 3 \times 10^{-6}$	---
Alexander ⁵⁰	CRDS	Liquid sheet	Pulsed laser	≥ 0.999	2545	628	2.3×10^{-3} 90	---	0.16	16200	7.1×10^{-8} (3 σ)	10
Vogelsang ⁵¹	CRDS	Soap film	Pulsed: Nd:YAG	0.9996	2600	355	9×10^{-4} 30	2.8×10^{-8} (detection)	4	10000	5.5×10^{-7}	0.8
Kiwanuka ⁴⁴	BBCRDS	Cuvette	SC	0.99	101	ca. 100 nm range in visible	5.4 42	2.7×10^{-3}	75	32	1.2×10^{-10}	11.6
Fiedler ^{15,16}	BBCEAS	Cuvette, normal incidence	Tungsten or Xe-arc lamp	0.99	---	450 - 650	1 (0.1) 8	---	9 (W lamp), 1 (Xe-arc lamp)	20 (W lamp), 3 (Xe-arc lamp)	---	---
Islam ³³	BBCEAS	Cuvette, normal incidence	LED	0.99, 0.9945 0.999	104 (CEF, $R = 0.999$)	420-670	0.2 5	---	4.5 – 100	51 – 1200	6.2×10^{-10}	10
McGarvey ⁶⁰	CEAS	Wet mirror	Ti:S	0.99998	ca. 10800 (CEF)	783	0.175 0.175	ca. 10^{-8} (detection)	ca. 0.5	ca. 7	2×10^{-10}	6
Seetohul ³⁴	BBCEAS	Cuvette, normal	LED	≥ 0.99	45 (CEF)	450 – 600	1 5	7×10^{-5}	8.3	19	2.71×10^{-10}	9.3
Seetohul ³⁵	BBCEAS	Wet mirror	LED	0.999	429 (CEF)	630–670	20 20	9.8×10^{-2}	2.4	0.28	1.2×10^{-11}	7.9
Kiwanuka ⁴³	BBCEAS	Cuvette	SC	0.99	---	480–700	5.4 30	2.7×10^{-3}	21	9.1	2×10^{-10}	2

Table 1.1 (continued) A comparison of previous liquid-phase absorbance measurements using optical cavity-based techniques.

1.6.2 Evanescent wave CRDS and CEAS

Evanescent wave variants of CRDS were some of the earliest (and most successful) attempts to harness the power of CRDS to study condensed-phase and interfacial phenomena. EW-based optical cavities are the subject of Chapter 3 and only the key principles of the technique are outlined below.

EW techniques, like attenuated total internal reflection (ATR) spectroscopy,⁶¹ rely on the evanescent field established when light undergoes total internal reflection (TIR) at the interface with an optically less dense medium. To achieve TIR, the angle of incidence (θ , measured from the normal to the interface) must exceed the critical angle, θ_c , defined by the refractive indices n_1 and n_2 of the two media (see Figure 1.8):

$$\theta_c = \sin^{-1} \left(\frac{n_2}{n_1} \right) \quad (1.26)$$

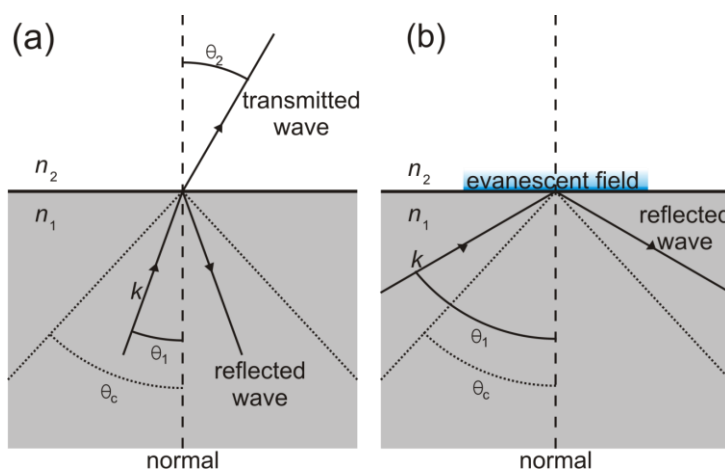


Figure 1.8 An interface comprising two media with refractive indices $n_2 < n_1$. If the angle of incidence, θ_1 , of an incoming electromagnetic wave, K , is smaller than the critical angle, θ_c , the wave is both reflected and transmitted at the interface (a). If $\theta_1 > \theta_c$, then the wave is totally internal reflected, generating an evanescent field beyond the interface (b).

If a cavity can be constructed incorporating TIR at a suitable interface, then any light circulating within the cavity will be sensitive to absorptive or scattering losses within the evanescent field. The amplitude of the evanescent wave decays exponentially beyond the interface (into the

optically less dense medium, n_2): The amplitude decays to $1/e$ of its maximum value at a distance d_p (the penetration depth), where:⁶²

$$d_p = \frac{\lambda}{2\pi \sqrt{n_1^2 \sin^2 \theta - n_2^2}} \quad (1.27)$$

d_p ranges smoothly from infinity (for $\theta \leq \theta_c$) to $\lambda/2\pi\sqrt{n_1^2 - n_2^2}$ as θ approaches $\pi/2$.

Depending on the wavelength of the source, the difference of the refractive indices of the two media and the angle of incidence, the penetration depth typically ranges from 50–500 nm (see, for example, Figure 1.9).

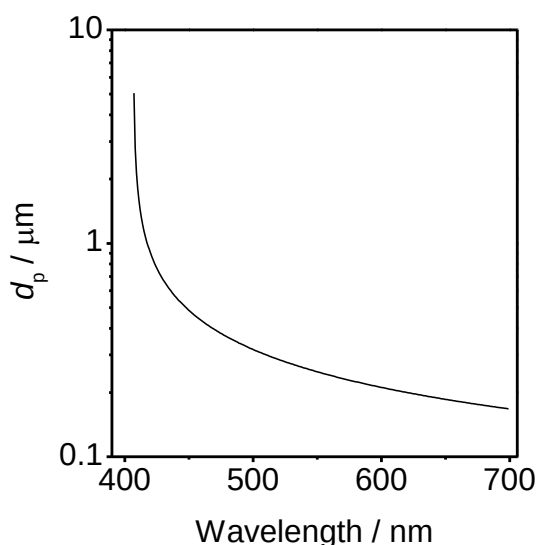


Figure 1.9 EW penetration depth as a function of wavelength, calculated using: $\lambda = 400 - 700$ nm, $\theta = 72^\circ$. n_1 and n_2 are the (wavelength dependent) refractive indices of fused silica and water (at 25°C), respectively.^{63,64}

n_1 , 400–700 nm = 1.47012....1.45529 and
 n_2 , 400–700 nm = 1.399....1.331

Of course, the total depth of sample probed is significantly greater than d_p . In EW-CRDS and EW-CEAS, as in ATR, it is convenient to consider the measured absorbance, as determined by Equations 1.13 and 1.21 respectively, as $A = \epsilon C d_e$ where d_e , the effective thickness, is the equivalent thickness of solution or film required to produce the same absorbance in a conventional transmission measurement at normal incidence. Under typical conditions $d_e \approx 3d_p$ (see also Section 3.2.2). The inherent interfacial layer sensitivity of EW-CRDS and EW-CEAS renders these techniques ideal for the study of surface processes. For this purpose a variety of cavity configurations, described in Chapter 3, have been employed.

1.6.3 Other condensed-phase applications

EW and solution-phase cavity-based techniques are the subject of the remainder of this thesis. However, for completeness it is first worth noting other condensed-phase applications in passing.

Several early manifestations of condensed-phase CRDS involved coating the cavity mirrors or intra-cavity elements with thin films of interest. Engeln *et al.* studied solid C₆₀ films on transparent ZnSe windows using the far infrared radiation of a free electron laser⁶⁵ while Kleine *et al.* coated the cavity mirrors directly with molecular films of iodine.⁶⁶ These techniques have been developed by several other groups for applications as diverse as structural determination of sub-monolayer dye films,⁶⁷ the study of defects in amorphous silicon films⁶⁸ and ultra-high vacuum studies of deposited metal clusters.⁶⁹ In many of these variants, placing the intra-cavity elements at Brewster's angle significantly reduced reflection losses and thereby enhanced sensitivity.

The condensed-phase variants described in the previous sections employ optical cavities based on conventional high reflectivity mirrors. However, the use of simple optical fibers is also possible. Light is confined within fiber-based cavities using either reflective elements (such as reflective coatings on the ends of fiber strands, or fiber Bragg gratings (FBGs)), or *via* fiber loops (in which light travels around a fiber, the ends of which are spliced together to form a loop). In both types of fiber-based cavity, a small gap in the fiber can be used as a sample cell. The gap size is typically on the order of tens of micrometers. Hence, fiber-based cavities are suitable for use with nanoliters or even picoliters of sample—considerably smaller than most volumes employed in conventional mirror-based cavities (see Table 1.1). However, at the present time, the sensitivity of such fiber-based approaches is relatively poor ($\alpha_{\min} \approx 10^{-1} \text{ cm}^{-1}$),

due to large per-pass losses at splice connectors and input and output couplers (although very recent developments are addressing these issues).⁷⁰ For the interested reader, a good review of fiber-based cavities is provided by Waechter *et al.*⁷¹

1.7 Thesis scope and objectives

This chapter has outlined the basic principles of CRDS and (BB)CEAS, with a focus on condensed-phase applications. The remainder of this thesis describes the development and application of novel CRDS-, CEAS- and BBCEAS-based techniques to condensed-phase systems. The overall concept of this project is to explore the use of cavity-based techniques in new liquid-phase applications. A large portion of this thesis focuses on the use of cavity-based techniques in detecting magnetic field effects in radical recombination reactions, as funding was obtained (from the Defense Advanced Research Projects Agency and the EMF trust) to explore these avenues. A brief summary of the proceeding chapters is given below.

Chapter 2 provides an overview of the cavity-based instruments used in this project. The various light sources, cavity mirrors and detection methods used are discussed, with a particular focus on the (atypical) instrumentation used in (EW-)BBCEAS.

In Chapter 3, EW-CRDS and EW-CEAS, introduced in Section 1.6.2, are covered in greater detail. The relative merits of different configurations for EW-CRDS and EW-CEAS are discussed, along with a review of previous EW cavity-based studies. The comparatively new, but complementary, cavity technique of EW-BBCEAS is introduced and its application to spectroelectrochemistry is demonstrated. Finally, the potential for extending the evanescent wave technique to probe other interfaces is exemplified with a description of initial interfacial absorbance measurements at a water|air interface.

Chapter 4 describes the first application of BBCEAS to the analysis of microfluidic samples flowing through a microfluidic chip. Proof-of-principle experiments are presented, demonstrating the technique's ability to provide full visible broadband spectral measurements with both high detection sensitivity and excellent temporal and spatial resolution.

Chapter 5 provides an introduction to radical recombination reactions and associated magnetic field effects (MFEs) in solution, which are studied using cavity-based techniques in later chapters. The physical origins and the chemical and biological importance of these field effects are discussed. Finally, this chapter outlines the need for a step-change in methodology—and the potential of cavity-based techniques—to extend MFE studies beyond model systems to biomolecular systems of interest.

Chapters 6, 7 and 8 describe the powerful capabilities of CEAS, CRDS and BBCEAS, respectively, in monitoring radical recombination reactions and associated MFEs in solution.

Chapter 6 describes the combination of CEAS with phase-sensitive detection, used to study MFEs in a chemical system. MFE data obtained using this modulated CEAS technique are shown to be superior to those obtained using conventional transient absorption (TA) flash photolysis methods typically employed for these measurements. In Chapter 7, CRDS-measured MFEs are observed in pump-probe experiments and discussed in terms of the sensitivity gains and sample-volume minimization afforded by CRDS when compared with single-pass TA methods conventionally applied. Finally, Chapter 8 describes a newly developed BBCEAS instrument and its use in making the first observations of a magnetic field effect in an animal cryptochrome.

Each chapter ends with a conclusion, summarising the work reported and looking to future developments in the (chapter-specific) field. A final conclusion is also given at the end of this thesis, outlining the present state of condensed-phase cavity-based techniques and making

some general comments about how the work described in this thesis might be applied/developed in the future.

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Chapter 2

Experimental

This chapter is designed to act as a guide to the numerous experimental schemes described in later chapters. It provides a summary of the various cavity-based techniques employed in this project, along with the cavity geometries used for specific experiments. Although the majority of experimental details are provided in later chapters, some of the apparatus (namely the light sources, cavity mirrors and photodetectors) used are also discussed in the present chapter.

2.1 Cavity-based techniques and geometries

Table 2.1 summarizes the design and application of the various cavity-based techniques and geometries used in this project. The table also includes citations to articles in which these various experiments are described.

2.2 Cavity light sources

Three different types of cavity light source were used in this project. Cavity techniques (a)-(c) in Table 2.1 used either CW (diode, linewidth *ca.* 1 nm) or Nd:YAG (pulsed) laser light sources, whereas for BBCEAS (technique (d) in Table 2.1) supercontinuum radiation sources were employed.

Diode and Nd:YAG lasers are commonly used in condensed-phase CRDS and CEAS (see Table 1.1) and those used in this project are described only briefly below and in the relevant chapters. However, the use of SC sources in condensed-phase BBCEAS is atypical and their use warrants the discussion in Section 2.2.1.

Two laser diodes were used interchangeably depending on the experiments being performed: both are Power Technology CW diodes operating nominally at 405 nm (*ca.* 1 nm broad linewidth). Diode 1 (model: 1Q1H50) emits approximately 50 mW, diode 2 (model 1Q1H350) approximately 350 mW. Both allow transistor-transistor logic (TTL), digital beam modulation from CW to 100 MHz, with rise/fall times of *ca.* 2 ns.

The Nd:YAG laser is a Continuum Minilite laser. The fundamental output of the Nd:YAG laser at 1064 nm is frequency-doubled to produce pulsed (~ 10 Hz, 5 ns pulsewidth) radiation at 532 nm, emitting approx 25 mJ at maximum power.

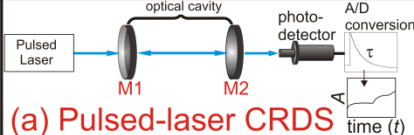
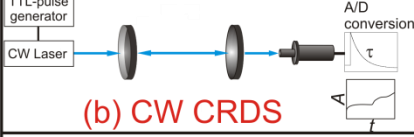
Cavity-Based Technique	Cavity Geometry			
	EW (prism)	EW (water-air)	Flow cell	μ L chip
 (a) Pulsed-laser CRDS			Monitoring magnetic field effects (Chapter 7) ¹	
 (b) CW CRDS		Monitoring interfacial absorbance of a Langmuir film (Chapter 3) ²	Monitoring absorbance of photochemically generated radicals (Chapter 6) ³	
 (c) CEAS		Monitoring interfacial absorbance of a Langmuir film (Chapter 3) ²	Monitoring magnetic field effects (Chapter 6) ³	
 (d) BBCEAS	Monitoring electrogenerated Ir(IV) complexes in a thin layer electrochemical cell (Chapter 3) ⁴	Monitoring interfacial absorption spectrum of a Langmuir film (Chapter 3)	Monitoring magnetic field effects, as a function of wavelength (Chapter 8)	Monitoring absorption spectrum of microfluidic droplets (Chapter 4) ⁵

Table 2.1 Project summary.

2.2.1 Supercontinuum source specifications

Three different commercial SC sources, summarized in Table 2.2, were used as light sources for BBCEAS instruments in this project. Their specifications differ slightly, although in practice this had little effect on their relative applicability to experiments reported in this thesis. For example, although there appears to be a significant difference in their output power, the spectral irradiance of all three sources is so high that each BBCEAS instrument was capable of

operating well-above the detector shot-noise level. Additionally, the detectors used in combination with the SC sources (based on CCDs and described in Section 2.5) limited the instrument's time-resolution to (at best) ten microseconds (see Section 3.2.1), meaning that individual SC pulses could not be temporally resolved and the individual SC source repetition rate and pulsewidth made no practical difference to the experiments reported in this thesis. In other words the SC sources were operated as pseudo CW sources.

However, for several of the experiments reported in this thesis, there was an advantage to having a blue-wavelength-enhanced SC broadband output ($\lambda < 450$ nm, see Table 2.2). For example, Section 8.3.3 describes the use of blue light (from the NKT SC source) for photoexciting an intracavity sample. Additionally, the relatively small diameter of the NKT source beam (< 1 mm) was advantageous in its application to a μL chip-based cavity (see Chapter 4). The emission spectrum (from 400-1750 nm, provided by the manufacturer) of the NKT SC source is shown in Figure 2.1.

	Fianium SC400-2	Fianium SC450-4	NKT SuperK Power Blue
Broadband output	<i>ca.</i> 400-2200 nm	<i>ca.</i> 450-2200 nm	<i>ca.</i> 425-2100 nm
Average output power	2 W	4 W	3.5 W (<i>ca.</i> 300 mW in visible)
Repetition rate	20 MHz	40 MHz	80 MHz
Pulsewidth	< 8 ps (fundamental)	< 8 ps (fundamental)	5 ps (fundamental), <i>ca.</i> 50 – 450 ps (exiting PCF)
Beam diameter	<i>ca.</i> 1.5 mm	<i>ca.</i> 1.5 mm	<i>ca.</i> 0.8 mm
Pump source and wavelength	1064 nm mode-locked fiber laser	1064 nm mode-locked fiber laser	1064 nm mode-locked fiber laser
Emission spectrum	See Appendix	See Appendix	See Figure 2.1
Experimental application	EW-BBCEAS solid liquid interface (Chapter 3)	EW-BBCEAS liquid air interface (Chapter 3)	BBCEAS microfluidic chips (Chapter 4) BBCEAS MFE detection (Chapter 8)

Table 2.2 Summary of SC light sources used in this project.

To some extent, there is a price to be paid for the intense broadband coverage described above: due to the non-linear processes involved in their operation, SC sources exhibit large

shot-to-shot intensity- and spectral-fluctuations. Therefore, as discussed later, in Section 2.7, it is no longer clear that signal averaging will increase measurement sensitivity.

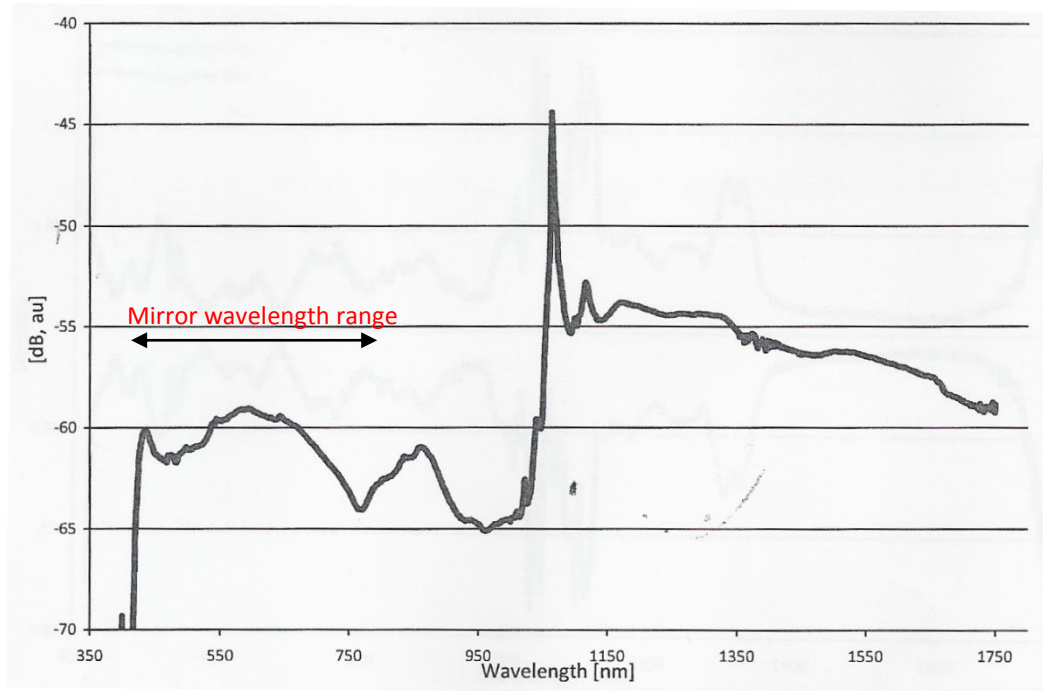


Figure 2.1 Emission spectrum of light exiting the NKT SC source photonic crystal fiber, measured using an ANDO 6315B optical spectrum analyzer and Ocean Optics integrating sphere. Figure taken from NKT SuperK Blue data sheet, provided by the manufacturer. The wavelength range of the cavity mirrors used for BB studies is shown (see also Section 2.4).

2.3 Beam delivery and Cavity alignment

The laser diode beams are typically collimated, shaped and polarized using various stand-alone optics and delivered *via* open beam paths to any one of a variety of different experimental arrangements, described later.

In all SC source applications, the master source and photonic crystal fiber of the SC source are entirely contained within the SC source unit. The (randomly polarized) light exiting the source's PCF was collimated (using an optic, provided by the SC source manufacturer and attached to the end of the PCF) and spectrally filtered to provide light within a specific range, typically *ca.*

425 – 800 nm (see later chapters for details), to match the reflectivity range of the cavity mirrors (see Section 2.4).

Spectral filtering of the Fianium sources was achieved with a combination of stand-alone hot mirrors (see Section 3.2.1) positioned in the open beam path before the cavity. Spectral filtering of the NKT source was achieved using an integrated fiber delivery system (FDS), which couples light from the photonic crystal fiber to a passive spectral filter (SpectraK Split). The SpectraK Split is a plug and play filter box used to split visible and near-IR radiation output from the SC source. It produces two simultaneous outputs, *ca.* 400-900 nm and 900-2100 nm, the second of which is safely dumped within the SpectraK Split unit. Stand-alone filters (hot mirrors) can also be positioned in the open beam path before the cavity, to further modify the wavelength range of the light injected into the cavity. The spectral output of the visible port of the NKT's SpectraK Split is shown in Figure 2.2.

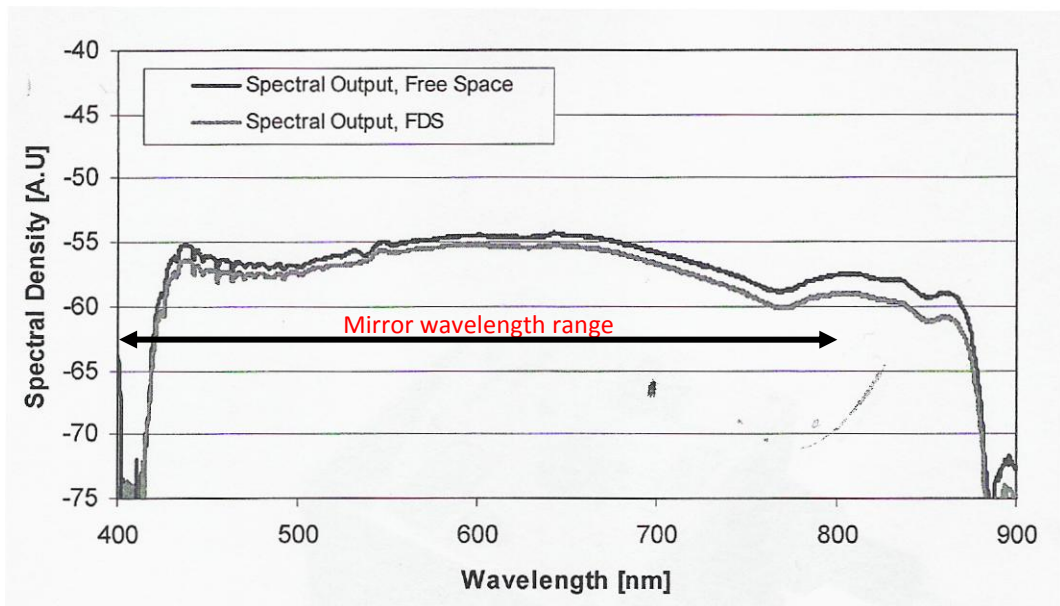


Figure 2.2 FDS and free space spectral outputs of the SpectraK split, provided by the manufacturer. The wavelength range of the cavity mirrors used for BB studies is shown (see also Section 2.4).

Cavity alignment was typically achieved in three steps. First, using a pair of suitably coated turning mirrors, or a multi-axis manual positioning stage, the laser beam is aligned through the

centre of a pair of pinholes and through the intracavity element of choice. The dimensions and positions of the pinholes are identical to those of the mirrors used to form the optical cavity. In the second step, the intracavity element is aligned in a normal incidence geometry, using a suitable manual positioning stage and observation of back-reflections from the element's surfaces. Finally, the pinholes are replaced with the cavity mirrors, which are then aligned (along with the intracavity element) in an iterative manner to maximize the signal at the detector. The necessary safety measures involved in these alignment steps are covered in the laboratory's Laser Safety documentation.

2.4 Cavity mirrors

All cavity mirrors used in this project rely upon interference effects between thin layers of dielectric coatings (on an optically transparent substrate) to produce an optimum reflectivity over a particular wavelength range. The reflectivity of cavity mirrors is thus highly wavelength dependent and typically drops off dramatically either side of the design wavelength.

Figure 2.3(a) shows the reflectivity profile of one of the traditional cavity ring-down mirrors used in this project (manufactured by Los Gatos Research and referred to throughout this thesis as 'mirror set 1'). The reflectivity spectrum is characterized by a relatively narrow region of extremely high reflectivity (in this case $R_{\text{pure}} \approx 0.999975$, four-9s mirrors), centered at 415 nm and spanning a wavelength range (to $R_{\text{pure}} = 0.99995$) of *ca.* 40 nm. While mirrors with such high reflectivity are frequently used in the gas phase, they are not necessarily the most appropriate choice for condensed-phase cavities: as discussed in Section 1.6, the additional absorption/scattering losses encountered in condensed-phase studies limit the effective mirror reflectivity. Additionally, using very high reflectivity cavity mirrors can be detrimental to the light intensity dependent signal-to-noise ratio at the detector. For these reasons it is often more appropriate (and significantly cheaper) to use mirrors with slightly lower reflectivity.

Mirror set 1 also has limited applicability to BBCEAS studies of condensed-phase species, whose absorption spectra can span hundreds of nanometers. Fortunately, however, custom mirror coating runs are now commercially available, which allow customization of the reflectivity profile.

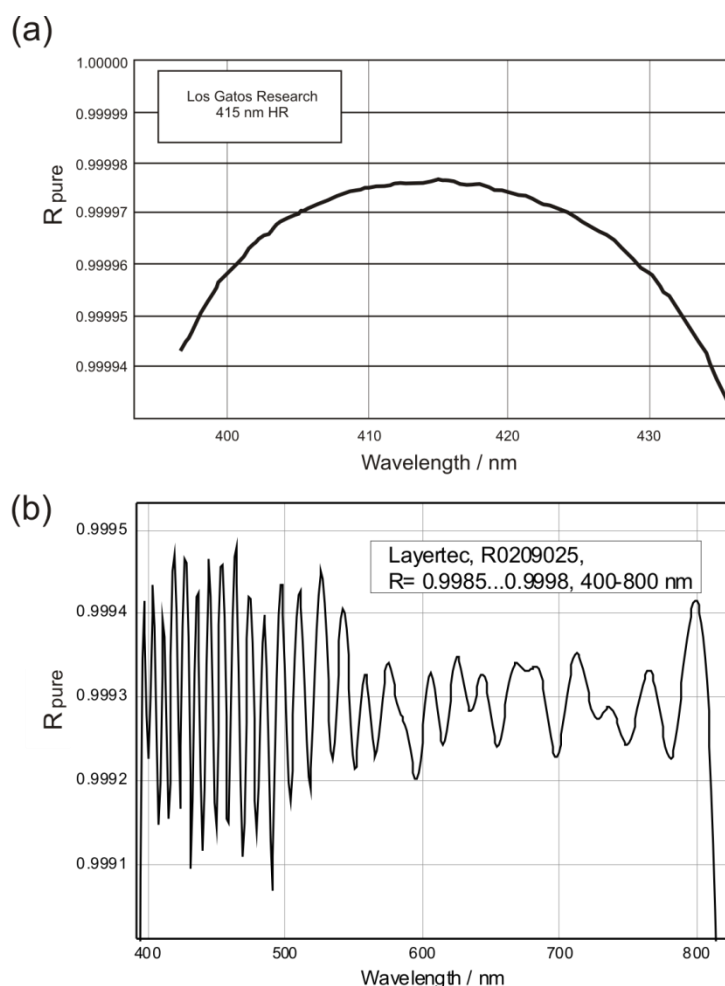


Figure 2.3 Reflectivity profiles (provided by the manufacturer) of (a) mirror set 1 and (b) mirror set 2 (see text for details).

The reflectivity profile of a custom-coated broadband mirror (manufactured by Layertec and referred to throughout this thesis as ‘mirror set 2’) used in this project is shown in Figure 2.3(b). The mirror is highly reflective ($R_{\text{pure}} > 0.999$) over the entire visible wavelength range with an average reflectivity of 0.9993. The reflectivity of mirror set 2 is also markedly lower than that of mirror set 1 described above (and, therefore, potentially better suited to

condensed-phase BBCEAS). However, as a result of the coating process, $R_{\text{pure}}(\lambda)$ has a significant amount of structure, which, if not correctly accounted for (by a calibration, such as that described in Section 1.6.1.2), can produce false structure in any absorbance spectrum calculated using Equation 1.23.

2.5 Photodetectors

For CRDS and CEAS experiments, which did not require spectral resolution, PMTs were employed as photodetectors. Two different PMTs were used (see later chapters): PMT 1 was an Electron Tubes photomultiplier (Q-9893), PMT2 was a Hamamatsu photomultiplier (R928). Both PMTs have blue-green sensitive photocathodes, high gain ($\times 10^6 - 10^7$) and a fast time response (*ca.* 2 ns rise time).

For BBCEAS experiments, spectral resolution was achieved using grating spectrographs and CCDs. Light exiting the cavity was focused (using an achromatic doublet pair) into an optical fiber (Thorlabs 400 μm , numerical aperture, NA, 0.39), coupled to the spectrograph, which dispersed its component wavelengths onto a CCD camera. The majority of BBCEAS experiments employed an Andor 303i spectrograph (303 mm focal length, 150 lines/mm, Czerny-Turner type, with motorized grating turret and input slit (10 μm to 2.5 mm) and a home-built lens-based F/# matcher for a NA 0.22 fiber): two CCD cameras were used independently, in combination with the Andor spectrograph. CCD 1 was an Andor iXon electron multiplying (EM)CCD (512 x 512 pixel array), which contains a frame transfer device, allowing entire images to be stored on the chip prior to readout, without further exposure to light, thus preventing additional charge accumulating in the image during the readout process, which would otherwise corrupt (temporally ‘smear’) the signal and reduce the time resolution of the instrument. CCD2 was an Andor Newton CCD (DU90P-BV, 1024 x 256 pixels). For a small number of experiments (see Section 3.3.1) an Ocean Optics spectrograph and CCD were used

(OO-DuUSB2000, wavelength range 400–850 nm). CCD and spectrograph settings are specified in later chapters for individual experiments.

The NKT's spectrum (after filtering *via* the SpectraK Split and a hot mirror to remove light at wavelengths > 800 nm, see Section 2.3) as measured by the Andor Newton CCD (in combination with the Andor spectrograph) is shown in Figure 2.4. The differences in the spectral profiles shown in Figures 2.4 and 2.2 are attributed to the hot mirror (causing the differences in the red wavelength region), as well as the quantum efficiency (QE) of the CCD and efficiency of the spectrograph (shown in Figure 2.5), which vary over the wavelength range shown and are reduced significantly at wavelengths toward the blue (below 500 nm).

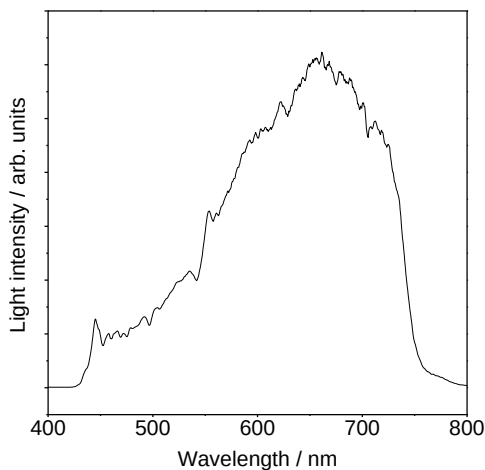


Figure 2.4 NKT SC source spectrum, recorded using the Andor spectrograph and Newton CCD.

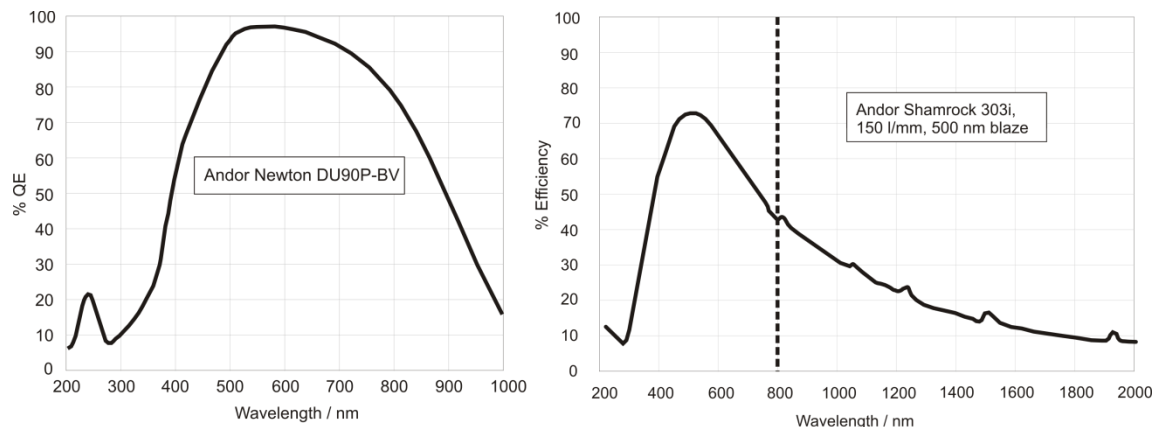


Figure 2.5 (left) the quantum efficiency of the Andor Newton CCD and (right) the efficiency of the Andor Shamrock spectrograph used in this project.

2.6 A basic BBCEAS setup

Figure 2.6 shows a BBCEAS setup in the laboratory, using some of the elements described in the sections above.

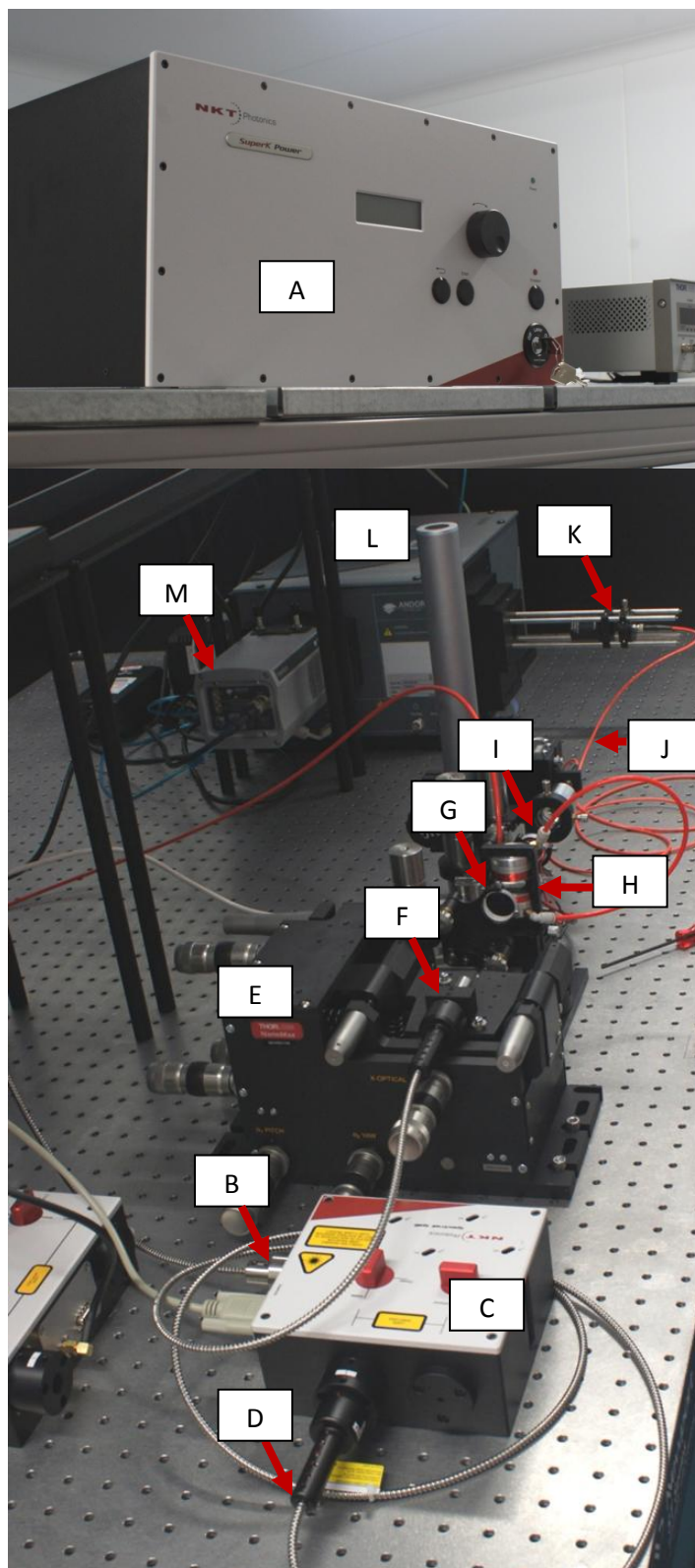


Figure 2.6 A BBCEAS setup in the lab. (A) SC source (NKT); (B) PCF input into (C) SpectraK Split; (D) FDS mounted to (E) a 6-axis mount; (F) collimating lens mounted to the FDS; (G) cavity input mirror; (H) intracavity cell in mount; (I) rear cavity mirror; (J) fiber optic cable leading to (K) an F/# matcher, (L) the spectrograph (Andor) and (M) the CCD (Andor Newton).

2.7 Instrument detection limits and Stability

For a series of N measurements of amplitude x_i ($i = 1, \dots, N$), an instrument detection limit can be characterized using the standard deviation (σ) of the data set:

$$\sigma = \sqrt{\frac{1}{N-1} \sum_{i=1}^N (x_i - \mu)^2} \quad (2.1)$$

where μ is the mean value of the data set.

If desired, the full data set can be split up into M subgroups containing y individual measurements (so that $M = \frac{N}{y}$). For each of the M subgroups the standard deviation (σ_g) is then written as:

$$\sigma_{g,n} = \sqrt{\frac{1}{y-1} \sum_{l=1}^y (x_{(n-1)y+l} - \mu_n)^2} \quad (2.2)$$

Where $\mu_n \left(= \frac{1}{y} \sum_{l=1}^y x_{(n-1)y+l} \right)$ is the average value of the n th subgroup. When measurements are dominated by random ‘white’ noise the resulting uncertainty in a measurement is expected to decrease with the square root of the averaging number (y). In reality, however, this square root relationship only holds for a finite size of y , as all instruments have instabilities: long term drifts, for example those mentioned in Section 2.2.1 for SC sources, limit the timescale (and, therefore, the size of y) over which white-noise-dominated measurements can be made (see, for example, Werle et al.6). Figure 2.7(a) shows raw temporal data of the light intensity recorded in ‘counts’ (9.4 electrons per count, see Andor CCD Test Data sheet) using an SC-BBCEAS instrument (described in the Figure caption). Each acquisition was recorded using a 3.66 ms integration time at the CCD (the shortest acquisition time possible for the camera used). Adjacent averaging of the data results in significant smoothing of the acquisition series (Figures 2.7(a) and (b)). However, in this averaged data it is clear that, over the entire acquisition series, the SNR is dominated by long term drift (which is

insignificant in the raw data, where shot-to-shot variations in light intensity dominate). The Allan deviation⁷ can be used to assess the instrument stability and its affect on measurement sensitivity, as described in the next section.

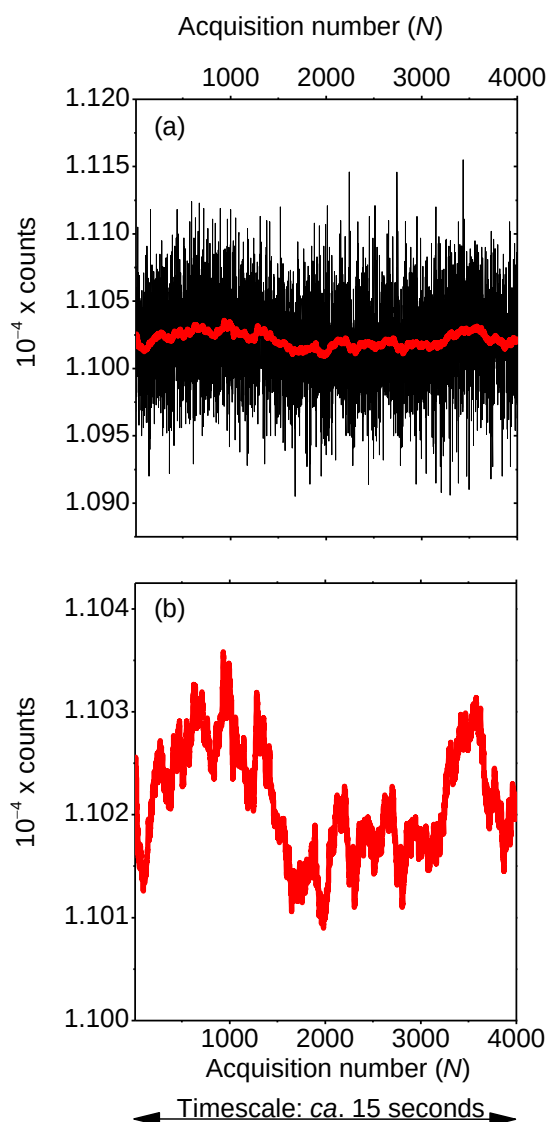


Figure 2.7 Baseline light intensity (I_0), recorded using SC-BBCEAS at 600 nm, as a function of acquisition number. The SC-BBCEAS instrument—similar to that pictured in Figure 2.6—employed a simple linear optical cavity arrangement ($L \approx 20$ cm) including an intracavity flow cell (Starna 45/Q, $l = 0.1$ cm), filled with water and positioned at normal incidence with respect to the cavity axis (using a goniometer stage). The cavity was excited using an SC radiation source (NKT SuperK Power Blue). Light exiting the cavity was dispersed into its constituent wavelengths (using a spectrograph, Andor Shamrock 303i, slit width $10 \mu\text{m}$) before detection, using a CCD camera (Andor Newton).

(a) Raw acquisition series (black line) and averaged acquisition series (red line, 100 point adjacent averaging of raw series). (b) enlarged view of averaged acquisition series.

2.7.1 Allan deviation

To minimize the influence of long-term drifts on data and optimize instrument sensitivity, the optimum number of measurements that can be usefully averaged together in any data subset must be considered carefully. y should be large enough to mitigate the effects of “high-frequency” spectral variations but not long enough to introduce excessive deviation due to long term drift. Typically, to determine the optimal averaging number, y_{opt} , the Allan

deviation, σ_{AD} , is calculated from a set of M independent measurements and compared for various values of y :^{6,7}

$$\sigma_{AD}(y) = \sqrt{\frac{1}{2m} \sum_{s=1}^m [\mu_{s+1}(y) - \mu_s(y)]^2} \quad (2.3)$$

Where $\mu_s \left(= \frac{1}{y} \sum_{l=1}^y x_{(s-1)y+l} \right)$, $m = M - 1$ and $s (= 1, \dots, m)$ is the subensemble number.

By way of example, the Allan deviation was used to determine y_{opt} and the affect of signal averaging on sensitivity for the SC-BBCEAS instrument described in Section 2.7.

A total of $N = 19950$ consecutive baseline light intensity spectra were recorded using the CCD, each with an integration time of 3.66 ms. The spectra were then processed to form subgroups of various size M : the 19950 spectra were formed into a series of $M = 9975$ spectra with an averaging number of $y = 2$ (the average of two adjacent 3.66 ms spectra), a series of $M = 6650$ spectra with an averaging number of $y = 3$ (the average of three adjacent 3.66 ms spectra) and so on and so forth for all integer values of $\frac{N}{y}$. $\sigma_{AD}(y)$ was then calculated (via Equation 2.3, at 600 nm only), and used to generate Figure 2.8, which also shows results of $\sigma_g(y)$ calculations using Equation 2.2.

Figure 2.8 shows that as y is increased from 1 to ~ 100 ($\log_{10} y = 0$ to ~ 2) the Allan deviation decreases with the square root of y : a linear fit of the data in this region results in the red line shown in the Figure, with gradient $= -0.5$. Within this region, the instrument sensitivity improves with signal averaging. However as y is increased beyond ~ 100 , $\sigma_{AD}(y)$ reaches a minimum and subsequently begins to increase. This behavior is attributed to the detrimental influence of long term drift on measurement sensitivity: as y is increased the (formerly white-noise-dominated) difference between adjacent subgroup values (x_{i+1} and x_i) is eventually dominated by long term drift and further signal averaging is no longer beneficial to measurement sensitivity.^{6,8}

In a separate experiment using the same instrument and procedure described above (except for $N = 9900$), $\sigma_{AD}(y)$ was determined as a function of wavelength. The results (not shown here) indicate that y_{opt} is not significantly wavelength dependent; y_{opt} is around 100, across the entire wavelength range studied ($\sim 425 - 700$ nm).

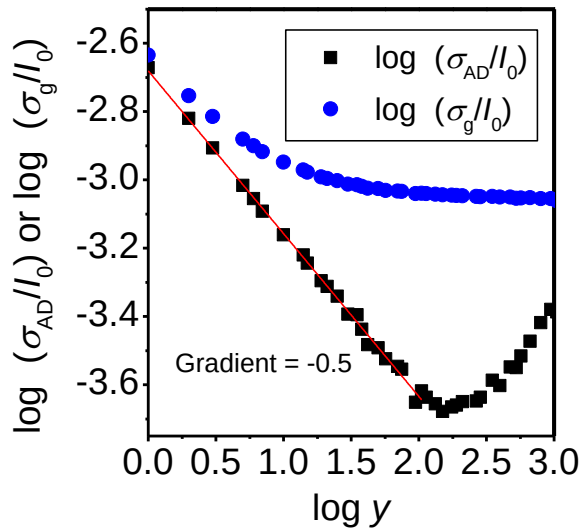


Figure 2.8 $\log_{10} \left(\frac{\sigma_{AD}}{I_0} \right)$ and $\log_{10} \left(\frac{\sigma_g}{I_0} \right)$ versus $\log_{10} y$ (see text for details). I_0 is the average of 50 baseline BBCEAS light intensity measurements acquired immediately before the main measurement series used to calculate σ_{AD} and σ_g . Solid red line: linear fit of the Allan deviation data between $y = 1$ and 100.

Interestingly, $\sigma_g(y)$ does not display a square root relationship (as seen for $\sigma_{AD}(y)$) in the region $y = 0$ to ~ 100 . Unlike $\sigma_{AD}(y)$, the standard deviation is not calculated using the difference between adjacent subgroup values, but instead using the difference between all subgroups and the mean across the entire series of data. Therefore, the standard deviation is influenced by long term drift at lower y than $\sigma_{AD}(y)$ and rapidly departs from the white-noise ideal. It appears that this departure occurs so rapidly in the present instrument that a white-noise-dominated regime for $\sigma_g(y)$ cannot be resolved (it occurs at averaging times less than 3.66 ms).

Regardless, both Allan deviation and standard deviation measurements indicate that, for this particular instrument, there is little sensitivity advantage to be gained by averaging together more than around 100 consecutive spectra with individual integration times of 3.66 ms. In other words, the optimum averaging time is around 0.366 s. The results shown in Figure 2.8

could be reproduced consistently (see, for example, Figure 2.9, below) and similar results have also been observed by others in the group, using an entirely separate BBCEAS instrument and different SC source (Fianium SC400-4).⁹ It seems likely that the long term drift in these instruments is caused by the shot-to-shot intensity and spectral fluctuations inherent to SC sources (mentioned in Section 2.2.1).

On the basis of the above results, in order to increase instrument sensitivity, signal measurements should be stopped once y_{opt} is reached and a new reference should be recorded before yet another signal measurement is taken. This method of alternating reference and signal measurements is employed (in Chapter 8) to monitor magnetic field effects in radical recombination reactions using BBCEAS. Another approach to increasing sensitivity and correcting for drifts in light intensity involves monitoring and comparing both the signal and background light intensities simultaneously (practically, this is achieved using a separate reference beam: see Fiedler *et al.*¹⁰ for example).

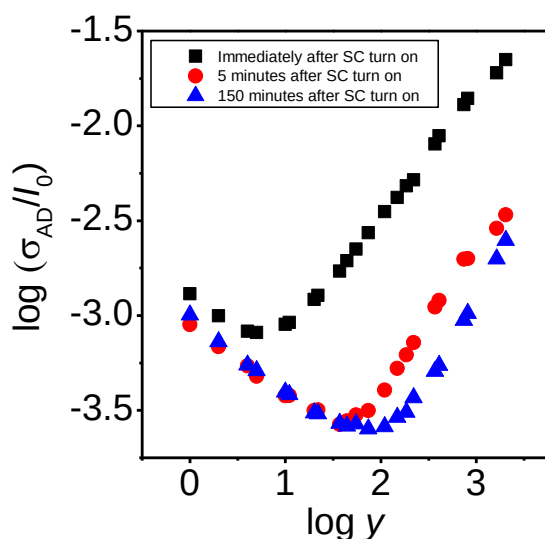


Figure 2.9 $\log_{10} \left(\frac{\sigma_{AD}}{I_0} \right)$ versus $\log_{10} y$, acquired at three different times after SC emission initiation (immediately after initiation, 5 mins after and 150 mins after). $N = 16280$. I_0 is the average of 50 baseline BBCEAS light intensity measurements (acquired at the beginning of the measurement series). See text for details.

Allan deviation experiments were also performed at various times following the initiation of SC source emission, the results of which (shown in Figure 2.9) indicate that the SC source should be left to warm up for at least 5 minutes (preferably longer) following initial firing, before

experiments are run. In this regard the NKT performs somewhat better than the Fianium SC source, in use elsewhere in the group, which requires longer to stabilize.

2.8 Conclusion

This Chapter has provided a general overview of the experimental schemes and apparatus used for work described in later chapters. The main components of the cavity-based instruments (light sources, cavity mirrors and photodetectors) used have been described, with particular attention paid to BBCEAS apparatus: supercontinuum light sources, ultra-broadband coated cavity mirrors and CCD-based photodetectors.

The Allan deviation and standard deviation, for assessing instrument stability and optimizing sensitivity, has been described and measured using a BBCEAS instrument employing an SC source. The results suggest that long term drift in these instruments is caused by fluctuations inherent to SC sources.

The remaining chapters of this thesis focus on the experiments summarized in Table 2.1.

2.9 Bibliography

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Chapter 3

Evanescent wave cavity-based spectroscopic techniques

This chapter expands upon the basic principles of the evanescent wave cavity-based spectroscopic techniques, discussed in Chapter 1. First, recent examples from the literature are used to illustrate how evanescent fields can be generated, at solid|liquid and solid|air interfaces, using a variety of different cavity configurations (each with their own merits and drawbacks), and exploited to investigate interfacial properties and processes such as adsorption and surface reactions. The bulk of this chapter is devoted to discussing results obtained using the comparatively new cavity technique of EW-broadband cavity enhanced absorption spectroscopy (EW-BBCEAS), as well as a discussion of the potential for extending EW cavity-based techniques to probe other interfaces—exemplified with a demonstration of initial interfacial absorbance measurements at a water|air interface. Finally, this chapter concludes by looking to future developments in the field. Much of the work in this chapter has been published within a paper ([van der Sneppen *et al.*, *Analyst* **135**, 133-139, \(2010\)](#)¹ - Reproduced by permission of The Royal Society of Chemistry) written in collaboration with Dr Lineke van der Sneppen (formerly a member of the Ritchie Group, Oxford), and in a recent review article ([Schnippering, Neil *et al.*, *Chem. Soc. Rev.* **40**, 207-220, \(2011\)](#)² - Reproduced by permission of The Royal Society of Chemistry), written in collaboration with Dr Mathias Schnippering (formerly a member of the Unwin Group, University of Warwick).

3.1 Introduction

The use of EW-CRDS to study the solid|gas interface was first realized more than a decade ago by Pipino *et al.*³ In the same year they described an EW-CRDS study of adsorbed I₂ films on silica using an intracavity Pellin–Broca prism (Figure 3.1(a)).⁴ Since this pioneering work, a wide

range of different cavity configurations have been developed, each of which have their own advantages and disadvantages in various applications. A comprehensive review of previous EW cavity-based studies would be excessively large within the framework of this thesis, therefore only a summary of common cavity configurations and applications is provided here. The interested reader can find an in-depth discussion in a recent review article we have written focusing on the collaborative work of the Mackenzie-Unwin groups (Schnippering, Neil *et al.*²).

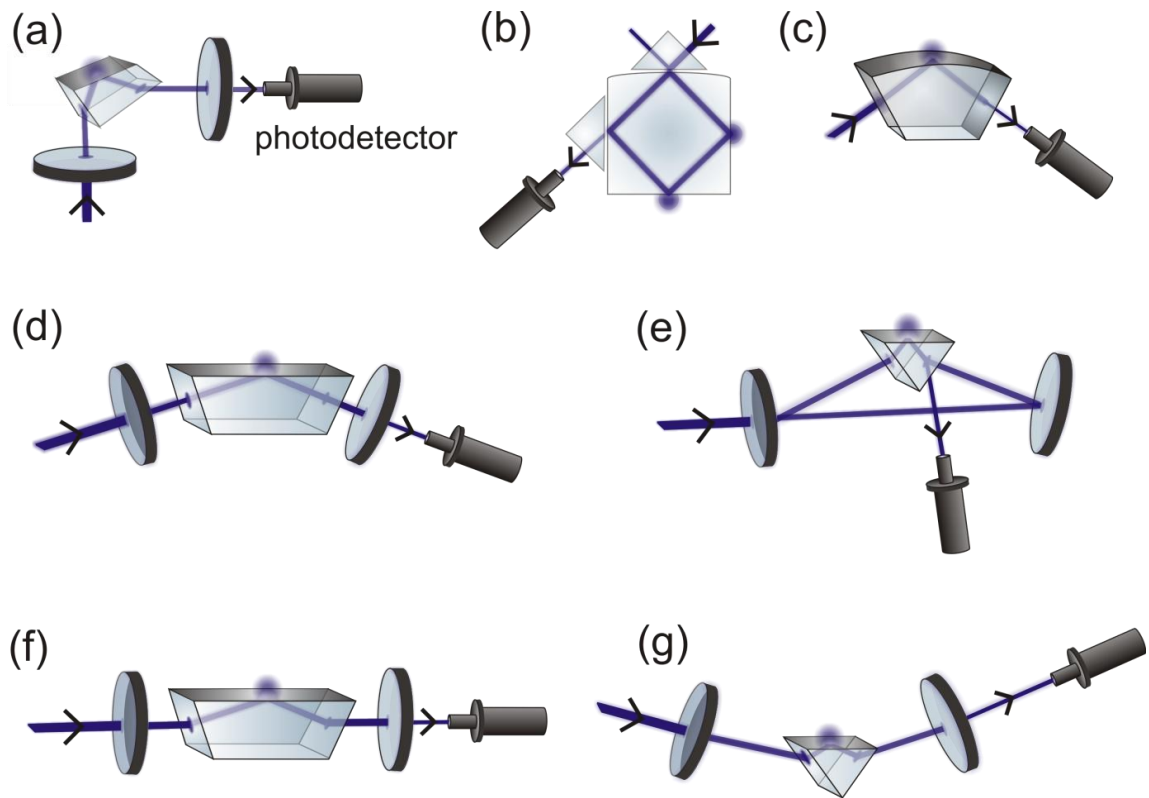


Figure 3.1 Schematics of (a) a Pellin–Broca cavity, (b) a polygonal monolithic cavity, (c) a monolithic folded cavity configuration, (d) a folded cavity, (e) a ring cavity, (f) a linear cavity employing a dove prism and (g) a right angle prism cavity in a Brewster’s angle configuration. Figure modified from Schnippering, Neil *et al.*²

3.1.1 Cavity geometries and applications

Monolithic cavities, pioneered by Pipino *et al.*,^{3,5} of which Figure 3.1(b) is an example, can have various polygonal geometries. In these variants the entire cavity lies within the material itself (typically silica) and thus there are no reflective or surface scattering losses due to entry and exit. Light is typically coupled in and out by photon tunneling using additional prisms. As a

result, these cavities generally lead to the longest ring-down times in the visible and near infrared and thus the highest sensitivities (e.g $A_{\min} \leq 1.6 \times 10^{-7}$ at 580 nm).⁵

A slightly simpler twist on the monolithic cavity is shown in Figure 3.1(c) in which light enters and exits the cavity at normal incidence to the planar faces of a prism, whilst the evanescent wave is generated at a third, convex face. The planar surfaces are high reflectivity-coated to provide a monolithic folded cavity arrangement whose high finesse has been used to study film growth and overtone spectroscopy of adsorbed species (in one of the relatively few examples of studies employing pulsed tunable light sources to acquire absorption spectra in EW-CRDS).^{6,7} In an extension of the Pellin-Broca-based work described above, Pipino *et al.* used the monolithic cavity depicted in Figure 3.1(c), in combination with polarization dependent measurements, to investigate the molecular orientation of I₂ molecules on the prism's silica surface.⁵

Despite the high finesse achieved by the geometry (e.g $F = 28500$ at 1650 nm using a monolithic folded cavity: the finesse, F , is the free spectral range divided by the full width half maximum of the cavity modes, as described in Section 1.3),⁶ monolithic cavities rely upon expensive custom-cut and polished optics in which at least one surface must be convex to ensure cavity stability; the Pipino group reported the first monolithic cavity measurements over two years after conceiving the geometry and the group remains the sole publisher of work using these configurations.

A variation on the Pellin–Broca cavity,⁴ the folded cavity (Figure 3.1(d)) comprises two high reflectivity mirrors and a trapezoidal-prism. For studies at the silica|air interface this arrangement can be used with regular right angle prisms⁸ or dove prisms, but the refractive indices are such that at the silica|water interface $\theta_c > 45^\circ$ and a larger incidence angle is required (typically $\sim 70^\circ$). The entrance and exit of the beam perpendicular to the prism faces ensures that any reflective loss is trapped within the cavity. By mounting a liquid cell on top of

the TIR surface of the prism, it is possible to investigate optical absorbance properties of the solution within the evanescent field.

The prisms used in the folded cavity are not typically coated with antireflective materials, thereby enabling measurements with polarised light which yield information on the orientation of molecular adsorption at the TIR surface. A polarization-dependent study of heamoglobin's molecular orientation during its adsorption on silica has been previously demonstrated using a Dove prism within a folded cavity.⁹ The Zare group have also employed polarization-dependent studies to characterise silanol groups on the fused silica surface of an intracavity prism¹⁰ and to determine the molecular orientation of methylene blue at a silica|air interface.¹¹

The folded cavity configuration has also been used for ultra-broadband cavity-enhanced absorption measurements at the silica|liquid interface using supercontinuum radiation, as described in Section 3.2.^{1,12}

The conceptually simple ring cavity configuration, Figure 3.1(e), has been used in several studies at the solid|liquid interface using right-angled prisms.¹³⁻¹⁵ The sides of the prism may be antireflection coated to minimise losses. A small but significant part of the beam is reflected specularly at the entrance face of the prism (see Figure 3.1(e)), providing a convenient measurement of the light circulating within the cavity. Whilst this reflection limits ring-down times to typically $< 1 \mu\text{s}$, the high light levels detected permit the use of cheap, low power light sources such as diode lasers (see Section 1.4.1.2). Furthermore, prisms for the ring cavity are cheap and readily available commercially. This configuration is versatile in terms of the range of interfaces and environments that can be studied and A_{min} of the order of 10^{-5} are readily achievable,¹³ which is sufficient for many purposes, some of which are described below.

Recent work using ring cavities has demonstrated that the surface chemistry studied using EW cavity-based techniques can be diversified and expanded, for example, by using polyelectrolyte

polymers or other thin materials.¹⁴⁻¹⁶ Mazurenka *et al.*¹⁷ within our group used EW-CRDS (with a ring cavity geometry) to monitor the adsorption of two different types of commercially available gold nanoparticles (NPs, with average sizes of 5 nm and 20 nm) onto silica that had been modified with a positively charged layer of poly-L-lysine (PLL).

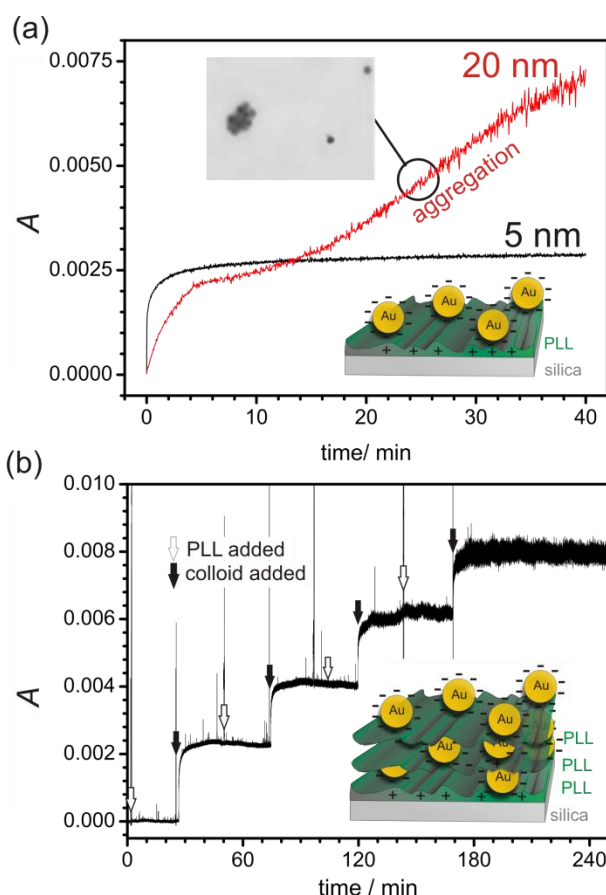


Figure 3.2 (a) Absorbance transients for the adsorption of 5 nm (black line) and 20 nm (red line) Au NPs onto a PLL-modified silica surface. (Inset) tapping mode atomic force microscope image of the surface, showing colloidal particle aggregation at 25 min. (b) Interfacial optical absorbance recorded, as a function of time, during the alternating sequential addition of PLL and 5 nm diameter Au NP solution to form a multilayer structure. Data from Mazurenka *et al.*¹⁷ Artist's representations are shown inset.

As shown in Figure 3.2(a) both the 5 nm and 20 nm Au NPs demonstrate a high affinity towards the PLL-modified surface. This can be explained by the electrostatic interaction between the negatively charged citrate shell of the Au NPs and the positively charged PLL layer. As seen by the EW-CRDS measured absorbance, the surface coverage of 5 nm Au NPs as a

function of time shows a monotonic increase and ultimately obtains a limiting value. In simple terms the data can be explained by neutralisation of the PLL film's charge during adsorption of the NPs, resulting in inhibition of further adsorption and attainment of a maximal surface coverage. The time-dependence of adsorption for the 20 nm Au particles was considerably different (Figure 3.2(a)). After initial rapid adsorption, similar to that with the 5 nm particles, the surface coverage attained a plateau quasi-steady value after *ca.* 10 min. However, the subsequent linear increase in optical absorbance indicates a change in the coverage and/or surface structure and the formation of aggregates at this stage was confirmed by an *ex situ* tapping mode atomic force microscopy study (Figure 3.2(a) inset).

As illustrated in Figure 3.2(b), the generation of multilayer structures, comprising alternate PLL and Au NP layers, could also be studied. Understanding the formation of films of this type is important as they have proven particularly valuable for sensing applications.¹⁸

The ring cavity geometry has also been used to implement electrochemistry in EW-CRDS: by positioning an electrode close to the TIR interface and evanescent field, a thin layer electrochemical cell can be configured and used in conjunction with EW-CRDS as a spectroelectrochemical technique to study a variety of interfacial phenomena.¹³⁻¹⁵

Although the ring cavity geometry has many advantages and potential applications, it does not lend itself to polarization-sensitive measurements due to the need for anti-reflective prism coatings and concomitant differential losses for different polarizations, nor does it permit broad spectral coverage as refraction at the prism surface is inherently wavelength dependent.

Linear cavity arrangements, such as that shown in Figure 3.1(f), have been used for EW-CRDS experiments.^{19,20} These are similar to the ring cavity described above and the sides of the prism are again typically anti-reflection coated. This typically restricts measurements to one polarization (that for which the coating is optimised) and can never eliminate surface reflective

losses completely, which limits the sensitivity compared to monolithic cavities. Like the ring cavity, however, the open architecture allows a diverse range of environments to be studied.

EW-CRDS using a right-angle prism in an arrangement shown in Figure 3.1(g) has also been demonstrated.²¹ In this configuration the light beam enters and exits the (uncoated) prism boundaries at Brewster's angle. Like the Pellin–Broca prism cavity, this geometry allows incorporation of a low cost off-the-shelf prism within a high finesse optical cavity and may, therefore, be well suited to use in a commercial instrument.

3.1.2 Scope of this chapter

In the first part of this chapter, Section 3.2 describes evanescent-wave broadband cavity-enhanced absorption detection of Ir(IV) complexes, electrogenerated in a thin-layer electrochemical cell arrangement. In these experiments the use of a supercontinuum radiation source and a sensitive CCD detector allow BBCEAS measurements with excellent signal to noise and spectral range, at repetition rates (> 600 Hz) that are easily fast enough to follow solution kinetics and interfacial processes in real time.

In the second part of this chapter (in Section 3.3), preliminary EW-CRDS, EW-CEAS and EW-BBCEAS experiments at a liquid|air interface are presented as an example of how EW cavity-based techniques can be diversified beyond silica surfaces. Finally, future developments and trends in EW-cavity based spectroscopy are predicted in Section 3.4.

3.2 Monitoring dynamical interfacial processes with EW-BBCEAS

As described in Section 1.4.1.1, the use of tuneable lasers in cavity-based spectroscopic techniques can, in principle, allow the acquisition of optical absorption spectra as a function of wavelength. However, inhomogeneous broadening of absorption bands in the condensed phase makes tuneable light sources unsuitable for monitoring rapid spectral changes in EW-

CRDS. Hence, examples of studies employing pulsed tuneable light sources to acquire absorption spectra in EW-CRDS are comparatively scarce^{5-7,22,23} and the technique may be considered somewhat one-dimensional. By contrast, broadband (BB) variants of CEAS (Sections 1.2.3, 1.4.2 and 1.6.1.2), allow simultaneous acquisition of spectral information over wide wavelength ranges (tens or hundreds of nanometres). Therefore, BBCEAS may prove particularly useful in identifying spectral changes, such as shifts or broadening effects, associated with dynamical surface processes, or reaction kinetics in which the loss of reactants and the growth of products may be followed simultaneously. Recently, and particularly pertinent to work described in this chapter, BBCEAS has been introduced for measurements in the condensed phase using EWs.^{1,8,12}

Sections 3.2.1 and 3.2.2 describe the development of an EW-BBCEAS instrument and its application to monitor electrochemical reactions at a solid|liquid interface.

3.2.1 Experimental

Figure 3.3 shows a schematic of the EW-BBCEAS setup, employing a folded cavity arrangement (similar to that pictured in Figure 3.1(d)), which is constructed using mirror set 2 (see Section 2.4 and Figure 2.3(b), 1 m radius of curvature) and a fused silica dove prism (custom made by Apex Services, specification tolerances: Dimensions ± 0.10 mm; Angles ± 3 arc min; Scratch/dig 10/5; Flatness $\lambda/20$) positioned at the centre of the cavity, with its entrance and exit faces at normal incidence to the cavity beam. A photograph of the folded cavity arrangement is also shown in Figure 3.3 and schematics of the dove prism and incidence angle used are shown in Figure 3.4.

The light source used to excite the cavity was a Fianium SC400-2 supercontinuum source (see Table 2.2). Before entering the cavity, the output of the SC source was collimated, and then spectrally filtered, limiting its wavelength range to the visible region, using hot mirrors and

suitable filters, e.g. a Comar 668 GK 50 short-pass filter (see also Section 2.3). Finally, the light was perpendicularly polarized (Thorlabs Glan-Taylor polarizer, GT5-A) before entering the cavity.

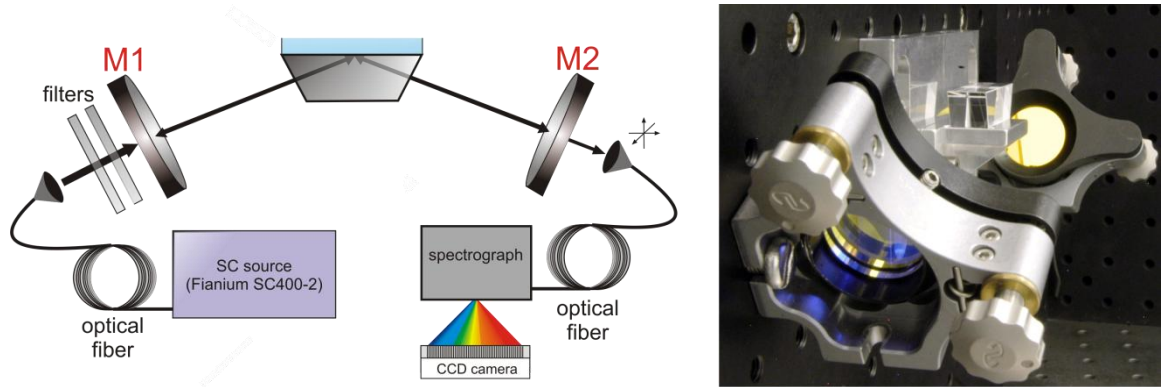


Figure 3.3 (left) schematic of the experimental setup for EW-BBCEAS studies using a folded cavity arrangement and (right) a photograph of the folded cavity setup in the lab

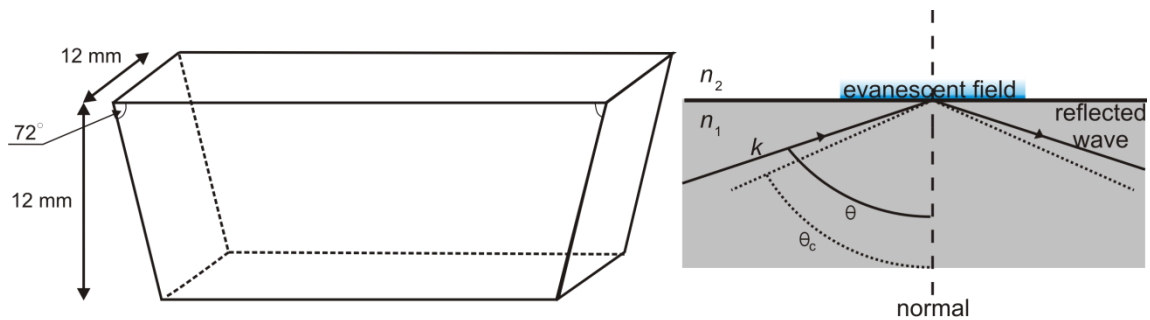


Figure 3.4 schematics of the dove prism (left) and the incidence angle ($\theta = 72^\circ$) employed in this project (right). The critical angle $\theta_c = 66^\circ$ for a fused-silica|water interface at $\lambda = 500$ nm ($n_2 = 1.335$, $n_1 = 1.46233$) is also shown. The prism and incidence angle are suitable for use in TIR generation at a wide range of interfaces (including the fused-silica|air interface, with $\theta_c = 43^\circ$ and interfaces with $n_2 \leq 1.39$).

Light exiting the cavity was fiber-coupled to an Andor Shamrock SR-303i imaging spectrograph (wavelength range 533 nm, centered at 546 nm, blazed for 600 nm, 50 μm slit width), attached to a cooled CCD camera (Andor iXon)—see Section 2.5 for details. The spectrograph's slit width was chosen to provide a spectral resolution of *ca.* 4 nm, significantly narrower than any spectral feature. Data were acquired over time periods of both 10 ms and 10 μs with vertical

(spatial) binning of the CCD charge performed either off-line (10 ms exposures) or on-chip (10 μ s exposures).

A thin-layer (100 μ m) electrochemical cell was created above the TIR interface by positioning a 2 mm diameter Pt disk (working) electrode at a height of 100 μ m above the prism (see Figure 3.5). The cell contained a solution of 10 mM $[\text{IrCl}_6]^{3-}$ in 0.1 M KNO_3 (aq) as a supporting electrolyte. $[\text{IrCl}_6]^{2-}$ was electro-generated by stepping the potential of the working electrode from +0.4 to +1.0 V vs. an Ag/AgCl reference electrode for 60 s, after which the potential was stepped back to +0.4 V. The thin layer cell was designed, constructed and operated by Mathias Schnipper and is described in further detail in Reference 24.²⁴ Calibration for these studies was provided by recording spectra of known Ir(IV) solutions as discussed in the next section.

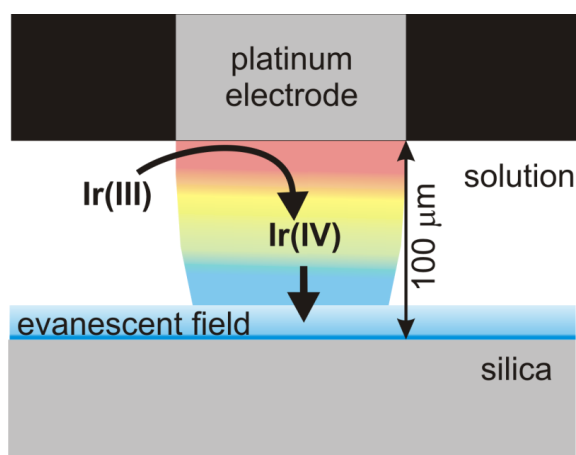


Figure 3.5 Schematic of the thin layer cell configuration for EW-BBCEAS, illustrating the potential step electrogeneration of Ir(IV), from Ir(III), $[\text{IrCl}_6]^{3-}$.

3.2.2 Results and Discussion

Figure 3.6(a) shows the signal (I) and reference (I_0) light intensity spectra obtained with 10 mM $[\text{IrCl}_6]^{2-}$ solution in the cell above the prism. Both spectra represent 200 x 10 ms spectral averages (i.e., a total accumulation time of 2 s each). The variations in the effective mirror reflectivity are clearly manifest in each spectrum with the transmitted light intensity greatest at the mirror reflectivity minima. The manufacturer's mirror reflectivity curve clearly shows these

variations, too. The broader structure in the signal and reference spectra reflects the characteristics of the source itself and the efficiency of the photodetector (see Section 2.5).

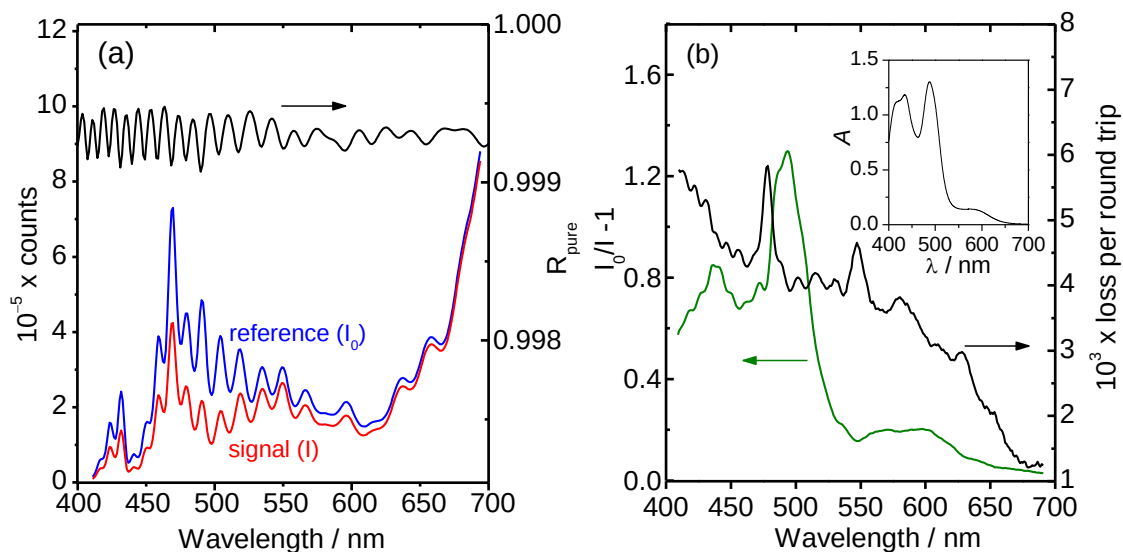


Figure 3.6 (a) (black line) The reflectivity profile of the cavity mirrors (see also Figure 2.3(b)) and raw reference (I_0) and signal (I) spectra, where I_0 and I are the light intensities recorded in ‘counts’ at the CCD with water and a 10 mM Ir(IV) solution in the sample cell, respectively. (b) The relative absorbance $\left(\frac{I_0}{I} - 1\right)$ spectrum for the Ir(IV) sample, extracted from the data shown in (a). The loss per round trip within the CEAS cavity was extracted by taking the ratio of the $\left(\frac{I_0}{I} - 1\right)$ spectrum to the single-pass Ir(IV) spectrum of 2 mM Ir(IV) (inset, acquired using a dual beam UV-Vis spectrometer—Varian, Cary 300 Bio, wavelength resolution 4 nm) scaled for the (wavelength-dependent) effective thickness (see Figure 3.7) and difference in concentration.

Figure 3.6(b) shows the cavity-enhanced absorption spectrum (reported as $\left(\frac{I_0(\lambda)}{I(\lambda)} - 1\right)$, see Section 1.2.3) which, when compared with a single-pass spectrum of Ir(IV) scaled for both the concentration difference and the spectral variation in the effective thickness (see below), provides the losses per round trip within the cavity (also shown, right hand scale), the reciprocal of which is the CEF (see Section 1.2.2) which varies over this range from 200 to 800. The effective thickness, $(d_{e\perp})$, for perpendicular polarization: see Section 1.6.2) as a function of wavelength, was calculated according to Harrick & du Pre²⁵ using:

$$d_{e\perp} = \frac{n_{21}\lambda \cos \theta}{\pi(1-n_{21}^2)(\sin^2 \theta - n_{21}^2)^{\frac{1}{2}}} \quad (3.1)$$

where $n_{21} = \frac{n_2}{n_1}$. $d_{e\perp}$ is shown as a function of wavelength in Figure 3.7.

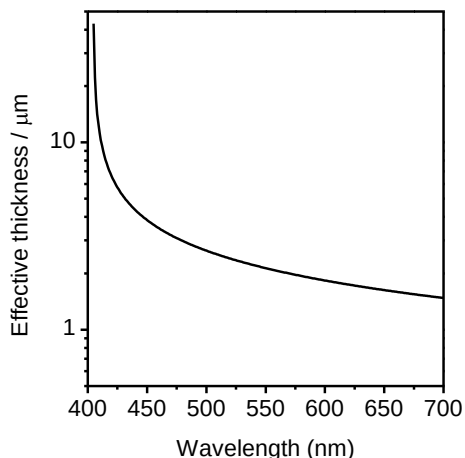


Figure 3.7 effective thickness as a function of wavelength, calculated using Equation 3.1.^{26,27}

$$n_1, 400-700 \text{ nm} = 1.47012 \dots 1.45529$$

$$n_2, 400-700 \text{ nm} = 1.399 \dots 1.331$$

As seen from Figure 3.6, the ripples in the mirror reflectivity are observed in the cavity losses, however it is clear that the cavity loss spectrum is dominated by other losses which increase significantly towards the blue. These additional losses almost certainly arise from scattering both at the prism surfaces and within its bulk. As well as the ripples due to the mirror reflectivity, the cavity loss spectrum exhibits additional structure, noticeably peaks at 478 nm and 547 nm, but also other less prominent features. The origin of these is unclear but they also appeared in data for other experiments (see Chapters 4 and 8), also performed using mirror set 2 but with different light sources and detectors, which suggests that absorption or etaloning within the mirrors themselves appears the most likely source, though this merits further investigation.

3.2.2.1 Following fast kinetics using EW-BBCEAS

Figure 3.8 shows the temporal evolution of the spectrum (400–700 nm) at the silica|aqueous interface measured using EW-BBCEAS during the potential step electrogeneration of Ir(IV) (via electro-oxidation of Ir(III)) in the 100 μm thick electrochemical cell. Here absorbance has been

determined as a function of wavelength using Equation 1.23, with $1-R(\lambda)$ determined from the results of the calibration described previously. During the experiment, the working electrode was stepped from +0.4 V to +1.0 V at 0 s and, following a *ca.* 10 s delay associated with diffusion of Ir(IV) to the silica surface, the interfacial absorbance increases, approaching its maximum as Ir(III) oxidation nears completion.

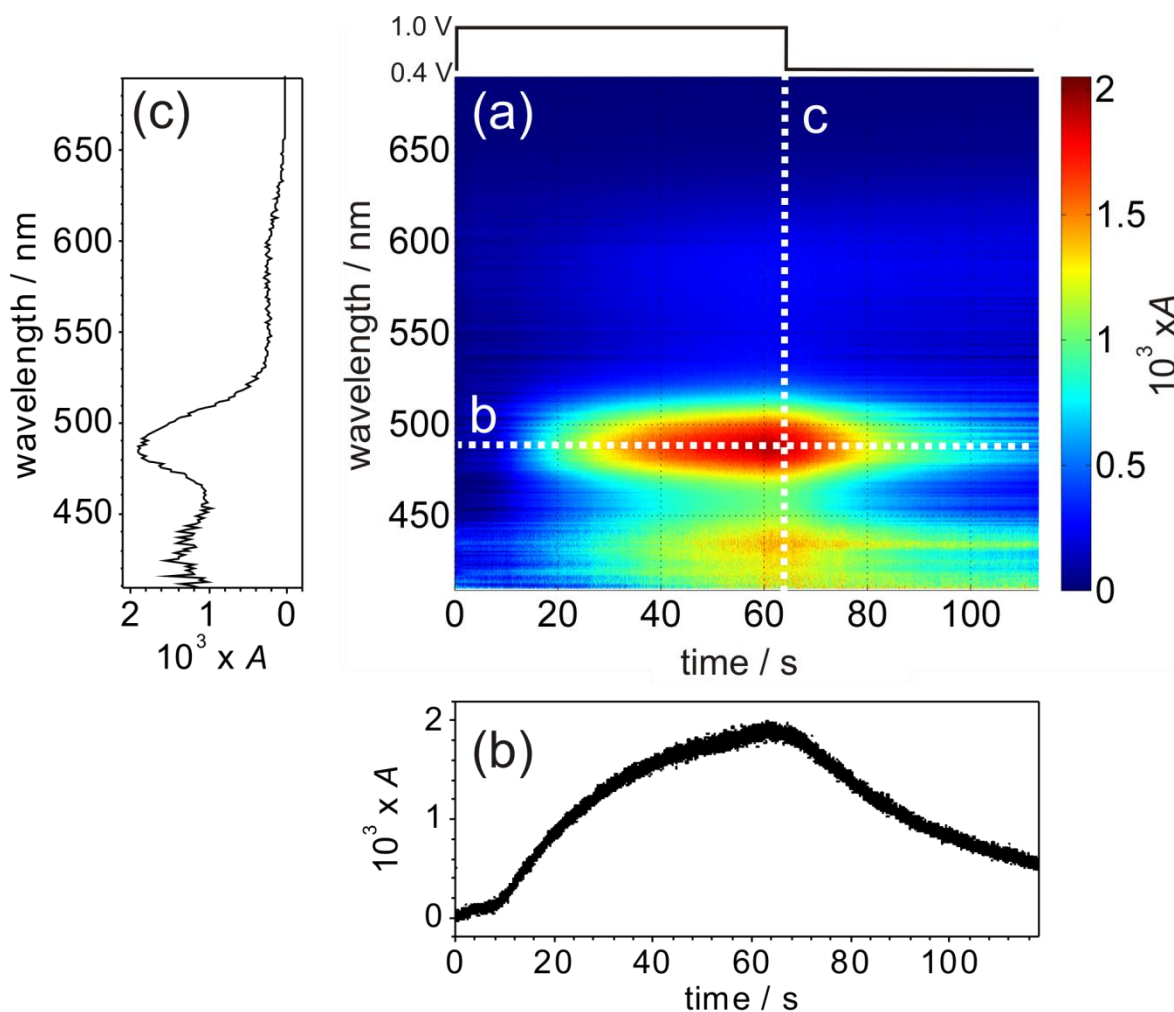


Figure 3.8 (a) Contour plot: interfacial absorbance spectrum as a function of time during the electrochemical generation of Ir(IV) from Ir(III): (b) a temporal cut through the contour plot at 487 nm and (c) a spectral cut through the contour plot at 65 s.

At 60 s the working electrode potential was stepped back to +0.4 V and, again following a short delay, the interfacial absorbance decreases as Ir(IV) is reduced. The data were recorded with 10 μ s acquisition times at a repetition rate (limited by data readout from the CCD array) of 606

Hz. This high acquisition rate—fast enough to follow, in real time, solution kinetics and interfacial processes—combined with the broadband capability of the technique and the excellent SNR achieved, is an extremely powerful combination.

One major advantage of SC radiation, compared with other excitation sources, is its superior combination of high spectral irradiance coupled with inherent collimation/directionality. Together, these permit rapid spectral acquisitions with a high SNR as illustrated by the temporal and spectral cuts through the contour plot shown in Figure 3.8; the minimum detectable absorbance was determined as 3.9×10^{-5} (at 487 nm, using Equation 1.22), from measurements made before $t = 0$ s. This initial work highlights the considerable potential of EW-BBCEAS for spectrally resolved dynamic measurements at interfaces. The technique's future applications and potential are discussed further in Section 3.4.

3.3 Diversification of environments: beyond silica surfaces

There is no practical reason for EW-cavity based spectroscopic techniques to be limited to studies at silica interfaces. To illustrate the potential for extending the technique to probe other interfaces, the remainder of this chapter describes EW-CRDS, EW-CEAS and EW-BBCEAS experiments at a liquid|air interface.

3.3.1 Experimental

The experimental setups used for EW-CRDS/EW-CEAS and EW-BBCEAS are shown schematically in Figure 3.9. Either a 405 nm diode laser (for EW-CRDS/EW-CEAS, see Section 2.2) or SC source (for EW-BBCEAS, Fianium SC-450-4, see Table 2.2, spectrally filtered as described in Section 3.2.1) was aligned, in a folded cavity arrangement, within a modified Langmuir trough (Nima Technology, see Figure 3.10 and Section 3.3.1.1) containing Milli-Q water (resistivity 18.2 MΩ cm at 25 °C).

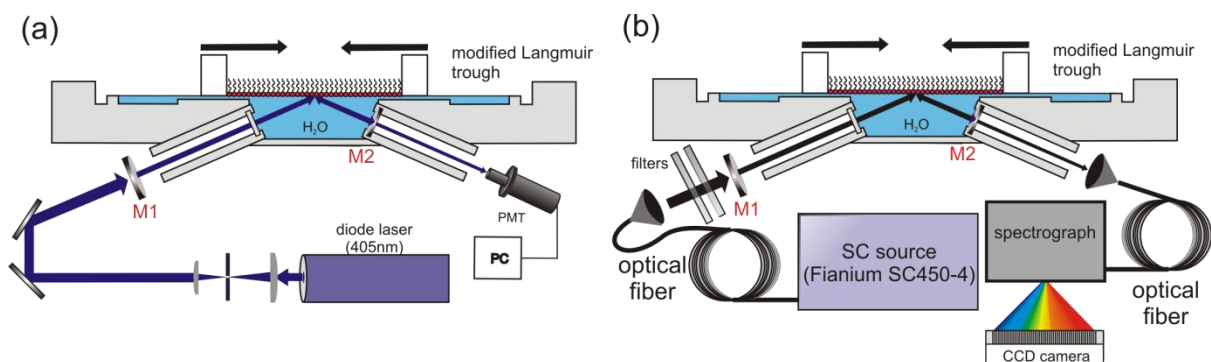


Figure 3.9 Cavity-based spectroscopy at the water|air interface: schematics of (a) EW-CRDS/EW-CEAS and (b) EW-BBCEAS experimental setups.

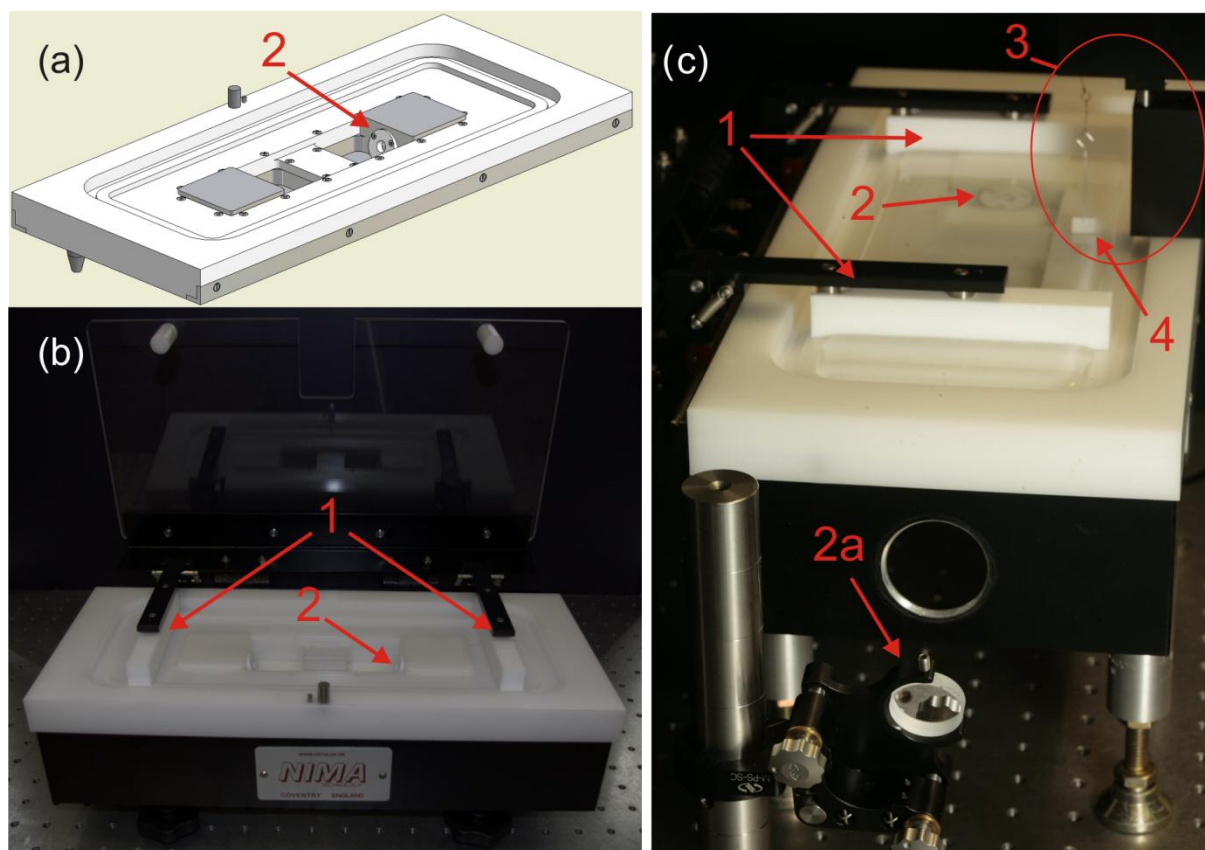


Figure 3.10 Schematic (a) and photographs (b,c) of the modified Langmuir trough: (1) Teflon barriers for varying the film's surface area. (2) output cavity mirror (M2) and mount, (2a) input cavity mirror (M1). (3) pressure sensor with (4) Wilhelmy plate attached.

Whilst both cavity mirrors have the same optical specifications (mirror set 2, see Figure 2.3(b)), one mirror is incorporated into the trough and the other manipulated externally for alignment;

an intracavity window is also employed, in the beam-path between the cavity mirrors, to contain water in the trough. This configuration yields a background ring-down time, $\tau_0 = 300\text{--}450$ ns with $L = 39$ cm (i.e. *ca.* 110–170 roundtrips per ring-down).

Light intensity transmitted through the cavity was monitored using either a PMT (for EW-CRDS/-CEAS experiments, PMT1, see Section 2.5), or an Ocean-Optics USB 2000 spectrometer (for EW-BBCEAS experiments, see Section 2.5).

The cavity arrangement is sensitive to the evanescent field established above the water interface and may thus be used to make *in situ* interfacial absorbance measurements of thin films supported at the water|air interface.

Langmuir films were deposited (from chloroform solution) at the water|air interface of the Langmuir trough, with the confinement area of the films controlled and varied using a pair of Teflon barriers, and the resulting changes in the molecular packing monitored via the interfacial absorbance. Surface pressure data were recorded simultaneously using a Wilhelmy plate balance for comparison with the optical data. The results of interfacial absorbance measurements are described later (in Section 3.3.2) but it is first informative to discuss conventional surface pressure measurements used for *in situ* monolayer study.

3.3.1.1 Surface pressure measurements and film deposition

The *in situ* study of monolayers at a liquid|air interface is often achieved by monitoring the surface tension (γ , the work per unit area required for isothermal expansion of the surface) during the monolayer's compression. In practice the 'surface pressure' (SP, the difference in surface tension in the absence and presence of the Langmuir film) is measured (in units of milli Newton per meter, mNm^{-1}) using a Wilhelmy plate and microbalance (see Figure 3.10), as the film is compressed using a pair of barriers:

$$SP = \gamma_{\text{abs}} - \gamma_{\text{inst}} \quad (3.2)$$

where γ_{abs} is the absolute surface tension of the liquid (72.8 mNm⁻¹ for water at 298 K and 1 atmosphere) and γ_{inst} is the instantaneous surface tension during compression. The surface pressure can then be plotted as a function of molecular area to give a so-called ‘pressure-area isotherm’ (PAI, see Figure 3.11), which is characteristic of the monolayer’s molecular composition.

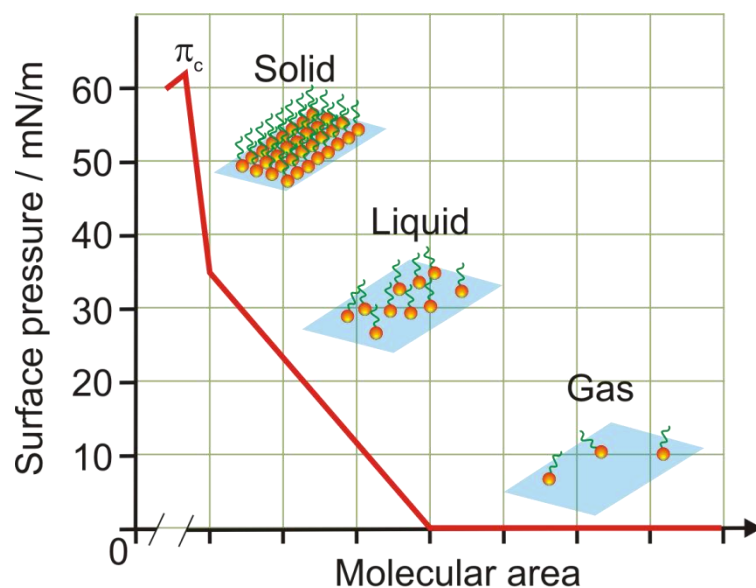


Figure 3.11 An idealized pressure-area isotherm of a ‘classical’ surface active material (with molecules comprising a hydrophilic head and hydrophobic tail), showing molecular distribution and orientation on the subphase surface. At large surface areas, very little change in the surface pressure is observed during barrier compression: the molecules are randomly distributed and unordered on the subphase surface and behave as a two-dimensional gas phase. As the surface area is further reduced, intermolecular spacing in the subphase enters the range of Coulomb forces and the surface pressure increases in a so-called ‘liquid phase’. At smaller surface areas still, the rate of surface pressure increase becomes even greater, as the isotherm enters a ‘solid’ phase, where molecules become highly ordered, with their hydrophobic tails standing nearly perpendicular to the subphase. Finally, the monolayer collapses (at π_c), losing its mono-molecular form. Figure adapted from Nima Technology Ltd. Operating Manual.

During this project, early work was performed using films doped with chromophores that absorb strongly at 405 nm (to match the cavity’s operating wavelength in EW-CRDS/EW-CEAS). Figure 3.12 shows an example of surface pressure data, recorded during compression of a mixed Langmuir film comprising the labelled phospholipid, Pacific blueTM 1,2-ditetradecanoyl-

sn-glycero-3-phosphoethanolamine, triethylammonium salt (PCB-DMPE, from Molecular Probes, $\lambda_{\text{max}} = 411 \text{ nm}$), and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC, Sigma Aldrich). More information on the practicalities of spreading Langmuir films and monitoring surface pressures can be found in the Langmuir trough's operating manual (Nima Technology Ltd).

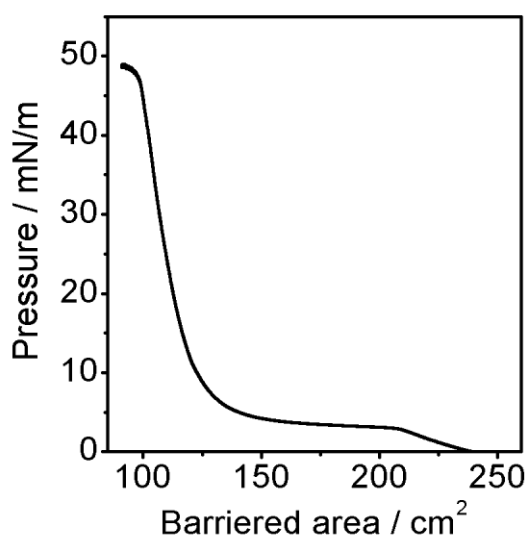


Figure 3.12 Isotherm recorded for a PCB-DMPE/DPPC hybrid film. The Barriered area, x-axis, can easily be converted to molecular area with knowledge of the volume and concentration of solution originally deposited on the subphase surface.

3.3.2 Results and discussion

Figure 3.13 shows preliminary EW-CRDS and EW-CEAS experiments at the liquid(water)|air interface. Figure 3.13(a) shows a typical example of the combined EW-CRDS/surface pressure response during a series of repeated film compressions and expansions of a mixed Langmuir film comprising the labelled phospholipid, PCB-DMPE and DPPC. The periodic and reproducible change in interfacial absorbance with time is due to a change in the number of adsorbed molecules within the EW region during film compression and expansion. This is reflected in the simultaneous surface pressure measurements (right hand scale of Figure 3.13(a)), which track the absorbance data. The interfacial absorbance data do, however, contain additional features which may yield information on surface processes. These additional features (for example the increase in absorbance and peak widths, as function of compression cycle, in Figures 3.13(a))

and 3.13(b) respectively) cannot currently be explained. One possibility is that the langmuir films investigated have an inhomogeneous distribution of the analyte at the interface: this could be confirmed *via* real time imaging of the film structure using a Brewster angle microscope.

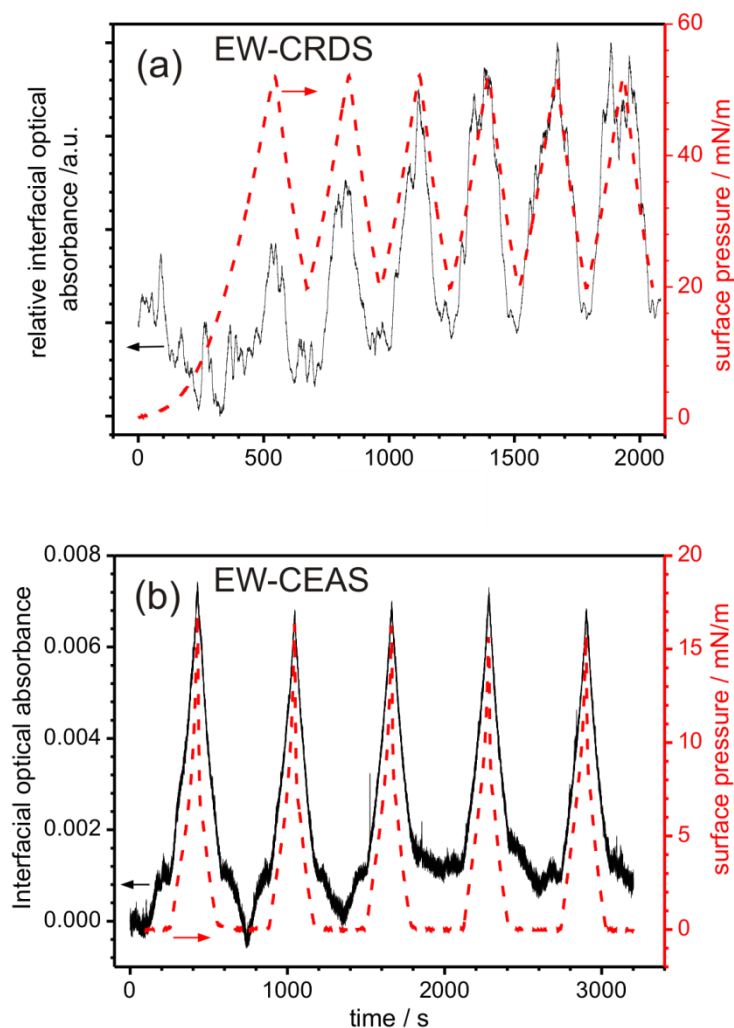


Figure 3.13 (a) surface pressure (red) and EW-CRDS-measured interfacial absorbance of a PCB-DMPE and DPPC Langmuir film, recorded during 6 barrier compression/expansion cycles. (b) Surface pressure (red) and EW-CEAS-measured interfacial absorbance of a zinc phthalocyanine derivative Langmuir film (see text for details), recorded during 5 barrier compression/expansion cycles. Interfacial absorbance is determined using the ring-down time for EW-CRDS, and using Equation 1.21 for EW-CEAS (with the effective mirror reflectivity and CEF determined from EW-CRDS τ_0 measurements with pure water, using Equation 1.12).

EW-CEAS and EW-BBCEAS may be used at the same interface: Figures 3.13(b) and 3.14 show EW-CEAS (at 405 nm) and EW-BBCEAS (450–700 nm) interfacial absorbance measurements, respectively, of a Langmuir film of a phthalocyanine derivative (zinc 2,3,9,10,16,17,23,24-octakis(octyloxy)-29H,31H-phthalocyanine, ZnPC, from Sigma Aldrich), during repeated film compression/expansion cycles.

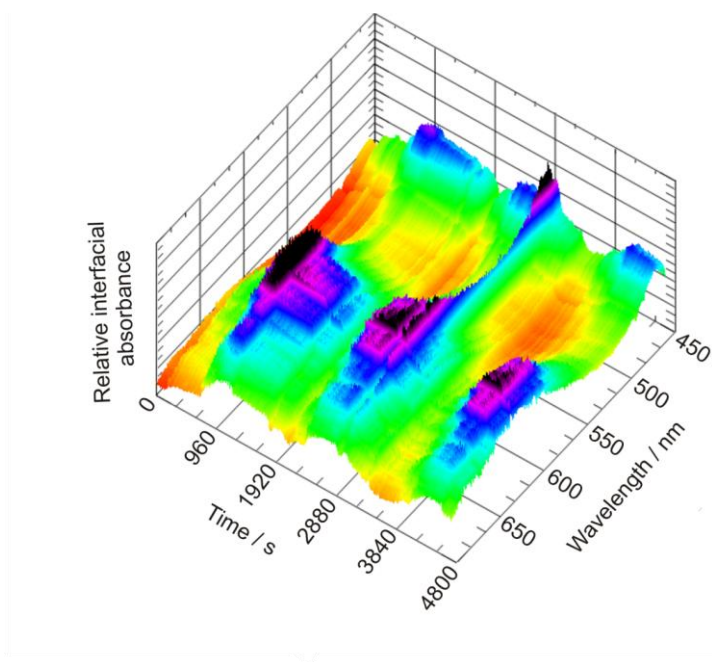


Figure 3.14 Surface plot showing the EW-BBCEAS-measured spectrum of a zinc phthalocyanine derivative Langmuir film (see text for details), as a function of time, recorded during 3 barrier compression/expansion cycles. The plot is made-up of 2000 individual spectra (1 second average per spectrum).

From Figure 3.13(b) it is clear that the surface pressure and interfacial optical absorbance changes are closely matched, whilst from the broadband data in Figure 3.14 Q-bands (above 600 nm) and ultraviolet bands (below 500 nm) are recognised. In a previous Langmuir Blodgett study of ZnPC films deposited on a solid substrate, Qui *et al.* used Q-band positions to identify the coexistence of three different types of aggregates within the film.²⁸ Therefore, in future, a similar study could be performed *in situ* on a ZnPC Langmuir film at the water|air interface using EW-BBCEAS.

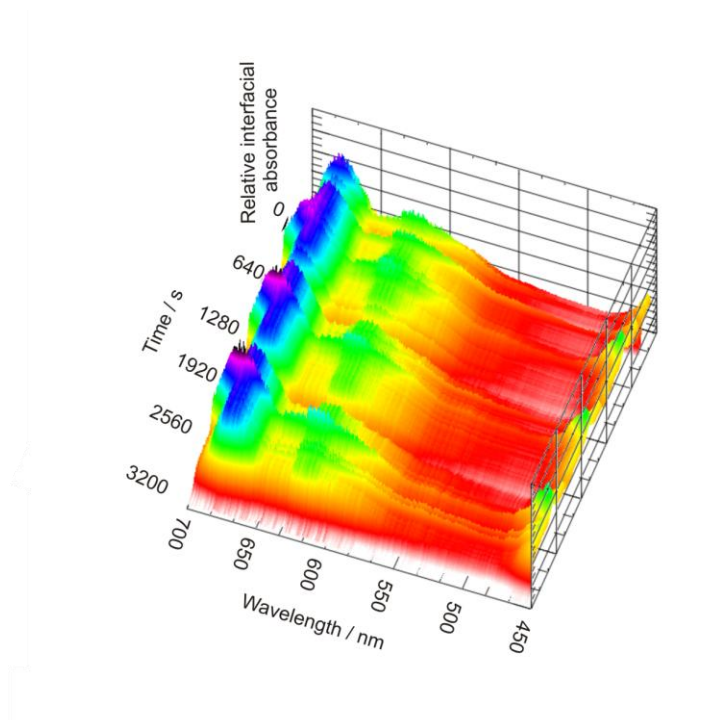


Figure 3.15 Surface plot showing the EW-BBCEAS-measured spectrum of a Chla Langmuir film, as a function of time, recorded during 3 barrier compression/expansion cycles. The plot is made-up of 2500 individual spectra (1 second average per spectrum).

In a second study, a chlorophyll a (Chla) film was studied by EW-BBCEAS, during film compression/expansion (see Figure 3.15). It was found that the interfacial absorbance peak position shifted during compression and expansion cycles (see Figure 3.16)—consistent with Chla aggregation.²⁹ Previous studies have suggested that many forms of chlorophyll found *in vivo* can be reproduced *in vitro* in a monolayer system.³⁰ These various chlorophyll species are spectrally distinct and it has been suggested that Chla monolayer compression results in increased Chla-Chla interaction, favoring formation of long-wavelength absorbing species.^{29,30,31}

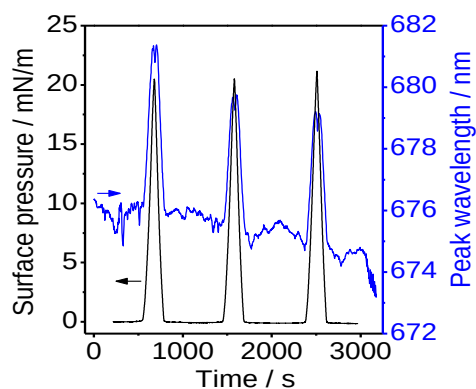


Figure 3.16 Plot of surface pressure and peak wavelength during compression of the Chla film.

3.4 Conclusions and outlook

As highlighted in this chapter, EW cavity based spectroscopic techniques are rapidly becoming established as a simple, yet sensitive, method for monitoring surface and interfacial phenomena. Although there are numerous ways of implementing EW cavity-based techniques, the principles of the various approaches and cavities derive from common principles.

The combination of a folded cavity configuration (using a dove prism to generate TIR), a supercontinuum source and a sensitive, fast readout CCD detector, permits the recording of the full visible spectrum (400–700 nm) of a (silica-liquid) interfacial layer, at rapid acquisition rates (> 600 Hz), sufficient to follow fast kinetics in the condensed phase, in real time. Even under such challenging conditions, spectra are acquired with a good signal to noise ratio and a minimum detectable absorbance of 3.9×10^{-5} is achieved. This sensitivity is comparable with previous EW-CRDS and EW-CEAS studies (see, for example, Table 5 in Ruth & Lynch⁸) but the spectral region accessed in this broadband variant is much larger.

As demonstrated previously in Section 2.7, SC sources experience a significant amount of drift over the timescales used in the EW-BBCEAS experiments described above, which will limit the measurement sensitivity. As it is not possible to rapidly alternate between I_0 and I measurements in these experiments, an alternative possibility for improving sensitivity might involve monitoring a signal and reference beam simultaneously (as mentioned in Section 2.7.1) and comparing the two to remove drift introduced by SC source fluctuations.

Additionally, it should be noted that the accuracy of the calibration method (Section 3.2.2), used to determine round trip losses and CEF within the cavity, has not been fully assessed or optimised. In the work described above it is assumed that the error in calibration is $\left(\frac{\Delta I}{I_0}\right)_c$, which adds in quadrature to the error in measurements made during the main (calibrated) experiment, $\left(\frac{\Delta I}{I_0}\right)_m$, to provide an ultimate sensitivity limit of:

$$\frac{\Delta\alpha}{\alpha} = \sqrt{\left(\frac{\Delta I}{I_0}\right)_c^2 + \left(\frac{\Delta I}{I_0}\right)_m^2} = \sqrt{2} \left(\frac{\Delta I}{I_0}\right)_{\min} \quad (3.3)$$

However, there will inevitably be other error sources, which have not been accounted for here, for example, errors associated with the UV-Vis measurement used in the CEF determination (see Section 3.2.2). Future experiments should seek to quantify and minimise these errors. Measurement sensitivity could also be improved by making multiple calibration measurements for each experiment.

The silica|aqueous interface studies described in this chapter were performed in parallel with others (in the collaborating Hancock and Ritchie Groups, Oxford), which compared SC and LED sources for use in EW-BBCEAS. Unsurprisingly, the SC source was found to provide better measurement sensitivity due to its higher spectral irradiance ($A_{\min}$ using LEDs and a 1 second signal integration time was on the order 10^{-4} , an order of magnitude higher than with the SC source).¹ Additionally, the smaller beam footprint provided by the SC source at the TIR surface allows better spatial resolution. However, the LED is clearly much more economic as a light source; the LEDs used cost around £100, whilst the SC source costs around £40,000! Additionally, LEDs are relatively stable, opening up the possibility of using long signal averaging times to improve sensitivity—unlike SC sources which (as discussed in Chapter 2) suffer from shot-to-shot fluctuations that limit the benefits of signal averaging to much shorter timescales. LEDs could, therefore, be used to provide a cheap and sensitive light source in EW-BBCEAS experiments where high temporal and spatial resolution is not needed.

The range of EW cavity applications is expected to continue expanding. In particular, the technique is expected to become increasingly popular when used in combination with other analytical techniques (electrochemical methods, HPLC, microfluidic procedures and scanning probe microscopy methods). In addition, recent trends toward diversifying and expanding the surface chemistry studied (using, for example polymer modified surfaces), are expected to

continue in future. In particular, applications to the life sciences (for example, using lipid bilayers, proteins and whole cells) are expected. Promising preliminary studies focusing on the use of EW cavities as biosensors,^{32,33} which detect specific analytes via their interaction with biomolecules immobilized on the TIR surface, suggest this application will find popularity in the near future.

As mentioned in Section 1.6.1.2 the multiplexing capabilities of broadband cavity-based spectroscopic techniques allow rapid detection and distinction of spectrally overlapping absorbing species. As demonstrated by Seetohul *et al.* this advantage lends itself well to HPLC detection.³⁴ By replacing the intracavity cuvette arrangement used by Seetohul *et al.*, with a TIR element, the sample volume in (EW-)BBCEAS could be reduced dramatically (to volumes in the nanolitre-picolitre range), developing further the technique's advantages in HPLC detection.

The excellent temporal resolution of SC-based EW-BBCEAS could be put to good-use in studying deposition (or desorption/dissolution) kinetics at the TIR surface. For example, Au NP adsorption (illustrated in Figure 3.2) could be monitored. Additionally, spectral shifts in the localized surface plasmon resonance of Au NP films, associated with changes in the external refractive index, could be monitored using EW-BBCEAS. This would complement previous EW-CRDS experiments performed within the group.¹⁷

Future cavity-based spectroelectrochemistry studies at the TIR interface might involve the broadband detection of redox-dissolution processes, such as those demonstrated by Schnippering *et al.* using EW-CRDS to monitor adsorbed silver NPs.¹⁴ Another possibility is that the electrode itself could be coated on the TIR interface and used for spectroelectrochemistry.

The prisms used in this project limit the attainable CEF. An improvement in the CEF and, therefore, in the instrument sensitivity could be achieved using a folded monolithic prism (see

Section 3.1.1). However, we are yet to find a supplier capable, or willing, to build such an optical element (the monolithic prisms used by the Pipino Group were provided by Research Electro-Optics, Boulder, Colorado. However, this company is no longer prepared to act as a supplier).

Much of the work described in later chapters of this thesis involves the photoexcitation of liquid-phase samples. Recently, Jing Li (Mackenzie Group) has begun to investigate the use of prisms with dual input and output faces (see Figure 3.17) for TIR of both photoexcitation and cavity (probe) beams. These prisms might prove useful in studying photochemical reactions at the prism's surface and magnetic field effects in the radical recombination reactions of surface-immobilized proteins (as discussed in Chapters 5 and 9).

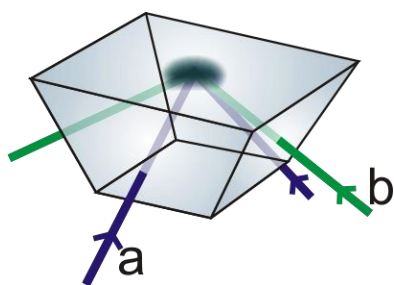


Figure 3.17 dual faced prism with input/output faces for generation of TIR using both cavity (a) and photoexcitation (b) beams

The possibility of studying liquid|air interfaces, illustrated here for the first time, greatly expands the applicability of EW cavity-based techniques. However, the current Langmuir-trough-based setup is extremely sensitive to vibration and the technique's CEF is limited by scattering and absorptive losses in the water subphase. It is likely that the instrument will be re-designed in the future to address these issues. However, these preliminary, illustrative studies demonstrate the potential of EW-cavity based spectroscopic techniques in probing liquid|air interfaces which are of wide interest for the formation and characterisation of ultra-thin (molecular) films. The major advantages of this application are foreseen to be in situations in which (i) surface tension measurements (using a Wilhelmy plate) are too obtrusive/perturbative, or where the surface area is too small to merit reliable quantification of coverage via surface tension measurements; (ii) in polarization-sensitive applications where

one might observe ordering and orientation of films as a function of surface pressure; and (iii) in kinetic studies at the liquid|air interface.

3.5 Bibliography

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Chapter 4

BBCEAS for real time, *in situ* spectral analysis of microfluidic droplets

Broadband cavity-enhanced absorption spectroscopy has been used to record, in real time, the absorption spectrum of microliter volume aqueous-phase droplets within a microfluidic chip assembly. Using supercontinuum radiation, broadband coated external mirrors, and a sensitive CCD detector, the full visible spectrum ($430\text{ nm} < \lambda < 700\text{ nm}$) of each passing droplet is acquired *in situ* at high repetition rates (273 Hz/3.66 ms acquisition time) and high sensitivity ($\alpha_{\min} < 10^{-2}\text{ cm}^{-1}$). The possibilities for further improvements in sensitivity and acquisition rate using custom designed chips are discussed. The work in this chapter represents a collaborative effort, where the expertise and instrumentation for BBCEAS and microfluidics were provided by the Mackenzie and Vallance groups respectively. This work has been published as: [Neil, S. R. T., Rushworth, C. M., Vallance, C. & Mackenzie, S. R. Broadband cavity-enhanced absorption spectroscopy for real time, *in situ* spectral analysis of microfluidic droplets. Lab on a Chip 11, 3953-3955 \(2011\)](#) – Reproduced by permission of The Royal Society of Chemistry.¹

4.1 Introduction

A wide variety of optical detection methods have been applied to microfluidic systems, including fluorescence, absorbance, chemiluminescence, surface plasmon resonance, and Raman spectroscopy.² In the analysis of macroscopic chemical samples, direct absorbance spectroscopy is considered a universally applicable analytical technique (see Chapter 1). Its application to microfluidic systems, however, presents significant challenges due to the dramatically reduced optical path lengths involved. In previous attempts to increase the optical

pathlength sampled, liquid-core waveguides and fiber optics have been employed to guide light through microchannels (as outlined below).^{3,4} However, whilst waveguide-based approaches are well suited to integration within microfluidic devices, paving the way for commercially viable “on-chip” techniques in which the light source, fluidic elements and detector are all incorporated within a single monolithic platform,^{2,5,6} they remain relatively insensitive compared with other on-chip optical detection methods. Furthermore, the integration of waveguides places constraints on the chip and channel geometry and can introduce significant light losses during coupling and detection.

On-chip absorbance detection methods typically use a liquid dye laser source, light from which is guided through a fluidic channel or a measurement cuvette and subsequently detected using a photodiode. The majority of the aforementioned components are monolithically integrated on a single substrate;^{2,6-10} a particularly elegant example of an on-chip absorbance detector, described by Balslev *et al.*,¹¹ includes an on-chip optically pumped liquid dye laser, waveguides and fluidic channels, all within one layer of the chip, as well as embedded photodiodes in the chip’s silicon substrate. This approach yields similar detection limits ($A_{\min}$ on the order $10^{-2} - 10^{-3}$) to ‘off-chip’ approaches, which use an optical infrastructure that extends beyond the chip itself.

Previous ‘off-chip’ approaches to direct absorbance detection have incorporated multipass cells, optical fibers, or a liquid core waveguide within the chip to guide light through the sample. In a simple fiber-based approach, Jindal & Cramer¹² coupled light between two optical fibers, the ends of which were positioned either side of a microfluidic channel. Using a tungsten lamp light source and CCD array detection, a minimum detectable absorbance of 2×10^{-3} was achieved during thiourea detection. A similar fiber-based design was used by Park *et al.*¹³ to monitor the concentration of ammonia during its reaction with indophenols: concentrations as low as 2 ppm ($A \approx 0.3$) were detected.

In a more sophisticated fiber-based approach, Ro *et al.*¹⁴ used a microlens embedded in the wall of a microfluidic channel to improve the coupling efficiency between the fibers. This allowed the use of a channel with a relatively long optical pathlength (500 μm), to detect fluorescein with a minimum detectable absorbance of 1×10^{-3} . In a different approach, Du *et al.*¹⁵ used a Teflon capillary, connected to a microfluidic chip, to act as a LCW with an effective optical pathlength of 1.7 cm. This instrument was used to demonstrate high-throughput (1000 samples h^{-1}) Fe(II) detection with a sensitivity comparable to that of conventional flow injection analysis techniques and a very low sample volume requirement (40 nL total cell volume).

Multipass techniques such as CRDS and CEAS represent potentially convenient ways to increase the effective pathlength. As discussed below, integration of multiple-reflection mirrors into microfluidic chips has been achieved in previous off-chip approaches using coated capillaries¹⁶ or, for protein/bulk solution detection, using integrated gold mirrors.¹⁷

Salimi-Moosavi *et al.*¹⁶ used lithographic fabrication techniques to produce a multipass cell, comprising a pair of aluminium mirrors, 200 μm apart, surrounding a 30 μm diameter flow channel (see Figure 4.1).

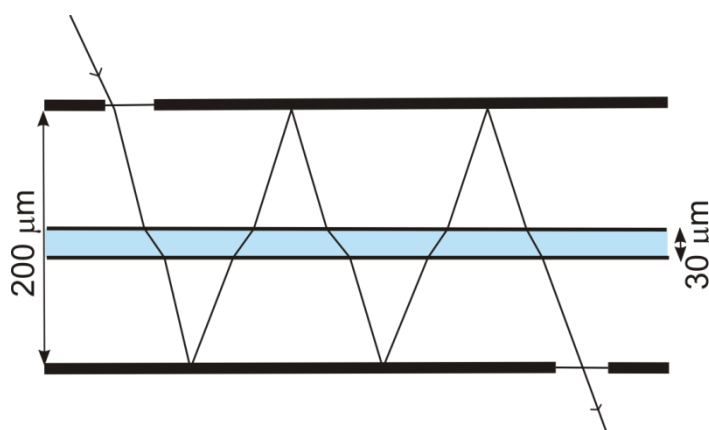


Figure 4.1 Schematic of the multipass cell design of Salimi-Moosavi *et al.* showing the light path, which enters and exits through apertures in two mirrors, spaced 200 μm apart and surrounding a 30 μm diameter flow channel.¹⁶

This multireflection cell (using a 633 nm He:Ne laser light source) allowed an effective optical pathlength of 272 μm through solution and a 5- to 10-fold sensitivity enhancement compared with single pass measurements.

Billot *et al.*¹⁷ demonstrated similar sensitivity enhancements using a patterned 50 μm -diameter microfluidic channel in a thin layer of polydimethyl-siloxane, sandwiched by two glass substrates onto which metallic layers had been deposited to form the mirrors of a multipass cell (see Figure 4.2).

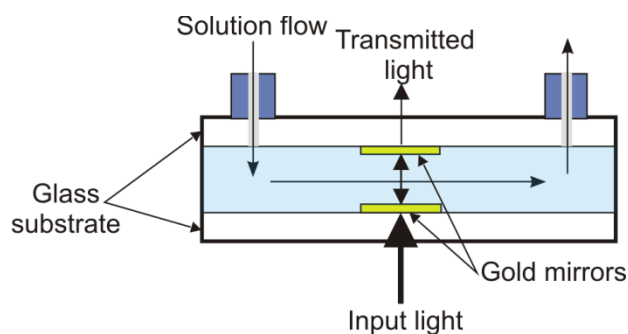


Figure 4.2 multipass cell design of Billot *et al.* See text for details.

Using 100 nm thick gold mirrors, a Rhodamine B concentration of 5 μM ($A \approx 2.5 \times 10^{-2}$) could clearly be detected and the authors claimed that, using slightly thicker Au mirrors (150 nm), an effective optical pathlength of 2.5 mm was achieved.

Compared with single pass measurements, relatively modest sensitivity gains are achieved using the multipass cells described above, due to difficulties in cell alignment. This chapter describes the first application of BBCEAS to *in situ* analyte detection within microfluidic droplets. The use of an optical cavity, comprising an intracavity microfluidic chip and a pair of broadband coated external cavity mirrors, in combination with an SC light source and sensitive detector allows the full visible spectrum ($430 \text{ nm} < \lambda < 700 \text{ nm}$) of each passing droplet to be acquired *in situ* at a higher repetition rate (273 Hz/3.66 ms acquisition time) and sensitivity ($A_{\min}$ of the order 10^{-4} , $\alpha_{\min} < 10^{-2} \text{ cm}^{-1}$) than that achieved in previous microfluidic chip-based studies.

4.2 Experimental

The experimental setup used here is shown schematically in Fig. 4.3(A). Two highly reflective mirrors, M1 and M2, (mirror set 2, 1 m radius of curvature, see Figure 2.3(b)) form a stable

linear cavity ($L = 12$ cm). A microfluidic chip is inserted into the optical cavity and oriented (using a micrometer-actuated goniometer-translation-rotation stage) at normal incidence to the optical beam such that the light within the cavity passes through a single microfluidic channel, Fig. 4.3(B).

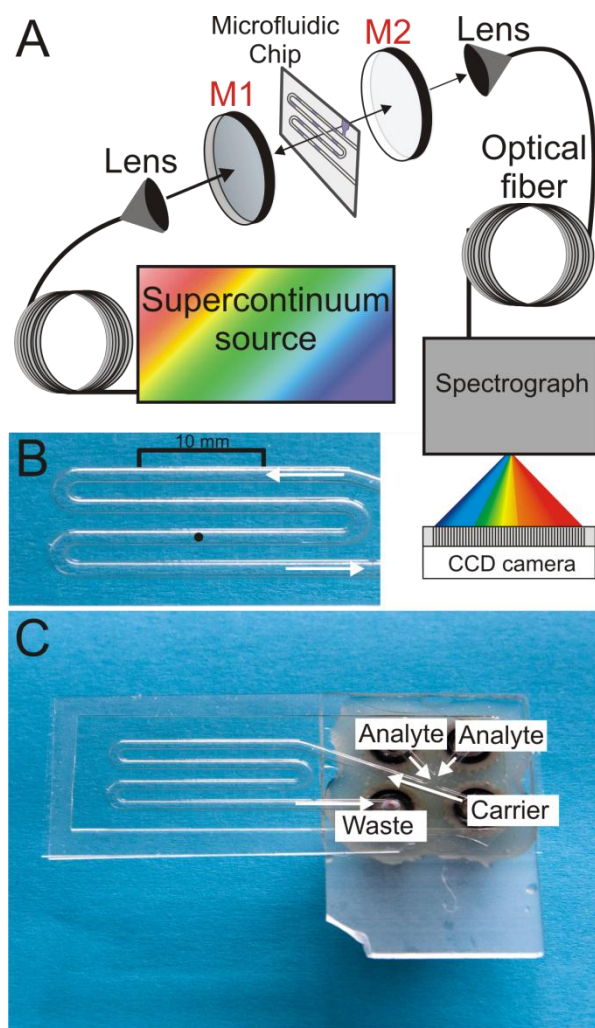


Figure 4.3 (A) Experimental BBCEAS setup. (B) The microfluidic channel (1 mm in width and 320 μm in depth), as viewed along the optical axis of the cavity. The position and estimated size of the optical beam is shown as a black dot. (C) Full view of the microfluidic chip and channels. Liquid flows into and out of the chip through nanoports (Upchurch Scientific). Dual carrier-analyte inlets allow for measurements to be made in continuous- or droplet-flow regimes, using one or two syringe pumps (Chemxy, Fusion 200) respectively. The two analyte nanoports are designed to allow injection of two different analytes (although this function was not used in the experiments presented here)

Figure 4.3(C) illustrates the simplicity of the microfluidic chip used in these experiments.

The chip was fabricated by Joanna Davies and Dr Joao Cabral at Imperial College London, according to the method described by Harrison *et al.*¹⁸ Channels of width 1 mm and depth 320 μm were fabricated in UV-curing optical adhesive (Norland NOA81), sandwiched between two ~ 140 μm thick microscope cover slides. After UV-curing (where a photomask was used to

generate the channel pattern within the adhesive), the channel was rinsed through with ethanol to remove uncured adhesive. This technique is designed for rapid and easy fabrication of relatively large channels, which are also compatible with most organic solvents. However, as discussed later, very little attention was paid to optimizing the optical quality of these chips.

An SC source (NKT, Koheras SuperK Power Blue, see Section 2.2.1) was used as the cavity light source. Before entering the cavity, the light was spectrally filtered (see Section 2.3) to yield a bandwidth of *ca.* 425-800 nm. At its intersection with the chip, the beam diameter is estimated (by observation of the light intensity exiting the cavity during chip translation) to be *ca.* 600 μm , which, with the per-pass optical pathlength through solution of 320 μm , yields a probed sample volume of ~ 90 nL.

Light exiting the cavity is fiber coupled to a spectrograph and CCD arrangement (Andor Technology, SR-303i, Newton, 150 line/mm grating, blazed at 500 nm, slit width = 100 μm , see also Section 2.5). Spectra are acquired with a 3.66 ms acquisition time, at a repetition rate of 273 Hz. Each acquisition is performed using full vertical (spatial) binning of the charge on the CCD array before readout. The data rate is limited only by the camera acquisition settings/transfer rate.

4.3 Results and Discussion

In this proof-of-principle demonstration, droplets or ‘slugs’ of aqueous K_2IrCl_6 solution (Ir(IV)) were monitored during their flow through the microfluidic chip, in a toluene carrier phase. Before droplet-flow measurements, the detection sensitivity was determined in a series of single-wavelength continuous flow measurements.

4.3.1 Continuous flow measurements

The absorption spectrum of Ir(IV), shown inset in Figure 4.4, displays a maximum at 487 nm. The intensity, I , of 487 nm light transmitted through the cavity was recorded as solutions

containing different concentrations of Ir(IV) were flowed continuously through the chip. The reference, I_0 , was determined from measurements with pure solvent (Milli-Q water, resistivity 18.2 MΩ cm) flowing through the chip. Figure 4.4 shows $\frac{I_0}{I} - 1$ as a function of Ir(IV) concentration. At low concentrations, $\frac{I_0}{I} - 1$ displays a linear dependence on concentration (i.e., $\frac{I_0}{I} - 1 \propto A$, see Equation 1.21). An error-weighted regression through this linear region provides a measure of the limit of detection (LOD) of 1.4 μM for Ir(IV), determined from the 3σ uncertainty on the intercept, giving $A_{\min} = 1.8 \times 10^{-4}$ (using $\varepsilon = 4 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$,¹⁹ for Ir(IV) at 487 nm) and a minimum detectable absorption coefficient, $\alpha_{\min}$, of $1.3 \times 10^{-2} \text{ cm}^{-1}$ under these experimental conditions.

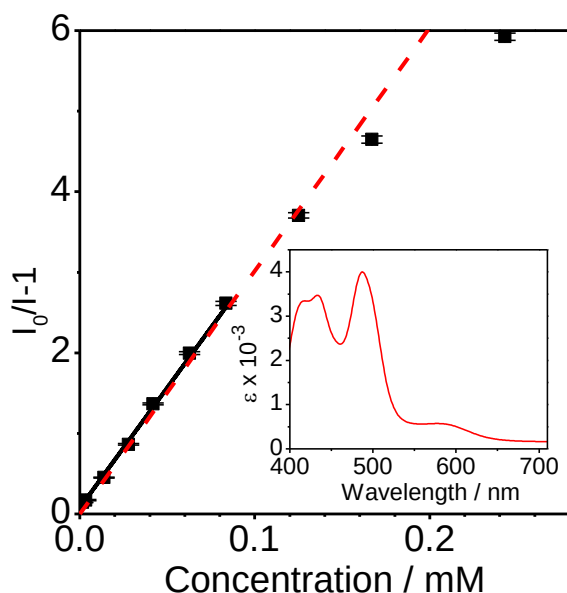


Figure 4.4 $\frac{I_0}{I} - 1$ (at 487 nm) versus Ir(IV) concentration. Data points represent the average of 15 measurements, the standard deviation of which is shown. The solid black line represents an error-weighted regression ($R^2 = 0.996$) through the linear region of the graph. The dashed red line represents a predicted relationship between $\frac{I_0}{I} - 1$ and concentration, calculated using equations 1.20 and 1.4, with $R = 0.9902$ (CEF = 102) and $\varepsilon_{487 \text{ nm}} = 4 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$.¹⁹ Inset: absorption spectrum of Ir(IV), with values of $\varepsilon(\lambda)$ determined using the known extinction at 487 nm.

To compare with single-pass measurements, Equation 1.21 can be used to determine values for the cavity enhancement factor (CEF, see also Section 1.2.2). At 487 nm, the slope of the linear fit in Figure 4.4 yields a CEF of 102, corresponding to an effective path length, $l_{\text{eff}} = \text{CEF} \times l$, of 3.26 cm. This CEF value is in agreement with the results of CRDS experiments, performed by Cathy Rushworth and Dean James (Vallance Group, Oxford) using the same chip, in which $\tau_0 \sim 350 \text{ ns}$ was achieved with $L = 1 \text{ m}$ (see also Equation 1.12).²⁰

The curvature at high Ir(IV) concentrations seen in Figure 4.4 is unusual. It appears that this feature cannot be explained by a breakdown in the approximations leading to Equation 1.21—Figure 4.4 shows that the same degree of curvature is not expected when $\frac{I_0}{I} - 1$ is predicted using Equation 1.20, which can be used for all ranges of R and A (see Section 1.2.2).^{12,13} It may be that photodegradation of the Ir(IV) solution resulted in a significantly reduced absorbance at the higher concentrations tested here, although, from experience, Ir(IV) appears to be quite photostable. A more likely cause of the curvature is that at these high concentrations (and absorbances) very little light is transmitted through the cavity and the CCD is operating outside of its linear dynamic range.

It is worth reiterating that the results shown in Figure 4.4 were achieved with a standard microfluidic chip—without any precision optical surfaces, or any care taken to ensure that the surfaces are precisely parallel to one another—in just a 55 ms acquisition time. However, it is clear even in these preliminary efforts, that the sensitivity of the BBCEAS technique surpasses that of previous absorption measurements in microfluidic chips (outlined in Section 4.1 and detailed in Kuswandi *et al.*² and Floquet *et al.*²¹ and references therein).

In the present work, the number of light passes through the cavity is primarily limited by the poor optical quality of the microfluidic chip itself. The optical quality of the chip seemed to vary along the length of the channel. Likely causes of the low optical quality include defects/damage in the glass slides and/or the presence of optical adhesive in the beam path. Whilst the channel should, in principle, have a rectangular cross section, it is possible that remnants of optical adhesive, left within the desired channel-boundaries after the curing process, reduced the optical quality of the chip.

In previous liquid-phase cavity-based studies, $\alpha_{\min}$ values in the range 10^{-3} – 10^{-7} cm⁻¹ have been achieved using high quality optical components (see Table 1.1), indicating that by improving the optical quality of the chip alone, there is scope to improve the measurement sensitivity by

several orders of magnitude. As the beam only passes through a small section of the chip, one option is to incorporate a high optical-quality detection chamber at a specific point along the microfluidic channel.

4.3.2 Droplet flow measurements

In addition to the small volumes probed and the high sensitivity, a major advantage of the BBCEAS approach is its spectral bandwidth and—with the aid of the high spectral irradiance of the SC source and sensitivity of the CCD detector—the excellent temporal resolution that may be achieved. To illustrate these combined attributes, Figure 4.5 shows the absorption spectrum of $\sim 6\ \mu\text{L}$ slugs of $40\ \mu\text{M}$ aqueous Ir(IV) solution flowing rapidly ($0.35\ \text{mL min}^{-1}$) through the chip in a toluene carrier phase.

The temporal and wavelength-resolved spectra are shown in Figure 4.5(a). For these measurements, I_0 was the signal recorded in the presence of the carrier phase only. Figure 4.5(b) highlights the excellent signal to noise achieved for an individual spectrum despite the short, 3.66 ms, acquisition time. The time resolution is demonstrated by the absorbance at 487 nm shown in Figure 4.5(c). Each individual spectrum contains a spectrally flat background component which is believed to arise from scatter at the interface between the slug and a thin lubricating layer of carrier phase at the walls of the chip. As the absorption spectrum of Ir(IV) is known, this scattering contribution is easily quantified and subtracted (see Figure 4.5(b)).

At both ends of the Ir(IV) slugs, lensing effects result in a large increase in the intracavity losses due to refraction (Figure 4.5(c)). Only as the main body of the slug passes into the beam is the absorbance spectrum of Ir(IV) recovered (Figure 4.5(b)). The large scattering-induced signals at the start and end of each droplet provide convenient signals for triggering acquisition during droplet flow, and could potentially be exploited for droplet shape analysis.

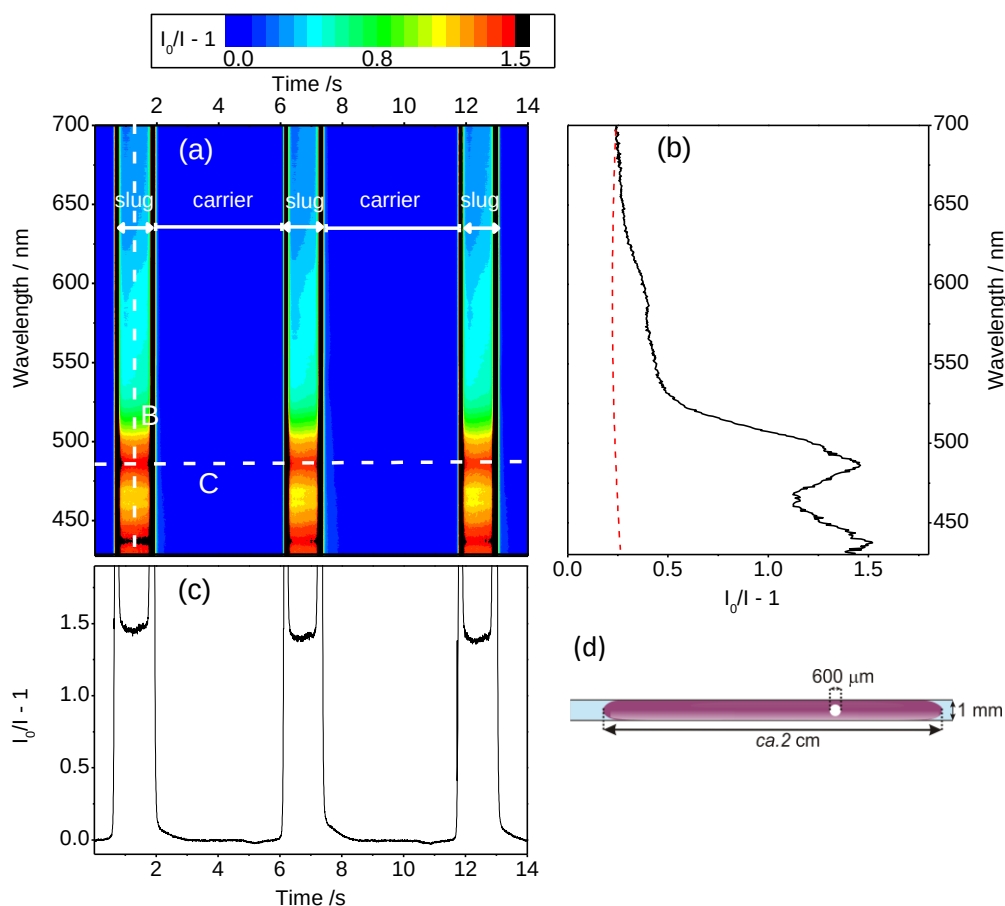


Figure 4.5 (a) Contour plot of the BBCEAS-measured spectra as a function of time during flow (0.35 mL min^{-1}) of Ir(IV) slugs through the microfluidic chip. (b) Spectral cut through the contour plot at 1.25 s (red dashed line represents a baseline scattering contribution) and (c) a temporal cut through at 487 nm – the microfluidic slugs and carrier phases are clearly visible with intense scattering observed at both ends of the slugs. A schematic of the estimated droplet dimensions and beam diameter is shown in (d).

The SNR achieved permits an alternative estimate of $\alpha_{\min}$. From the 3σ uncertainty in the baseline of the plot in Figure 4.5(c), $\alpha_{\min}(487 \text{ nm})$ is estimated to be $9.5 \times 10^{-3} \text{ cm}^{-1}$, close to the value obtained from the fit to the data in Figure 4.4.

4.4 Conclusion

In summary, results presented in this chapter represent the first application of broadband cavity-enhanced absorption spectroscopy to the analysis of microfluidic samples. The technique provides full visible broadband spectral measurement with both high detection sensitivity and excellent temporal and spatial resolution.

Given the proof-of-principle nature of the study described in this chapter, it is pertinent to discuss the future potential, and the limitations, of BBCEAS for microfluidic sensing. One of the key factors (discussed previously in Chapter 2), which restricts the detection limit of the technique, is the stability of the SC source and of the instrument as a whole. Due to drift in the BBCEAS signal (Section 2.7), increasing the signal integration time by a factor of 100, to 0.366 s, only results in a factor of two improvement in $\alpha_{\min}$ (487nm); the instrument stability is clearly worse than that achieved using the BBCEAS instrument described in Section 2.7 (where the same increase in signal integration time resulted in an order of magnitude improvement in sensitivity). This is probably due to the presence of flow-induced light intensity fluctuations, present during the microfluidics experiments, which might be caused by sample scattering or movement of the chip during sample flow. Therefore, the drift may be reduced by improving the chip and cavity design. However, a significant component is inherent in the SC source itself. Rapid switching between I_0 and I (mentioned previously in Section 2.7.1) may militate against long term drift and this approach would be well-suited to the study of rapidly flowing droplets.

It should also be noted that in the droplet flow measurements discussed in Section 4.3.2, I_0 was the signal recorded in the presence of the carrier (toluene) phase only. A better approach would be to record I_0 —between each I measurement, as discussed above—in the presence of water-only droplets. This would make simple the removal of any additional background losses (which result in the baseline scattering contribution shown in Figure 4.5) associated with the passage of droplets through the beam.

The sensitivity of the technique is expected to improve markedly with modifications to the chip design. As discussed above, the channel's optical quality seemed to vary along its length and there were very few sections of the channel that were suitable for use in BBCEAS: a number of initial experiments failed due to the poor CEF achieved. Future chips should be manufactured to higher standards of optical quality, even if this results in a slight increase in the fabrication

time/cost. Incorporating high quality optical surfaces could dramatically increase the CEF, as could altering the channel geometry to ensure a well-defined rectangular cross section. The large slug volumes used here could also be reduced substantially without losing sensitivity by using chip designs and dimensions that more closely match the total droplet/slug volume with that probed by the cavity beam. The slug volumes in this work also tended to vary throughout the experiment—better quality channels, with fewer defects, could help solve this problem. Additionally, the chip's mounting within the cavity could be improved, to increase stability and prevent changes in alignment during experiments.

Recently, Dean Sheppard (Mackenzie group) has begun a detailed theoretical and experimental analysis of beam diameter in a variety of linear cavity geometries. Initial experiments have used a razor blade, translation-stage-mounted within the cavity and moved incrementally to block the beam and determine its spatial profile.

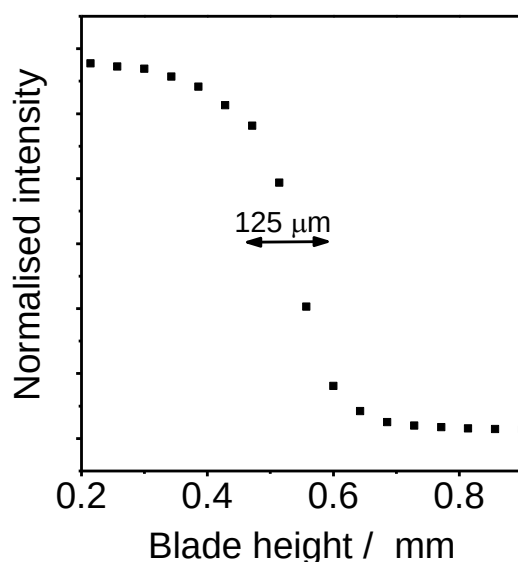


Figure 4.6 light intensity (at 550 nm) recorded at various razor blade heights (see text for details).

Figure 4.6 shows the result of a typical razor-blade beam-block experiment, for a cavity with the same length and mirrors as those used in the microfluidics experiments above. In this particular experiment the blade was positioned under the cavity, at the midpoint between the

cavity mirrors and mutually perpendicular to the cavity axis, then translated vertically to gradually block the cavity beam.

The razor-blade experiment described above suggests the beam has a near-Gaussian profile with a full width half maximum *ca.* 125 μm . This value is smaller than the beam diameter estimated in the presence of the chip (*ca.* 600 μm) although, given the chip's optical quality as well as the relatively crude estimation of beam diameter in its presence, this result is unsurprising. Further analysis of such beam-block experiments, in combination with theoretical simulations of the beam profile within the cavity, will allow cavity geometries to be optimized for particular sized microfluidic chips (and *vice versa*), allowing miniaturization of the total droplet volume.

A key advantage of the BBCEAS technique is that the broadband character of the absorption measurements allows the separation of contributions from species whose absorption spectra overlap.²² This may be potentially useful for probing reactions within droplets and, coupled with the fast temporal response, it is envisaged that the technique will be of considerable value in studying intra-droplet reaction kinetics. A simple demonstration of this might involve the study of the Belousov-Zhabotinsky reaction, which exhibits periodic oscillations in absorption that can be monitored in time.²³ By monitoring droplets at different points along the channel, or altering the droplet flow rate, the intra-droplet reaction could be studied as a function of time. This concept, along with some of the others described above, is illustrated in Figure 4.7.

Another possible route for research would be the development of an intracavity evanescent-wave-based chip. As described in Chapters 1 and 2, EW cavity-based techniques are only sensitive to processes within hundreds of nanometres of a TIR interface and therefore have very low sampling volumes. In principle a microfluidic channel with a very shallow depth (a few micrometers) could be fabricated on the upper surface of an intracavity TIR element and used, in combination with an EW cavity-based technique, to sensitively monitor the absorbance of a

sample within the channel. Due to the high surface-sensitivity of EW-based techniques, this approach would be particularly well-suited to studying adsorption processes along the microfluidic channel. However, the challenge in these experiments would be minimizing the beam footprint at the interface, to constrain it within the channel.

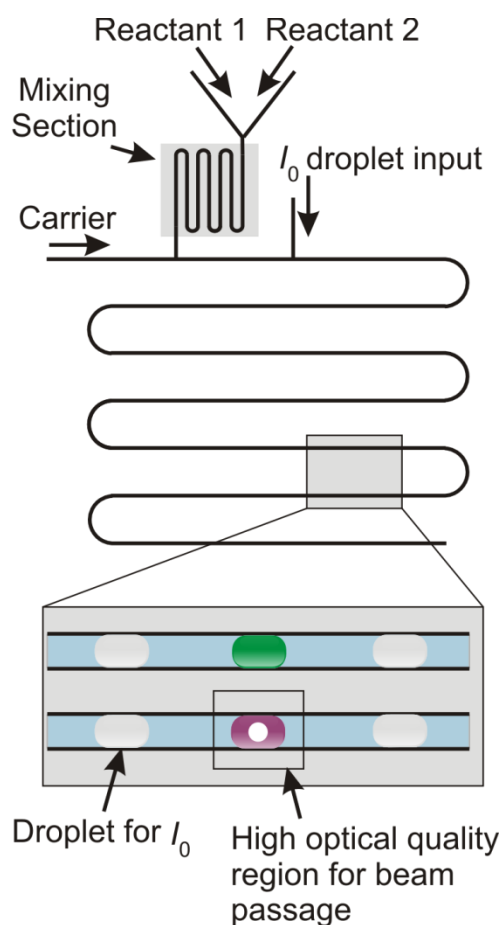


Figure 4.7 An intracavity microfluidic chip design, which improves on the chips used in this project. Droplets for I and I_0 measurements could be alternately injected (via separate inputs) using pre-programmed syringes. Absorption changes as a function of time could be monitored via droplet observation at different points along the channel, or at a fixed point whilst varying the flow rate. A high optical quality region (with a well-defined rectangular cross section and super-polished glass surfaces) would allow high CEFs. White dot indicates beam spot.

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Chapter 5

An introduction to magnetic field effects on radical recombination reactions

Spin correlated radical pairs exist as short-lived intermediates in numerous photolytic, thermolytic and radiolytic reactions, and have been studied widely for over 40 years.¹⁻³ It is well established that the application of external magnetic fields, either static or oscillating, can significantly affect the outcome of reactions proceeding *via* spin correlated RPs by perturbing the kinetics and/or yields of particular reaction paths.⁴ The yield of radical recombination reactions can even be affected by the application of magnetic fields as weak as that of the Earth ($\sim 50 \mu\text{T}$).⁵ These facts, at first, seem surprising as they cannot be explained on thermodynamic grounds. For example, even a one Tesla field produces a splitting of the electronic states of a radical (*ca.* 11.2 J mol^{-1}) which is three orders of magnitude smaller than RT ($\sim 2500 \text{ J mol}^{-1}$) at room temperature.^{1,6} However, as discussed in this chapter, the physical origins of these field effects are well understood within the framework of the radical pair mechanism (RPM) and the effects of magnetic fields on chemical reactions can be explained by changes in reaction kinetics, provided that a spin correlated RP is present as a reaction intermediate.⁴

Chemical magnetic field effects (MFEs) have been observed and studied in a variety of different contexts, including in biological systems and, as described in this chapter, an understanding of the RPM may help identify mechanisms governing bird migration. Crucially, however, the study of RP intermediates in biological systems has been hampered by the low sensitivity of the optical techniques usually employed to investigate these highly reactive species. Later chapters

(6-8) demonstrate the powerful capabilities of optical cavity-enhanced techniques (including CRDS, CEAS and BBCEAS) in monitoring radical recombination reactions and associated MFEs in solution. In particular, the high-sensitivity of these techniques is harnessed to study MFEs in biologically relevant model systems and biological samples. Whilst the work described in the coming chapters places a strong emphasis on the cavity-based techniques used, it is necessary to provide a brief introduction to the basic spin chemistry that underlies the radical pair reactions and magnetic field effects studied.

5.1 Spin

The principles that underlie MFEs fall within an area of study known as spin chemistry. Spin is a form of intrinsic angular momentum, purely quantum mechanical in nature and distinct from the orbital angular momentum of a classical particle. An electron has a spin quantum number $s = \frac{1}{2}$ and two possible m_s sub-states (representing different spin projections on a fixed z-axis): $m_s = +\frac{1}{2}$ and $m_s = -\frac{1}{2}$.⁷⁻¹⁰ In the absence of external fields these states are degenerate. This degeneracy is, however, lifted by the application of a magnetic field (Section 5.2).

As an electron is charged, its spin angular momentum has an associated spin magnetic moment, μ_s , which is determined by the particle's spin angular momentum vector $\mathbf{s}$:

$$|\mathbf{s}| = \hbar \sqrt{s(s+1)} \quad (5.1)$$

$$\mu_s = -\frac{g_e \mu_B s}{\hbar} \quad (5.2)$$

where $\hbar$ is the reduced Planck constant, the dimensionless proportionality constant g_e ($=2.002319304$) is the g-value for a free electron and μ_B is the Bohr magneton defined by:

$$\mu_B = -\gamma_e \hbar = \frac{e\hbar}{2m_e} \quad (5.3)$$

where γ_e and m_e are the magnetogyric ratio and mass of the electron, respectively, and e the elementary charge.

Furthermore, the spin angular momentum vector's projection, s_z , on a fixed, arbitrary (z) axis is given by $s_z = m_s \hbar$ and the z component of the spin magnetic moment itself is quantized according to:

$$\mu_z = -g_e \mu_B m_s \quad (5.4)$$

5.2 Interaction of electron spin with a magnetic field

In the absence of a magnetic field the two m_s states are degenerate. However, in the presence of an applied (external) static magnetic field, the spin states are split in energy by interaction of this field with the spin magnetic moment. This phenomenon is called the Zeeman effect (or Zeeman interaction) and can be understood by considering the interaction energy of a classical magnetic moment, $\boldsymbol{\mu}$, with an external static magnetic field, $\mathbf{B}$:

$$E = -\boldsymbol{\mu} \cdot \mathbf{B} \quad (5.5)$$

The Hamiltonian for the interaction of the electron spin angular momentum with the external magnetic field can therefore be written as:

$$\hat{H} = \frac{g_e \mu_B \hat{\mathbf{S}} \cdot \mathbf{B}}{\hbar} \quad (5.6)$$

where $\hat{\mathbf{S}}$ is the doublet spin operator. Defining the field corresponding to $\mathbf{B}$ as lying along the z-axis and having a field strength B_0 , Equation 5.6 can then be simplified to give:

$$\hat{H}_z = \frac{g_e \mu_B \hat{S}_z B_0}{\hbar} \quad (5.7)$$

with eigenvalues (Zeeman energies) given by:

$$E_z = \frac{g_e \mu_B B_0 m_s}{\hbar} \quad (5.8)$$

The Zeeman-splitting is then defined as the difference in energy, ΔE_z , between the two m_s levels:

$$\Delta E_z = \frac{g_e \mu_B B_0}{\hbar} \quad (5.9)$$

This Zeeman effect is shown in Figure 5.1. The figure also shows a vector representation for the spin angular momentum of an electron and its associated magnetic moment, which, in the presence of an applied static field, B_0 precess about the field direction at the Larmor frequency:

$$\omega_L = \frac{g_e \mu_B B_0}{\hbar} \quad (5.10)$$

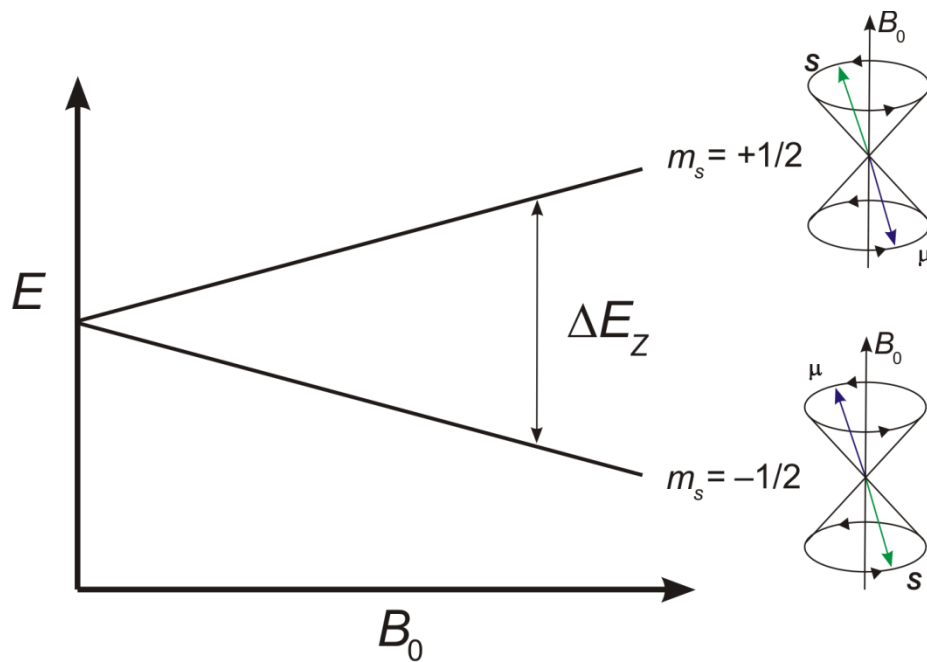


Figure 5.1 The Zeeman effect, causing the two spin states of an electron to split in energy, by ΔE_z , in an applied magnetic field, B_0 . Vector representations of the electron's spin angular momentum, $\mathbf{s}$, and its associated magnetic moment, $\boldsymbol{\mu}$, are also shown. N.B. the vector lengths shown are not to scale ($|\mathbf{s}| = \frac{3}{4}\hbar$ and $|\boldsymbol{\mu}| = -\frac{g_e \mu_B}{\hbar}$).

The treatment above is appropriate for free electrons. However, when considering an unpaired electron in an atom or molecule, B_0 in Equation 5.9 should be replaced by an effective magnetic field, B_{eff} , equal to the sum of the applied external magnetic field and an induced magnetic field, B_I , that depends on the specific local magnetic environment (resulting from spin-orbit coupling and orbital Zeeman interactions):

$$\Delta E_z = \frac{g_e \mu_B}{\hbar} B_{\text{eff}} \quad (5.11)$$

$$= \frac{g_e \mu_B}{\hbar} (B_0 + B_I) \quad (5.11a)$$

By convention any deviation of B_{eff} from B_0 is accounted for by adjusting the g -value away from g_e , so Equations 5.11 and 5.11a may be re-written as:

$$\Delta E_z = \frac{g \mu_B B_0}{\hbar} \quad (5.12)$$

However, for most organic radicals, such as those dealt with in this project, $g \approx g_e$.

5.3 A pair of coupled spins

The sections above have been concerned with single unpaired electrons. However, as will become apparent later, magnetic field effects require interactions between a pair of spins.

A pair of electron spins (a 'radical pair', RP) may either be correlated or uncorrelated depending on the spin state of the radicals. An uncorrelated RP has electron spins that are randomly oriented with respect to one another (this is the case in so-called *F*-pairs, described in Section 5.4, which result from chance encounters in bulk solution). Spin-correlated RPs, on the other hand, are created under conservation of total spin angular momentum, with their electron spins mutually oriented in a particular way. From this point forward, the term RP is used to describe a spin-correlated radical pair.

For a pair of electron spins 1 and 2, the total spin angular momentum quantum number, S , may take on values of 0 or 1 (as $S = (s_1 + s_2), (s_1 + s_2 - 1), \dots, (s_1 - s_2)$, where s_1 and s_2 are the spin quantum numbers of spins 1 and 2). For $S = 1$, there are three possible magnetic quantum states, referred to as ‘triplet’ states, described using the notation $|S, M_S\rangle$: $|1, -1\rangle$, $|1, 0\rangle$, $|1, +1\rangle$ and labelled T_- , T_0 and T_+ respectively, where M_S can take on values $M_S = S, (S - 1), \dots, -S$. With $S = 0$, only one (‘singlet’) state is possible: $|0, 0\rangle$, labelled S .

A simplified vector representation of these four spin states is shown in Figure 5.2. In this picture, the vectors, $\mathbf{s}_1$ and $\mathbf{s}_2$, representing the spin angular momenta of electrons 1 and 2, precess about the field (B_0) direction at the Larmor frequency (Equation 5.10, replace g_e with g)

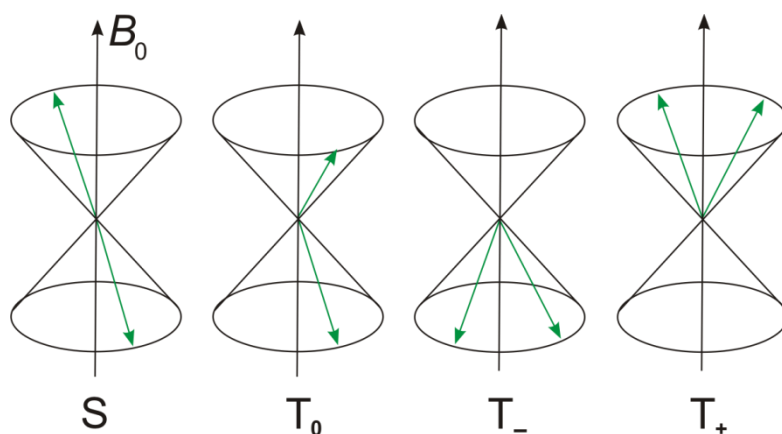


Figure 5.2 A simplified vector representation of singlet (S) and triplet (T_+ , T_0 , T_-) spin states. The spin vectors of electron spins 1 and 2 ($\mathbf{s}_1$ and $\mathbf{s}_2$, green arrows) precess about the field (B_0) direction (black arrows) at the Larmor frequency. Upward- and downward-pointing green arrows represent the $m_s = +\frac{1}{2}$ and $-\frac{1}{2}$ states, respectively. It should be noted that the vector model depicted here is related to a more robust (but less intuitive) vector model described by Syage.¹¹

By analogy with the Zeeman energy for a single electron case (Equation 5.8), for a pair of spins the Zeeman energy can be re-defined as:

$$E_z = \frac{g\mu_B B_0 M_S}{\hbar} \quad (5.13)$$

Therefore, the triplet states $T_{+/-}$ (with $M_S \neq 0$) are affected by applied magnetic field strength and become energetically separated (by $\Delta E_Z = \frac{g\mu_B B_0}{\hbar}$) from the S and T_0 states (as $M_S = 0$, these states have no component of spin angular momentum in the field direction and are unaffected by field strength). This behavior is shown in Figure 5.3(a).

Whilst the vector representation in Figure 5.2 is purely classical in nature, it is useful in showing that processes which provide a change in phase between the two spins can allow S- T_0 interconversion, and processes that cause spin-flip will allow interconversion of the S and $T_{+/-}$ spin states. The mechanisms that might cause such S-T interconversion are discussed in Section 5.4.1. However, before moving on, it is important to recognise that for the spins to form a RP they must interact with one another as described below.

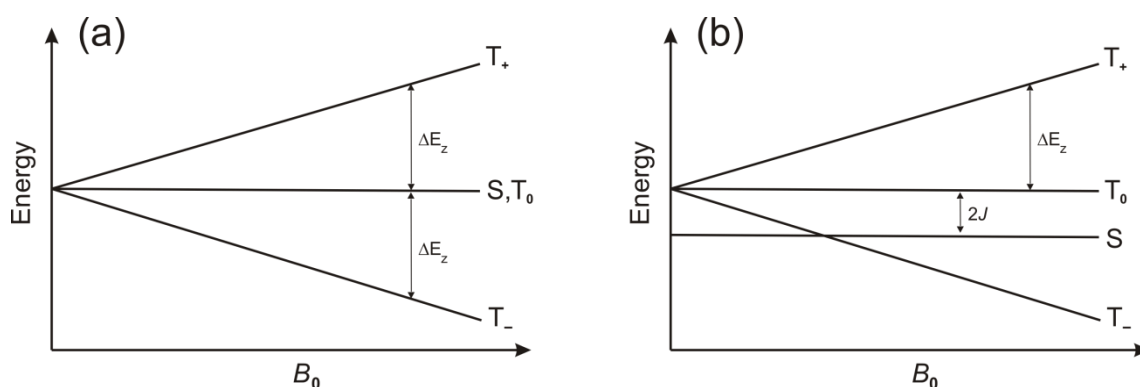


Figure 5.3 Energy of the spin states as a result of (a) the Zeeman interaction only and (b) the Zeeman and exchange interactions ($J > 0$), described in Section 5.3.1. In both cases dipolar interactions are ignored ($D = 0$, see Section 5.3.2).

5.3.1 Exchange interaction

The exchange interaction, which arises from the Pauli exclusion principle, is purely quantum mechanical in origin (with no classical analogue) and splits the energy levels of singlet and triplet states, removing their degeneracy even at zero field. The exchange Hamiltonian, $\hat{H}_{ex}$, for this interaction may be expressed as:¹²

$$\hat{H}_{ex} = -2J(r)\hat{\mathbf{s}}_1 \cdot \hat{\mathbf{s}}_2 \quad (5.14)$$

where $2J$ is the exchange energy (as a function of the RP's inter-radical separation, r) and $\hat{\mathbf{s}}_1$ and $\hat{\mathbf{s}}_2$ are the triplet spin angular momentum operators for electrons 1 and 2. The singlet and triplet state energies due to the exchange interaction (and the Zeeman interaction) are shown in Figure 5.3(b). The exchange interaction is strongly distance dependent and is assumed to decrease exponentially as r is increased—for $r > 1$ nm the exchange interaction is usually considered negligible.¹³

5.3.2 Dipolar interactions

The dipolar interaction describes the interaction of the magnetic dipoles associated with the electrons in a RP. The dipolar Hamiltonian, $\hat{H}_{dip}$ can be expressed as:

$$\hat{H}_{dip} = \hat{\mathbf{s}} \cdot \mathbf{D} \cdot \hat{\mathbf{s}} \quad (5.15)$$

where the dipolar tensor, $\mathbf{D}$, describes the size of the interaction and $\hat{\mathbf{s}} = \hat{\mathbf{s}}_1 + \hat{\mathbf{s}}_2$. The interaction is anisotropic in form and, even at zero field, causes a splitting of the triplet states; however, it is assumed that in the liquid-phase systems studied in this project rapid molecular motion averages out the dipolar interaction and it can be ignored.

5.3.3 Hyperfine interaction

In addition to the inter-electron interactions described above, the (hyperfine) interaction of an electron with nearby magnetic nuclei (with non-zero nuclear spin angular momentum, I) must be considered. There are two possible types of this hyperfine interaction: an electric and a magnetic hyperfine interaction. However, the distance dependence ($1/r^5$) of the electric hyperfine interaction makes it negligible at typical radical electron-nucleus separations and therefore only the magnetic hyperfine interaction is relevant to this thesis.

The hyperfine interaction is due to two spin coupling mechanisms: the dipole-dipole interaction and the Fermi contact interaction.⁸ The dipole-dipole interaction, between magnetic nuclei and electrons, is analogous to the inter-electron dipolar interaction described in the previous section—it is anisotropic and averages to zero with rapid molecular motion in solution. Therefore, in the liquid-phase, the only important component of the hyperfine interaction is the Fermi contact interaction, which is dependent on the electron spin density at a magnetic nucleus (i.e. it only applies to molecular orbitals with some s-orbital character), it is an exclusively isotropic interaction and hence does not average out in solution.

The hyperfine interaction provides an additional local magnetic field (B_{HF}), which modifies the effective field experienced by the radical electron:

$$B_{\text{eff}} = B_0 + B_{\text{HF}} \quad (5.16)$$

$$= B_0 + m_I a \quad (5.16a)$$

where $m_I (= I, (I - 1), \dots, (-I + 1), -I)$ is the nuclear magnetic quantum number and a is the isotropic hyperfine coupling constant.

5.4 The Radical Pair Mechanism

The radical pair mechanism describes how an applied magnetic field can influence S-T interconversion (also referred to as S-T mixing) and ultimately alter the recombination probability in radical pair reactions.

The RPM was discovered independently by Kaptein & Oosterhoff,⁴ and Closs,¹⁴ in 1969 and the majority of chemical reactions known to be affected by magnetic fields (including those studied in this project) proceed *via* the RPM.¹ The RPM is introduced below and the S-T mixing

mechanisms—crucial to the RPM—are discussed later in Sections 5.4.1. The details of RPM reaction schemes for the individual systems used in this project are given in later chapters. For the interested reader, the RPM is covered in depth by Salikhov *et al.*¹³ and Nagakura *et al.*¹⁵, as well as in an excellent review by Steiner & Ulrich.¹

The RPM has three important features: (1) it involves spin correlated RPs, which (2) can undergo S-T mixing. Finally, (3) RP recombination is state-selective: recombination-rates and/or -products differ for singlet and triplet RPs.

A simplified example of a RPM reaction scheme is shown in Figure 5.4. Here the precursor (A) is photoexcited, to produce a singlet excited state ($^1A^*$), which subsequently accepts an electron from a donor (D), forming a radical pair, comprising two radicals with opposite charge and correlated electron spins: $^1A^* + D \rightarrow ^1[A^{\bullet-} + D^{\bullet+}]$. A and D could be separate molecules, or they could be different moieties of the same molecule. The example here is for a singlet-born RP system: the RP is formed under conservation of total electron spin angular momentum so that a singlet precursor leads to a singlet arrangement of the spins of the two radicals. However, other systems can involve triplet born RPs, where photoexcitation, followed by inter-system crossing, leads to $^3A^*$ and then $^3A^* + D \rightarrow ^3[A^{\bullet-} + D^{\bullet+}]$.

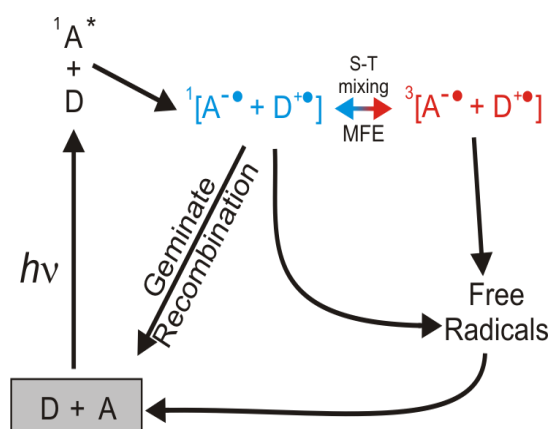


Figure 5.4 RPM reaction scheme for a singlet-born RP, $^1[A^{\bullet-} + D^{\bullet+}]$, formed *via* photoexcitation of A and subsequent electron transfer from a donor, D (see text for details). The magnetic field dependence of the S-T mixing process is indicated by 'MFE'.

Following formation, the pair of radicals are present as a 'geminate pair' (G-pair)—the radicals are very close to one another at this stage. Crucially, S-T mixing may occur at the G-pair stage and can be influenced by external magnetic fields, affecting the kinetics and product yield of radical recombination reactions, as discussed below.

In the (singlet or triplet) geminate pair state, the radicals have a high chance of re-encountering one another. Importantly however, the probability of recombination on radical re-encounter is spin-state dependent—as with radical formation, radical recombination also occurs with conservation of spin angular momentum and, for the systems used in this project, recombination from the triplet RP is spin forbidden. Therefore, upon re-encounter only the singlet-state RP may recombine, to give a geminate product (GP, which, in the scheme shown in Figure 5.4, is the RP's ground state precursor, $D + A$).

If a reactive re-encounter does not occur at the geminate-pair stage then the radicals may eventually separate and diffuse freely in solution. Each of these free radicals may encounter another freely diffusing radical to form a freely diffusing (F-)pair. Recombination at the F-pair stage results in a so-called escape product, EP—as for the G-pair stage, recombination at the F-pair stage is dependent on the spin state of the RP. In the example shown in Figure 5.4 EP is the same as GP, i.e. the ground state precursor. Whilst this simple scheme provides a sufficient introduction for the work in this thesis, it should be noted that EP may not necessarily be the same as GP, but can be formed from any combination of the free radicals present in solution (which might also include radicals formed from the solvent, for example by hydrogen abstraction). Additionally, in some systems, the geminate product may differ from the RP's ground state precursor.¹⁶

Crucially, as a result of the spin-state dependence of radical recombination, any process that alters the spin-state distribution can affect the reaction kinetics and product yield. For

example, an acceleration of S-T mixing in the singlet-born RP system shown in Figure 5.4 leads to a decrease in the (singlet-formed) recombination product and a concomitant increase in the number of triplet-formed free radicals (the opposite is expected for a triplet born RP). The rate of S-T mixing can be changed (affecting the kinetics and product yield of the recombination reaction) depending on the strength of an applied magnetic field, as discussed in the next section.

5.4.1 S-T mixing mechanisms

In order for appreciable magnetic field effects to occur, the RPs must undergo S-T mixing and must also have a high probability of later re-encounter. The latter condition might be achieved by trapping the radicals in close proximity to one another using i) a viscous solution or restricting medium such as a polymer or protein skeleton, ii) Coloumbic interactions or iii) biradicals.

For the purposes of work described in this thesis, the important S-T mixing mechanisms are the hyperfine coupling mechanism and the low-field hyperfine mechanism. However, before these are described (in Sections 5.4.1.3 and 5.4.1.4) it is informative to outline two other S-T mixing mechanisms: the Δg and relaxation mechanisms.

5.4.1.1 The Δg mechanism

As seen from Equation 5.10 and Section 5.3, the angular (Larmor) precession frequency of an electron spin in an external magnetic field is g -value-dependent. Therefore, in an RP, a difference in the g -values of electrons 1 and 2 ($\Delta g = g_1 - g_2$) results in a B_0 -dependent difference in precession frequencies, $\Delta\omega_L$:

$$\Delta\omega_L = \omega_{L,1} - \omega_{L,2} = \frac{\Delta g \mu_B B_0}{\hbar} \quad (5.17)$$

which can, in turn, drive mixing between S and T₀ states. However, for the carbon-centred RPs used in this project Δg is very small and, at the relatively weak applied field strengths employed ($B_0 \leq 25$ mT), the Δg mechanism's influence on the rate of spin mixing is negligible.

5.4.1.2 Relaxation mechanisms

Incoherent spin mixing acts to destroy the spin polarization that exists upon RP formation, *via* spin-lattice and spin-spin relaxation. Spin-lattice relaxation acts to return the system to an equilibrium spin polarization, *via* random spin flips that result in interconversion between S/T₀ and the T_± states. These spin flips are induced by varying local magnetic fields (oscillating at an appropriate frequency) that result from molecular motion and tumbling. Spin-spin relaxation is induced by static local magnetic fields and acts to destroy the phase coherence between the RP's electron spins and thus contribute to S-T₀ interconversion.

The influence of incoherent mixing is typically ignored as spin-lattice and spin-spin relaxation occurs on timescales (10^{-6} - 10^{-4} s) that are longer than both the lifetime of the spin correlated radical pairs (10^{-10} – 10^{-7} s) and the timescales for coherent spin mixing precesses (10^{-9} – 10^{-8} s), discussed in the following sections.¹⁵ However, for some of the systems studied in this project spin relaxation mechanisms do have a significant effect on S-T mixing (see, for example Chapter 7).

5.4.1.3 The hyperfine coupling mechanism

Consider a RP in which both radicals have identical g values, but only one electron spin, $\mathbf{s}_1$, is coupled (*via* an isotropic hyperfine interaction, described in Section 5.3.3) to a single nuclear spin with $I = \frac{1}{2}$. At high external magnetic field strengths ($B_0 \gg a$), the magnetic moments of both electron and nucleus are quantised, along the external field direction, and the magnetic moment of the nucleus modifies the effective field experienced by $\mathbf{s}_1$ (see Equations 5.16 and 5.16a), so that $B_{\text{eff}} = B_0 \pm \frac{a}{2}$: the additional hyperfine field either adds to, or subtracts from B_0

depending on the sign of m_I (i.e. the orientation of the nuclear spin). As a result, the precession frequencies of $\mathbf{s}_1$ and $\mathbf{s}_2$ will differ. $\mathbf{s}_2$ precesses at the unperturbed Larmor frequency, given by Equation 5.10 (replace g_e with g), whilst $\mathbf{s}_1$ precesses at:

$$\omega_{L,1} = \frac{g\mu_B B_0}{\hbar} \pm \frac{a}{2} \quad (5.18)$$

This field-independent difference in precession frequencies allows S- T_0 (but not S- $T_{\pm}$) interconversion.

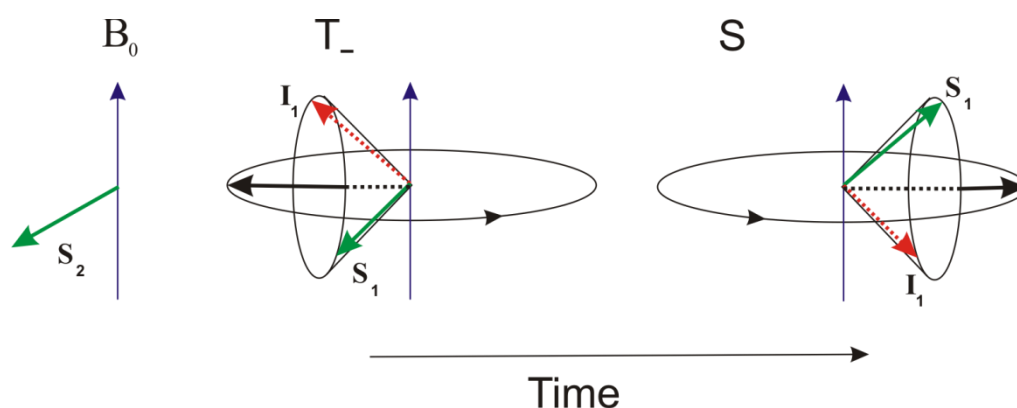


Figure 5.5 A simplified illustration of how the hyperfine interaction can drive S-T mixing between S and all three triplet states, at weak/zero external magnetic fields. $\mathbf{s}$ and $\mathbf{I}$ represent electron spin and nuclear spin, respectively (see text for details).

However, at weaker external magnetic field strengths ($B_0 \leq a$), the spins are no longer quantized along the external field axis, but instead are quantized around the resultant of B_0 and B_{HF} . This can result in the hyperfine interaction inducing mixing between S and all three triplet states, as described below.

As illustrated in Figure 5.5, when observed in a rotating frame of reference (in step with $\mathbf{s}_2$, which precesses about the field direction at the Larmor frequency and is seen as static), the electron spin, $\mathbf{s}_1$, and nuclear spin, $\mathbf{I}_1$, precess about their resultant, which itself precesses about the field direction. As in the high-field case described above, the precession frequency of $\mathbf{s}_1$ is perturbed by the hyperfine coupling between electron and nuclear spins. Therefore, in time, the projection of $\mathbf{s}_1$ onto the applied field direction changes and interconversion

between S/T_0 and the $T_{\pm}$ states becomes possible (T_{-} to S state interconversion is shown in Figure 5.5).

5.4.1.4 The Low-field hyperfine mechanism

At weak external field strengths ($0 \leq B_0 \lesssim \frac{a\hbar}{2g\mu_B}$) a so-called low field effect (LFE, first predicted by Brocklehurst¹⁷) is sometimes observed, in which the S-T mixing efficiency initially increases with field strength before decreasing at higher field strengths (as expected due to the Zeeman effect).^{18,19}

To understand the LFE, consider again a one proton ($I = \frac{1}{2}$) RP. If coupling to the magnetic nucleus is taken into account, the RP can form 8 possible states, listed in Table 5.1.

State	$S\alpha$	$S\beta$	$T_0\alpha$	$T_0\beta$	$T_+\alpha$	$T_+\beta$	$T_-\alpha$	$T_-\beta$
F	$\frac{1}{2}$	$\frac{1}{2}$	$\frac{1}{2}$	$\frac{1}{2}$	$\frac{3}{2}$	$\frac{3}{2}$	$\frac{3}{2}$	$\frac{3}{2}$
M_F	$+\frac{1}{2}$	$-\frac{1}{2}$	$+\frac{1}{2}$	$-\frac{1}{2}$	$+\frac{3}{2}$	$+\frac{1}{2}$	$-\frac{1}{2}$	$-\frac{3}{2}$

Table 5.1 Possible spin states of a one proton ($I = \frac{1}{2}$) RP. α and β represent the nuclear spin state.

This table also lists the total spin angular momentum quantum number, F ($= S + I$), (S and I representing the electron and nuclear spin angular momenta, respectively), and the projection spin angular momentum quantum number, M_F , for each state.

The LFE can be thought of as a change in the conditions for conservation of angular momentum as the system moves from zero to weak static fields. At identically zero field both F and M_F must be conserved (i.e. $\Delta F = 0$ and $\Delta M_F = 0$), whereas, when $B_0 \neq 0$, only M_F must be conserved. The consequences of this additional zero field selection rule on S-T mixing can be explained using the one-proton RP example in Table 5.1. At zero field only two transitions

($S\alpha \leftrightarrow T_0\alpha$ and $S\beta \leftrightarrow T_0\beta$) are possible. However, for $B_0 \neq 0$, additional transitions (to the $T_+\beta$ and $T_-\alpha$ states) become possible. Therefore, S-T mixing efficiency increases as soon as a weak field is applied. However, at higher magnetic field strengths the Zeeman splitting (Section 5.3) restricts S-T mixing of $S\alpha \leftrightarrow T_+\beta$ and $S\beta \leftrightarrow T_-\alpha$, due to energetic separation.

Theoretical studies by Timmel *et al.*^{18,20,21} conclude that for a RP to exhibit an appreciable LFE it must: 1) undergo spin-selective recombination, 2) be sufficiently long-lived (as a geminate pair) to experience significant S-T mixing at low field strengths, 3) have radical-radical exchange interactions that are weak compared to hyperfine coupling and 4) have a relaxation rate that is slow compared to RP recombination and S-T mixing.

5.5 Monitoring magnetic field effects

In this project, the effects of magnetic fields on analyte concentration were studied using several variants of direct absorption spectroscopy; both cavity-based and conventional transient absorption (TA) based spectroscopic techniques have been used to monitor MFEs. These techniques are discussed in later chapters, but at this point it is appropriate to discuss briefly how MFEs can be quantified using absorbance measurements.

In the work described in this thesis, the change in a sample's absorbance, ΔA , is measured upon photochemical generation of radicals. By measuring this absorbance change in the presence, $\Delta A(B_0)$, and absence, $\Delta A(B_0 = 0)$, of a magnetic field, an absorbance-independent percentage MFE can be defined as:

$$\% \text{ MFE } (B_0) = \frac{\Delta A(B_0) - \Delta A(B_0=0)}{\Delta A(B_0=0)} \times 100 \quad (5.19)$$

$$= \frac{\Delta \Delta A(B_0)}{\Delta A(B_0=0)} \times 100 \quad (5.19a)$$

5.5.1 Magnetically altered reaction yield spectroscopy

The magnetic field effect can be monitored as a function of field strength to provide a MFE spectrum (also referred to as a magnetically altered reaction yield (MARY) spectrum).

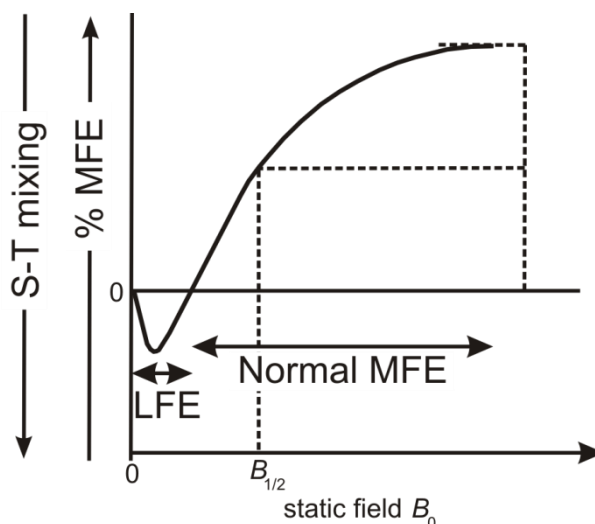


Figure 5.6 A typical magnetic field effect spectrum shown for a triplet born RP. The static field strengths (B_0) used in this project range from -25 mT to $+25$ mT. The decrease in % MFE in the low field region and the increase at higher field are explained by the LFE and 'normal' MFE mechanisms, respectively. $B_{1/2}$ is defined as the field at half of the saturation value of the % MFE.

A generic MARY spectrum, for a triplet born RP is shown in Figure 5.6. As discussed below, the spectral features arise from the various MFE mechanisms discussed in previous sections. The example of a triplet-born RP is used here, as an introduction to the triplet-born RP systems described later chapters, however the MARY spectrum for a singlet-born RP can easily be imagined by inverting the spectrum.

At zero field and in the absence of exchange and dipolar interactions, the S- and three T-levels are degenerate and interconversion is driven efficiently by the hyperfine interaction between the electron spins and surrounding magnetic nuclei. At low field strengths, the LFE initially causes an increase in the S-T mixing efficiency and the % MFE decreases. As the applied field is increased further the Zeeman effect dominates; magnetic fields exceeding the average hyperfine coupling separate the S/T_0 sub-levels from the $T_{\pm}$ manifold, thereby reducing the

efficiency of S-T interconversion. As a result, in this triplet-born RP system, radicals may become trapped in the $T_{\pm}$ states from which no recombination is allowed, leading to an increase in % MFE. This effect is referred to as the ‘normal’ MFE (see Figure 5.6).

5.6 MFEs in biologically relevant systems

With the notable exception of the short-lived radical pair intermediates involved in the initial steps of photosynthesis,²²⁻²⁴ little attention has been focused on the investigation of magnetosensitive reactions in biologically relevant systems *in vitro*.²⁵ However, the recent discovery—in organisms ranging from plants to insects, birds and humans^{26,27}—of cryptochrome, a *ca.* 55 kDa, blue light receptor protein, and the suggestion of its key role in the processes controlling the avian magnetocompass²⁸ have resulted in the observation of magnetic field dependent radical reactions in the cryptochrome-related photolyase protein^{29,30} and, most recently, a plant (*Arabidopsis thaliana*) cryptochrome (AtCry).³¹ To put these studies in perspective, a very brief overview of current avian magnetic sensing hypotheses is presented in the next section (for more detail, see Mouritsen & Hore).³²

5.6.1 Animal magnetoreception

Behavioral experiments have demonstrated that a wide variety of animals—including several invertebrate groups (molluscs, crustaceans and insects) and all five classes of vertebrate—can sense the Earth’s magnetic field and use it as a cue in navigation.³³ Much attention has been paid to the ability of migratory birds to navigate vast distances using (among other means) the Earth’s magnetic field. However, little is known about the processes that underlie avian magnetoreception (including the biophysical detection mechanism(s), signal transduction mechanism(s) and information processing events in the brain).

Over the past thirty years, a number of animal magnetoreception and avian magnetic compass mechanisms have been proposed. However, in birds, only two magnetoreception mechanisms

are currently supported by robust data: those based on a magnetodetection sense associated with the ophthalmic branch of the trigeminal nerve (V1, possibly innervating iron-containing structures in the beak)³⁴⁻³⁷ and those based on magnetic field dependent radical pair reactions.^{5,28,31,38,39} These hypotheses are discussed in more detail below. Recently, two other hypotheses have also been presented. The first is a RP-based model in which magnetosensitivity is conveyed *via* a physical (as opposed to a chemical) signature of the RP: a light- and magnetic field-induced transient electric dipole moment, the electric field of which could be observed by a bird.⁴⁰ The second hypothesis proposes that the inner ear lagena are involved in magnetoreception.⁴¹⁻⁴⁴

There is strong evidence that V1 transmits magnetic information to the brain of some migratory birds.^{37,45} However, the sensory structures and biological functions associated with V1 are still unclear. It has been suggested that the magnetic mineral magnetite (Fe_3O_4) could act as a beak-based magnetoreceptor in conjunction with V1 in some birds.^{35,36} However, a recent study, showing that magnetite is absent in the neurons in pigeon beaks, implies that magnetite is not, in fact, part of the avian magnetosensitive system.⁴⁶

Other species of bird (such as the European robin) are known to experience light-activated magnetic sensing.⁴⁷⁻⁴⁹ These results have helped encourage the popularity of a RPM-based hypothesis for magnetic sensing,^{5,28,49-51} supported by the recent observation of bird disorientation by weak oscillatory fields at frequencies close to the resonant frequency for an electron in the Earth's field.^{38,49} Cryptochromes—so named for their importance in cryptogamic plants and their cryptic nature—⁵²were first proposed as the primary magnetoreceptors in this RPM-based model in 2000.³⁸ Since then, four different cryptochromes have been identified in the eyes of migratory birds (see Mouritsen & Hore³² and references therein). Behavioural experiments also suggest that cryptochromes elicit magnetosensitivity in *Drosophila*.^{53,54} However, whilst cryptochromes remain the only candidate sensory molecules for RPM-based

avian magnetoreception,^{50,55,56} little concrete biophysical evidence exists in support of this hypothesis (as discussed in the next section).

5.6.1.1 The cryptochromes as potential chemical magnetoreceptors

The majority of support for the cryptochrome hypothesis of magnetoreception (first proposed by Ritz *et al.*²⁸) is based on circumstantial or indirect evidence (this point is discussed in detail by Mouritsen & Hore³²).

It has been established that cryptochrome contains the chromophore flavin adenine dinucleotide (FAD) in its fully oxidized state and photoinduced electron transfer along a nearby conserved triad of tryptophan (Trp) residues results in a flavosemiquinone and tryptophan radical pair.⁵⁷ Additionally, FAD-Trp radical pairs have been shown to exhibit electron spin-correlation on the (microsecond) timescales required for magnetic sensitivity. If the probability of flavosemiquinone formation was to depend on the Earth's magnetic field direction then cryptochrome could, in principle, provide birds with navigational cues.^{28,50,58}

The possibility (first proposed by Schulten *et al.*⁵⁹) that magnetic fields comparable with that of the Earth might affect reactions proceeding *via* radical pairs has attracted considerable attention in recent years. However, to date, only one experimental study has shown a true Earth-field effect, in a model system consisting of covalently linked fullerene, porphyrin and carotenoid moieties—structurally unrelated to cryptochrome.⁵ Crucially for the chemical compass hypothesis, this triad molecule also exhibits sensitivity to the direction of a magnetic field (albeit with a strength over 10 times greater than that of the Earth's).

Further supporting evidence for cryptochrome's magnetic sensing abilities has come from studies on the related photolyase proteins, discussed below.

Cryptochromes and photolyases share significant structural (and moderate sequence) similarities.^{60,61,62} Both cryptochromes and photolyases are found throughout the natural

world, acting as photoreceptors that mediate adaptive responses to blue and UV light (see Zoltowski *et al.*⁶⁰ and references therein). Photolyases (activated by blue/UV light) act predominantly to repair damaged DNA in many organisms⁶² and it is widely acknowledged that cryptochromes are the evolutionary descendants of DNA photolyases.^{55,63} Recent *in vitro* studies have observed the presence of a long-lived (microsecond lifetime) FAD-Trp radical pair with an associated magnetic field effect in photoactivated *Escherichia coli* photolyase (albeit at fields considerably stronger than that of the earth).^{29,30} The results of these (and other photolyase-based) studies are described in detail in Chapters 7 and 8.

Very recently, and in strong support of the cryptochrome chemical magnetoreceptor hypothesis, Maeda *et al.*³¹ demonstrated that photo-induced FAD-Trp radical pairs in *Arabidopsis thaliana* cryptochrome are sensitive, *in vitro*, to weak applied fields. The results of this study, which include a description of the mechanistic origin of the field effects in AtCry, are discussed further in Chapter 8 (Section 8.3.3) in relation to breakthrough work in this project demonstrating the first observations of a magnetic field effect in an animal (*Drosophila melanogaster*) cryptochrome.

5.7 MFE measurement techniques—the need for a step change in sensitivity

In contrast to the relatively small number of *in vitro* MFE studies on biological systems outlined above, the effects of both static and oscillating magnetic fields have been studied in a plethora of chemical systems involving RP intermediates.^{1-3,64} Measurements of the MFEs on either the yield or the kinetics of the radical recombination reactions are, with the exception of photoconductivity or HPLC measurements, nearly exclusively based on optical detection methods.

Investigation of RPs recombining *via* a fluorescing exciplex allows the direct detection of the reaction yield by a zero-background technique whilst facilitating the employment of modulation techniques and lock-in detection, rendering these experiments exceptionally sensitive. Despite the deep physical insight yielded by this technique, however, magnetosensitive measurements of radical reactions have, on the whole, been limited to a handful of emissive chemical systems.^{1,3}

The vast majority of RP reactions have been studied using absorption techniques. These methods frequently suffer from low sensitivity as the absorption signal is usually observed as a small change in light intensity measured against a large background (see Section 1.1). Typically, flash photolysis experiments are employed which facilitate time-resolved studies, but exclude the employment of modulation techniques combined with lock-in detection to improve the sensitivity of measurements. In order to compensate for the limited detection sensitivity, most studies to date have used high concentrations and long signal averaging times. To mitigate against the effects of photodegradation, large sample volumes are also typically required. The biological samples of most interest, however, such as the photolyases and cryptochromes mentioned above, cannot be produced in the (millilitre) volumes and (millimolar) concentrations needed to obtain high quality data. Concentrating these protein solutions frequently leads to precipitation and clouding of the solution. Furthermore, any *in vitro* studies at concentrations reflecting typical *in vivo* conditions are simply not feasible. There is, therefore, an acute need for high-sensitivity detection methods to facilitate meaningful magnetic field effect studies of biologically relevant systems.

The forthcoming chapters (6-8) describe a variety of complementary cavity-based spectroscopic techniques, applied to measure sensitively solution-phase photoinduced reactions and their associated magnetic field effects.

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Chapter 6

Cavity enhanced detection methods for probing the dynamics of spin correlated radical pairs in solution

The previous chapter introduced the concept, and spin chemistry, of magnetic field effects on radical recombination reactions and outlined the need for high-sensitivity absorbance-based MFE detection methods. The current chapter describes how narrow-band cavity-enhanced absorption spectroscopy, combined with phase-sensitive detection, has been employed to study the effects of static magnetic fields on radical recombination reactions. This work was designed as a proof-of-principle study to establish the efficacy of CEAS as a high-sensitivity MFE detection method, before effort and resources were spent on custom-built experiments

The chemical system studied comprises the photochemically generated thionine semiquinone radical and a 1,4-diazabicyclo[2.2.2]octane (DABCO) cationic radical, in a micellar solution of sodium dodecyl sulphate (SDS). Data obtained using the modulated CEAS technique, describing the MFE on reaction yields, are shown to be superior to those obtained using conventional transient absorption flash photolysis methods typically employed for these measurements. The high sensitivity afforded by modulated CEAS detection is discussed in terms of the new possibilities it offers, such as the measurement of magnetic field effects in real biological systems, which have hitherto been largely beyond the detection capabilities of existing techniques. This chapter has been published as: Neil *et al.* *Mol. Phys.* **108**, 993 (2010).¹ The experimental work was performed in collaboration with Dr Kiminori Maeda (Timmel Group, Oxford) who had previous experience of this photochemical system in TA measurements.

6.1 Experimental

6.1.1 The Photochemical System

The chemical system chosen for this study comprises the photochemical dye thionine (TH^+) and 1,4-diazabicyclo[2.2.2]octane (DABCO) (Figure 6.1(a)) in a micellar solution of SDS. Green light irradiation of the solution produces the excited singlet state of thionine, $^1\text{TH}^{+*}$ (see Figure 6.1(b) for the absorption profile of TH^+ featuring a strong band at 600nm with a weak tail at 532 nm). Figure 6.1(c) shows the photochemical reaction scheme. Following production of the $^1\text{TH}^{+*}$ excited state, intersystem crossing to the $^3\text{TH}^{+*}$ state occurs on a picosecond timescale.² Quenching of $^3\text{TH}^{+*}$ with DABCO produces the spin-correlated radical pair, $^3[\text{TH}^\bullet + \text{DABCO}^{+\bullet}]$, under conservation of total spin angular momentum. The efficiency of the subsequent $^3[\text{TH}^\bullet + \text{DABCO}^{+\bullet}] \leftrightarrow ^1[\text{TH}^\bullet + \text{DABCO}^{+\bullet}]$ interconversion and hence the radical yield of the photochemical reaction, is magnetic field dependent as discussed in Section 5.4.

The intense absorption band of the $\text{TH}^\bullet$ radical, centered around 400 nm, allows selective detection of this species using a 405nm diode laser without spectral overlap with the TH^+ species, see Figure 6.1(b).

The RP molecular precursors are dissolved in a micellar solution of SDS to prolong the lifetime of the geminate radical pair thereby maximizing the observed magnetic field effect as discussed in Section 5.4.1.

The exact location of the molecules/radicals with respect to the micelle (core/Stern layer/bulk) is not known with certainty. Previous work on the related DABCO/Xanthone(Xa) system indicates that, at the moment of creation from its molecular precursor, $\text{DABCO}^{+\bullet}$ is located just outside the micelle, on the opposite side of the phase boundary to the hydrophobic Xa which inhabits the micelle interior.³ The surface charge of the micelle leads to $\text{DABCO}^{+\bullet}$ being trapped

within the negatively charged Stern layer of the SDS micelle whilst $\text{Xa}^{\bullet-}$ escapes the negatively charged micro-reactor only slowly.

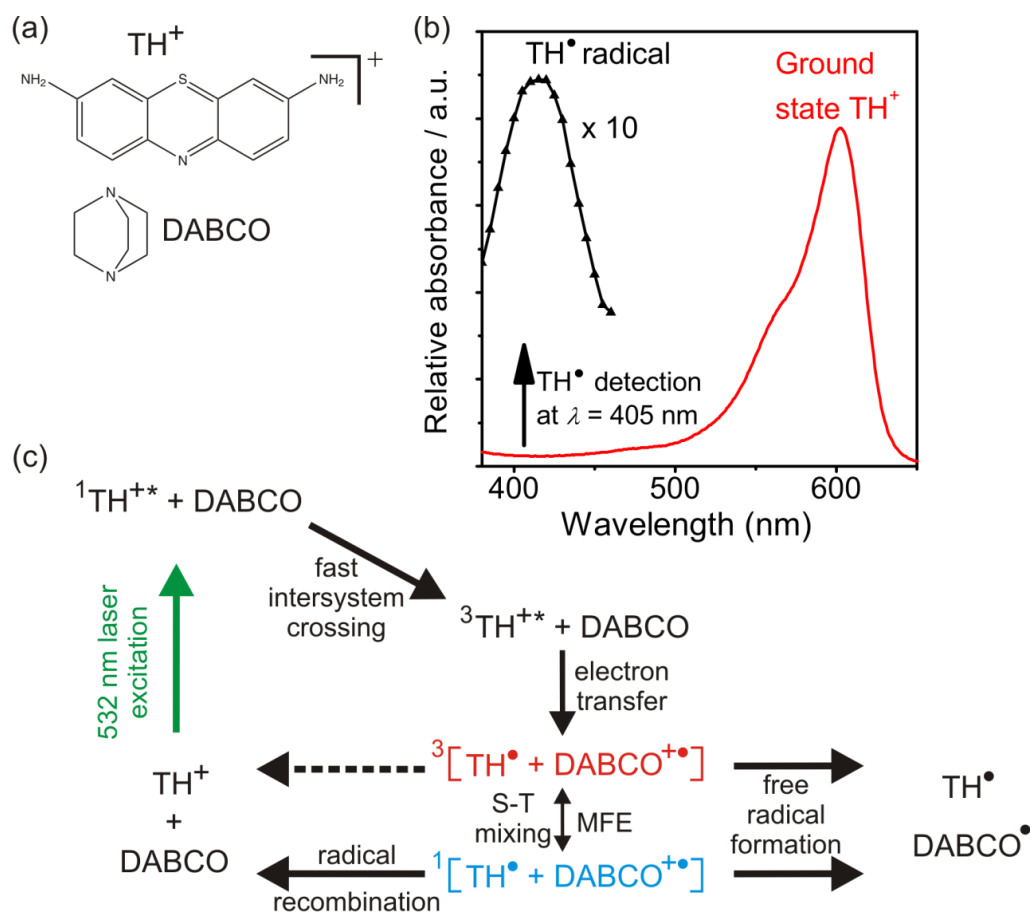


Figure 6.1 (a) Structures of the RP molecular precursors, thionine and DABCO. (b) Relative absorption spectra for the thionine ground state and the neutral $\text{TH}^{\bullet}$ (free) radical. (c) Photochemical reaction scheme for the TH, DABCO system in a micellar solution of SDS.

For the DABCO/ TH^+ system used here, the situation at the moment of the birth of the radical pair is likely to be very similar because the positively charged ground-state sensitizer TH^+ will initially be located on the negatively charged micelle surface. The uncharged and slightly hydrophobic sensitizer-derived radical enters the micelle, whilst the DABCO radical cation is trapped in the Stern layer. It is thus likely that both radicals are found in or near the Stern layer leading to a much prolonged lifetime of the geminate RP compared with that in homogeneous solution and hence a significant MFE.

6.1.2 Materials

Solutions were made up in ultrapure Milli-Q water (resistivity 18.2 M Ω cm). A small aliquot of a stock solution of thionine (Aldrich) in water was added to a solution of 10mM SDS, followed by addition of aqueous DABCO (0.2 M). The thionine concentrations used for individual experiments are specified later. During experiments, all solutions were continually purged with nitrogen to eliminate dissolved oxygen, thereby minimizing the quenching of the thionine triplet state in solution. The pH of the (unbuffered) solution was approximately 11. No filtering of the solution was performed prior to any of the experiments.

6.1.3 CRDS and frequency modulated CEAS

The experimental apparatus and optical arrangement are shown schematically in Figure 6.2, and comprise four main components: (i) an optical cavity, (ii) an excitation source for radical generation (iii) a 405nm diode probe laser and photomultiplier tube (PMT) for radical detection, and (iv) a Helmholtz coil arrangement for applying modulated magnetic fields. The heart of the experiment is the short path-length intra-cavity cuvette through which the solution is continually flowed such that the cavity axis, magnetic field and the flow direction are mutually perpendicular.

The high-finesse optical cavity was formed using two highly reflective concave mirrors (mirror set 1, see Figure 2.3(a), radius of curvature 1 m) mounted 12 cm apart on individual precision gimbal mounts. The sample cell (Starna Scientific, 45/Q, UV Quartz, 1 mm path length) was carefully oriented normal to the optical cavity by mounting it on a rotation stage. This geometry ensures that any reflections from the surfaces of the cell are not lost from the cavity. Attempts were initially made to mount the cell at Brewster's angle to the cavity axis in order to minimize these reflective losses, but for these experiments such an arrangement proved less satisfactory than the normal configuration.

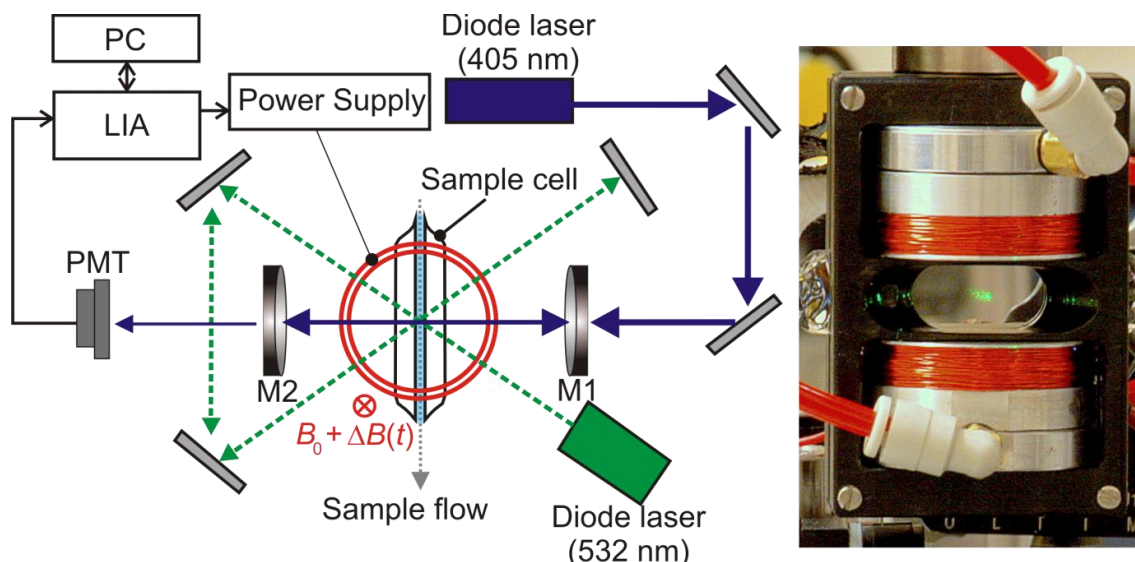


Figure 6.2 Schematic of the experimental arrangement for modulated CEAS. A pair of Helmholtz coils (indicated by concentric circles) used to generate the magnetic field, $B = B_0 + \Delta B(t)$, surround the sample cell which lies at the centre of an optical cavity formed by mirrors M1 and M2. The 532 nm photo-excitation beam (green dashed arrows) is arranged in a 4-pass bow-tie arrangement. LIA= lock-in amplifier. Inset: The mounted sample cell, with Helmholtz coils above and below (cooled by water, delivered through the tubes shown), as viewed along the optical axis of the high finesse cavity. The 532 nm laser beam spot is visible in the centre of the cell.

Light from a CW diode laser (Laser Quantum, Torus, 532 nm) was used to generate the electronically excited $^1\text{TH}^+$ radicals. Various laser powers, specified for individual experiments, were used. A four-pass bow-tie arrangement of the photoexcitation laser through the sample (see Figure 6.2) increased the concentration of radicals generated and thus the SNR achieved. Optical alignment of the 532 nm laser path was ensured by maximizing the CRDS-monitored absorbance within the sample cell (see below).

The detection light source was a 405 nm diode laser (Power Technology, 1Q1H350, see also Section 2.2) and the light transmitted through the cavity was detected using a PMT (either Electron Tubes, Q-9893 or Hamamatsu R928, see Section 2.5). Switching between CRDS and modulated CEAS experiments could be done without modifying the optical arrangement. For CRDS measurements, the detection laser was pulsed at a frequency of 3 kHz using an external pulse generator (TTi, TGP 110). Every time the laser was switched off, the light intensity within

the cavity decays exponentially with a ring-down time, τ , characteristic of the losses per round trip as described in Section 1.2.1. As well as surface and bulk scattering losses, these losses include the absorbance within the sample itself. Ring-down traces were recorded on a 12 bit 200 MS/s PC data acquisition (DAQ) card (National Instruments PCI-5124) from which τ was extracted *via* a fast Fourier transform (FFT) method using LabVIEW (developed by Dr Mikhail Mazurenka, formerly Mackenzie Group), as described below.^{4,5}

The FFT method maps the ring-down traces recorded by the DAQ card into a function of frequency, $Fr(\omega)$, *via*:

$$Fr(\omega) = P(Ye^{-\tau t} + D) \quad (6.1)$$

Where P is the fourier transform operator and Y and D are the amplitude and baseline offset of the ring-down trace, respectively. The ring-down time can then be calculated using the ratio of the real and imaginary components of $Fr(\omega)$ at any non-zero frequency ω :

$$\frac{1}{\tau} = -\omega \frac{\text{Real}[Fr(\omega)]}{\text{Imaginary}[Fr(\omega)]} \quad (6.2)$$

Thus, τ is calculated without the need for extraction of D . Additionally, the FFT method is non-iterative and, therefore, very fast.

Before every modulated CEAS experiment, CRDS was used to determine the absolute absorbance of TH^* within the intra-cavity cell at $B = 0$. The cavity and sample cell were aligned to maximize the ring-down time with Milli-Q water flowing through the cell. The DABCO, thionine and SDS solution was then flowed through the cell using a gravity-fed arrangement between two reservoirs. An alternative, peristaltic pump system was found to introduce additional noise. A flow rate of 3 mL/min, chosen to maximize the CRDS SNR, was maintained by means of a tap placed in the flow path after the sample cell and the solution was recycled between the reservoirs. Continual sample flow was essential to militate against the effects of

photodegradation. The cavity ring-down time, recorded in the absence, τ_0 , and presence, τ , of the 532 nm photoexcitation light, was used to calculate the $\text{TH}^\bullet$ absorbance per pass, A (in conventional $\log_{10}$ scale) using Equation 1.13. The corresponding $\text{TH}^\bullet$ concentration, $[\text{TH}^\bullet]_{B=0}$, was calculated *via* the Beer–Lambert law, using the known radical extinction coefficient ($11426 \text{ M}^{-1} \text{ cm}^{-1}$, in methanol) at 405nm.²

Since τ_0 reflects all cavity losses except the $\text{TH}^\bullet$ absorption, it is strongly dependent on (i) the alignment of the entire cavity, (ii) the cleanliness of the cell and (iii) any additional losses (scattering and absorbance) within the aqueous DABCO, SDS and thionine solution not due to radical absorption. τ_0 was typically 150–185 ns which represents 375–460 sample passes before the light intensity in the cavity drops by a factor $1/e$. For comparison, the ring-down time obtained with pure water in the cell was typically 300–400 ns (750–1000 passes). The additional losses arising from the sample were probably dominated by scattering of the micelles with minor absorption losses due to reactant precursors.

Following CRDS measurements, and without change to the cavity alignment, modulated CEAS was performed; the pulse generator was turned off and the 405 nm diode laser operated in continuous wave mode. Light exiting the cavity was directed, using a liquid light guide, onto a PMT, the output of which was connected to signal and auxiliary inputs on a lock-in amplifier (LIA, Stanford Research Systems SRS510) to permit phase-sensitive detection and direct monitoring of the PMT output, respectively. The principle of phase-sensitive detection of MFEs is shown schematically in Figure 6.3 and discussed further in Section 6.1.3.1.

To enable the recording of modulated CEAS MFE spectra, the sample cell was mounted between a pair of water-cooled Helmholtz coils (see Figure 6.2), which provided swept and modulated homogeneous magnetic fields in the sample region. The applied field was varied from -15 to +15 mT, through zero. In addition, the magnetic field was audiofrequency-modulated, at 404 Hz with an amplitude of 0.52 mT, controlled by the LIA which drove the

power supply to the Helmholtz coils. The whole experiment was controlled using custom-written LabVIEW software (Dr Chris Wedge & Dr Kiminori Maeda, Timmel Group, Oxford).

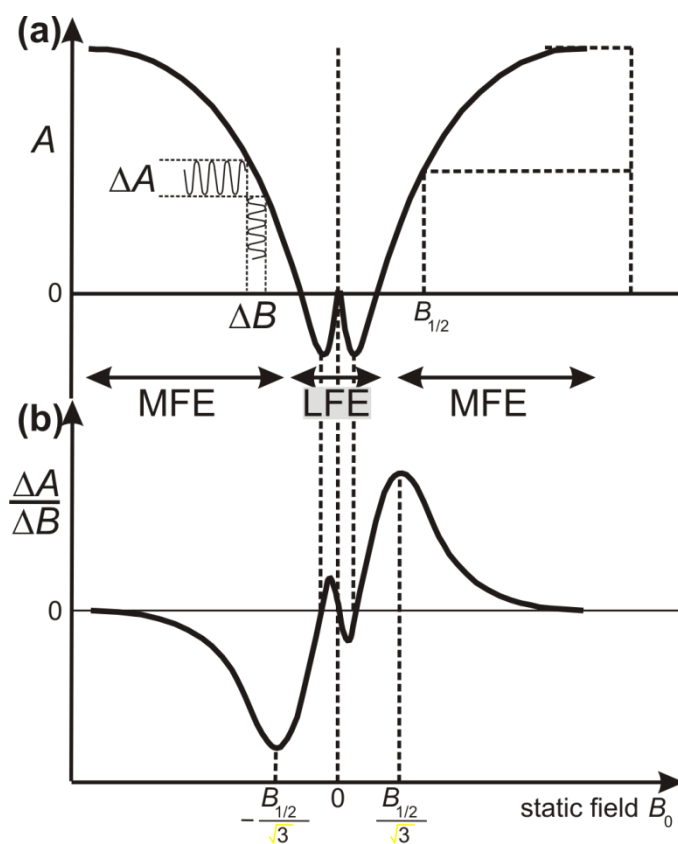


Figure 6.3 (a) A typical MFE (MARY) spectrum shown for a triplet born RP (the features of the MFE spectrum, including the low field effect, LFE, are discussed in Chapter 5—see, in particular, Section 5.5.1). The signal, A , might correspond to the absorbance of one of the radicals. The field is modulated, typically at audio frequencies, and with modulation depth, ΔB , leading to a modulation in the resulting signal, ΔA (b) Phase-sensitive detection records the first derivative ($\Delta A/\Delta B$) of the expected MFE graph. For a Lorentzian function, peaks in the first derivative spectrum occur at $\pm B_{1/2}/\sqrt{3}$.

As described in the next section, the use of field modulation and the LIA improves significantly the SNR of the MFE measurements by specifically recording the absorbance oscillating only at the field modulation frequency, thereby discriminating against low frequency noise. However, use of modulation methods precludes the determination of absolute absorbance values (and thus radical concentrations) during the modulated CEAS experiments. All absolute absorbance measurements were thus made using CRDS, as described above, at $B = 0$, immediately before

modulated CEAS. Unless otherwise stated, the MFE curves shown in later sections were collected in a sequence of 20 repeat scans to improve the SNR.

6.1.3.1 Lock-in amplifiers

Lock-in amplifiers are used to sensitively detect an experimental signal at a particular frequency. In essence, only those experimental signals which oscillate at the same frequency as a reference signal are amplified and detected, whilst all other background (noise) signals are filtered out.

In the present experiment, a reference generator within the LIA is used to generate an identical pair of sine waves, with a particular frequency, ω_x . One of these is used to provide the LIA output—a voltage that is supplied to the Helmholtz coils' power supply, modulating the magnetic field strength (at $\omega_x = 404$ Hz, see Section 6.1.3). Assuming that the absorbance of the sample is field-sensitive, this field modulation will result in a modulation (again, at ω_x) in the absorbance (ΔA) (see Figure 6.3(a)) and in the experimentally measured PMT voltage, V_{exp} . Using a phase sensitive detector (PSD) within the LIA, V_{exp} is then compared to a reference signal, V_{ref} , which is produced from the second sine wave of the reference generator.

V_{exp} contains not only the desired signal (V_x) oscillating at ω_x , but also a significant amount of background noise (signals with different amplitudes, frequencies and phases to V_x). However, by multiplying V_{exp} with V_{ref} and subsequently applying a low-pass filter, the PSD allows only those signals oscillating at ω_x and, in principle, attenuates other frequencies. For practical reasons (such as coil inductance) there is a phase lag between the applied modulation and that detected at the PMT. To increase sensitivity the phases of the reference and PMT-detected signals can be matched by applying a phase-shift within the LIA. Finally, the size of the detected V_x signal is further increased using a DC amplifier and read out of the LIA. As illustrated in Figure 6.3 this DC signal (Figure 6.3(b)) is the first derivative of the true MFE spectrum (Figure 6.3(a)).

6.1.4 Transient absorption measurements

One of the key purposes of the studies described here was to compare the performance of the modulated CEAS experiment with more conventional experiments for detecting magnetic field effects. Therefore, TA measurements were performed, by Dr Kiminori Maeda and Dr Kevin Henbest (Timmel Group, Oxford), on the same DABCO, SDS and thionine system. The laser flash photolysis system is described elsewhere.⁶ In brief, the sample flowed through a 1 cm square section Hellma quartz flow cell and was photoexcited with 532 nm laser pulses from a Continuum Surelite I Nd:YAG laser. The monitoring beam from a 300 W Xenon lamp was focused through the sample cell at right angles to the laser excitation and the radical absorbance measured at 405 nm using a monochromator (Oriel) and a Hamamatsu R928 PMT. An in-house C++ software program controlled a home-built magnetic field controller, optical shutters, and a digital oscilloscope (LeCroy), and recorded the TA signals at each magnetic field in random order. A home-made macro program (written by Dr Kiminori Maeda) is used to calculate the field effects, MFE, according to:

$$\text{MFE} = \frac{1}{t_2 - t_1} \int_{t_1}^{t_2} (A(B, t) - A(0, t)) dt \quad (6.3)$$

i.e. the difference in TA signals in the presence and absence of an applied magnetic field, B , integrated over a time interval $\Delta t (= t_2 - t_1)$.

6.2 Results and discussion

6.2.1 Modulated CEAS

Figure 6.4 shows the TH^* absorbance at zero-field measured by CRDS as a function of photoexcitation laser power. The right hand axis shows the corresponding thionine radical concentration, $[\text{TH}^*]_{B=0}$. The absorbance signal shows signs of reaching a plateau at the higher laser powers corresponding to the saturation onset of the thionine absorption.

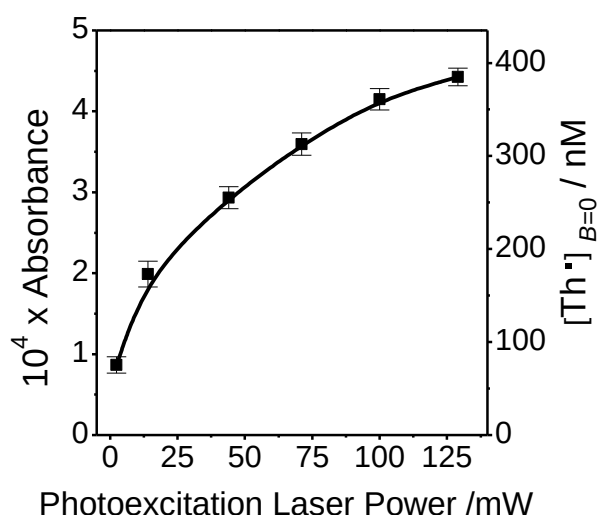


Figure 6.4 CRDS-measured absorbance of a solution of 2.5 μM thionine in DABCO, SDS, at 405 nm as a function of photoexcitation laser power, showing the onset of optical saturation.

Figure 6.5(a) shows modulated CEAS MFE spectra measured with different 532 nm photoexcitation laser powers for a DABCO, SDS and 20 μM thionine solution. As the data are obtained using phase-sensitive detection, the graphs depict the first derivative of the actual magnetic field effects as shown in Figure 6.3. As expected from Figure 6.4, at higher photoexcitation laser powers, more $\text{TH}^\bullet$ is generated resulting in larger observable signals and improved SNR. The spectra are symmetrical with respect to the total magnetic field, the slight offset observed in the figure arises from the presence of the Earth's magnetic field (~ 0.05 mT). The spectra exhibit high SNR illustrating the benefits of using modulated CEAS in these studies. Both the 'normal' MFE and the LFE (both discussed in more detail below) are clearly observed for 532 nm powers of 14 mW and higher, and, as expected, the graphs are (apart from a normalization factor) superimposable as the magnetic properties of the system are laser power independent.

While Figure 6.4 shows the dependence of $[\text{TH}^\bullet]_{B=0}$ on the photoexcitation laser power, Figure 6.5(b) shows how the modulated CEAS signal varies with $[\text{TH}^\bullet]_{B=0}$ (top axis) and $\text{TH}^\bullet$ absorbance (bottom axis); the figure demonstrates the linearity of the modulated CEAS signal with $[\text{TH}^\bullet]$, both at the maximum (at 4.8 mT) in the MFE spectrum and integrated over the LFE region.

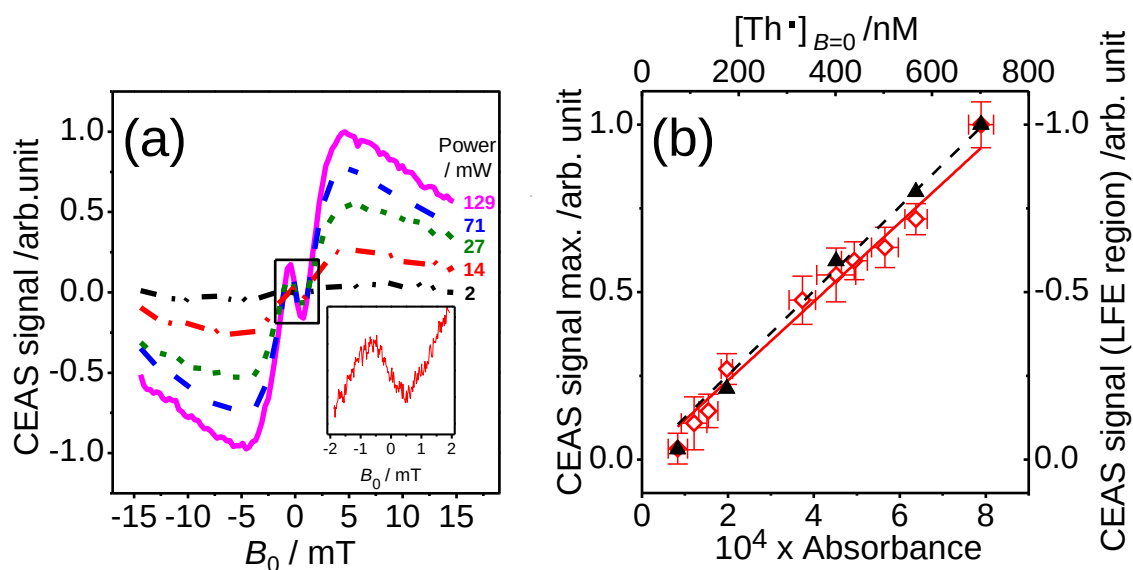


Figure 6.5 (a) CEAS-MFE spectrum of a solution of 20 μM thionine in DABCO, SDS for various 532 nm photoexcitation laser powers. The rectangle in the middle of the figure encloses the low field effect and the inset shows the LFE at 14 mW. (b) The CEAS MFE signal maximum at 4.8 mT (open diamond, left hand scale) and integrated over a 1.1mT interval in the LFE region (triangle, right hand scale), as a function of $[\text{Th}^\bullet]^+_{B=0}$ determined by CRDS. The linear fits are forced through the origin and uncertainties represent one standard deviation (1 s average).

The improvement in SNR afforded by the use of a high finesse cavity is illustrated in Figure 6.6, which compares the modulated CEAS signal with that for a single-pass measurement. This simple comparison provides a compelling justification for CEAS's development and use as a high-sensitivity MFE detection technique. While no MFE is detected using the single-pass experiment, the modulated CEAS data shows both MFE and LFE with sufficiently high SNR that spectral features are easily quantifiable. In turn this facilitates the extraction of kinetic and spin relaxation information for the spin system studied (see below).

It is an interesting feature of CEAS that, as the losses within the sample represent a significant fraction of the overall loss per round trip, a reduction in the concentration of the sample solution can actually increase the number of passes within the cavity, and hence the sensitivity. The CEF increases as a result of the higher overall finesse of the cavity due to lower scattering and precursor absorption. In these experiments, up to 460 passes per ring-down time are achieved with 500 nM thionine solution flowing through the cell. An increase in SNR is best

achieved by decreasing the thionine concentration and increasing the photoexcitation power to compensate (within the saturation constraints shown in Figure 6.4). The use of high 532 nm photoexcitation powers (100 mW) in the experimental configuration employed here, converts a significant fraction of the thionine within the sample volume to the radical form (*ca.* 20%).

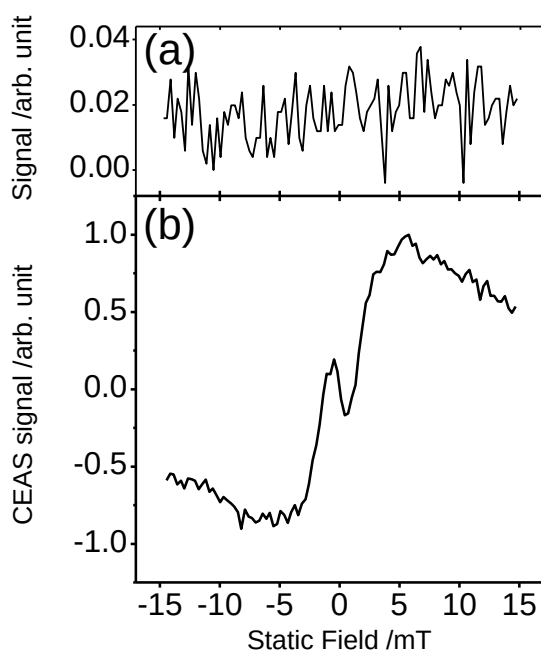


Figure 6.6 Comparison of (a) modulated single-pass (same setup as modulated CEAS, minus the cavity mirrors) and (b) modulated CEAS-MFE spectra using the same sample solution (DABCO, SDS and 1 μ M thionine), photoexcitation power (100 mW) and averaging (50 scan average).

6.2.2 Comparison of modulated CEAS with conventional transient absorption

A major objective of this project was to test the dynamic range of CEAS, with the aim of developing a technique whose superior sensitivity would allow the characterization of MFEs on smaller and less concentrated samples than are possible using the TA techniques employed previously in the study of such effects. Clearly, though, it is important to establish that the cavity enhanced methods yield the same MFE result as TA under similar conditions. Such comparisons are non-trivial, as a result of the differences in the methods themselves, but Figure 6.7(a) depicts the MFE spectra obtained using both modulated CEAS and single-pass TA for relatively high sample concentrations of thionine (20 μ M for CEAS, 50 μ M for TA) typical of those used in TA measurements. The gratifyingly close agreement between the two sets of

data indicates that both methods sample the same MFEs produced by the same radical species. The high SNR in both graphs allows for a straightforward interpretation of all features. Typically, MFEs are characterized by the $B_{1/2}$ value (defined as the field at half the MFE saturation, see Figure 6.3(a)) which is often predicted using the Weller formula:⁷

$$B_{\frac{1}{2}} = \sqrt{3} \frac{\langle a_A^2 \rangle + \langle a_B^2 \rangle}{\langle a_A \rangle + \langle a_B \rangle} \quad (6.4)$$

with

$$\langle a_X \rangle = \sqrt{\frac{4}{3} \sum_i a_{iX}^2 (I_{iX} + 1) I_{iX}} \quad (6.5)$$

where the sum in Equation 6.5 runs over all hyperfine couplings, i , of the radical X to define a_X , the effective hyperfine couplings of radical X . Weller's formula is a 'rule-of-thumb', holding to within a factor of 2 provided the radicals live for neither too long nor too short a time compared with the S-T mixing processes.⁸ It fails badly, however, if the radicals are subject to fast spin relaxation in which case substantial broadening of the MFE graph is observed. For the hyperfine couplings of thionine⁹ and DABCO¹⁰ the estimated $B_{1/2}$ value of approximately 7 mT does not agree with the experimental results, which suggest a much larger $B_{1/2}$ value: the MFE does not appear to be saturating even at 15 mT. Such behavior is often observed for radical species confined to micelles and is typically explained by a contribution of both the hyperfine mechanism^{2,11} and the relaxation mechanism^{2,11,12} leading to a broadening of the MFE spectrum far beyond that predicted by the Weller formula. Further information as to the role played by both mechanisms may be extracted through sophisticated analysis of MFE data obtained by time-resolved and pulsed techniques¹³ but is beyond the scope of this thesis.

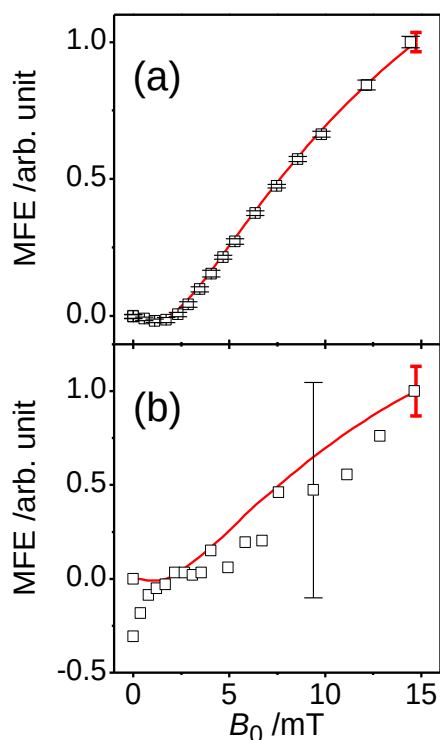


Figure 6.7 (a) Comparison of modulated CEAS and TA MFE spectra for high concentrations of thionine in DABCO, SDS, showing close agreement of the two techniques. The solid line is the 20 scan average of the modulated CEAS data with $[\text{TH}^+]_{B=0} = 690 \pm 14$ nM. The TA data (open squares) were obtained by integrating the flash photolysis data using a boxcar window with $\Delta t = 6$ μs centered at 5 μs after the 20 mJ laser flash and represent 1000 shot averages per point. (b) Comparison of modulated CEAS and TA MFE spectra for 1 μM thionine in DABCO, SDS. The solid line is the modulated CEAS data, collected with $[\text{TH}^+]_{B=0} = 157 \pm 12$ nM, 50 scan average. The TA data (open squares) were obtained as in (a) but with 170 shot average per point for more direct comparison with the CEAS data (see text). The TA spectrum (MFE as a function of B_0) shown is a 20 trace average. Representative experimental uncertainties are shown (see text for details).

Figure 6.7(b) provides a comparison of the two techniques at low precursor concentrations (1 μM TH^+). To allow as reliable a comparison between TA and CEAS as possible, the data are obtained under as close to identical recording conditions as possible; *via* the number of repeat scans averaged it is ensured that the total integrated photoexcitation energy used during the course of the TA experiment (1360 J) is comparable to that used in modulated CEAS (1380 J). As a result, solutions in the two experiments are subject to similar numbers of photons and the results thus provide a reasonable comparison of the relative sensitivities of the two methods. The standard deviation in the integrated CEAS data is displayed on the right hand edge of the curve. Judging from the SNR in Figure 6.7(b), the quality of the data from the modulated CEAS experiment is demonstrably superior to that achieved using the traditionally employed single pass TA apparatus.

As this is the first application of cavity-based techniques to the study of magnetic field effects it is worth considering the improvements they provide and the sensitivity which might ultimately be achieved. The minimum detectable absorbance achieved using CRDS may be determined using Equation 1.14. Determining $\Delta\tau_{\min}$ from three standard deviations in the baseline noise over a 1 s time, yields a minimum detectable absorbance per pass of 2×10^{-5} , when flowing a solution of 500 nM thionine in DABCO, SDS solution. The sample solution introduces scattering, which, as well as producing instability in the measurements (*i.e.* noise, which increases markedly the smallest detectable change in the ring-down time), reduces the base ring-down time, τ_0 , by approximately a factor of two as discussed in Section 6.1.3. It follows from Equation 1.14 that this significantly affects $A_{\min}$. For comparison, the minimum detectable absorbance change in the TA experiment is estimated to be 1.5×10^{-4} .

By virtue of the use of modulation techniques employed, it is less easy to be quantitative regarding the limits of detection using the modulated CEAS. Ultimately, the improvement modulated CEAS provides over TA is illustrated by the improved SNR in Figure 6.7(b) and the fact that an MFE could be observed using modulated CEAS in more dilute solutions than were amenable to TA measurements. Using modulated CEAS, it was possible to clearly observe the MFE (including LFE component) for a solution of 500 nM thionine (generating $[\text{TH}^*]_{B=0} = 98 \text{ nM}$, detectable by CRDS), which was comfortably beyond the detection limits of the TA measurements.

By comparing data recorded for similar solutions, it is possible to estimate the relative sensitivities of CRDS, CEAS and modulated CEAS as implemented here. For a comparison of CRDS and un-modulated CEAS the TH^* absorbance was measured at zero field for a solution of $1 \times 10^{-6} \text{ M}$ thionine (generating $[\text{TH}^*]_{B=0} = 306 \text{ nM}$). In this comparison, unmodulated CEAS proved the more sensitive by a factor of 5.3. This is initially surprising as CRDS is usually considered the (slightly) more sensitive of the two techniques. However, at the light levels and

ring-down time used here, detector noise and uncertainties in fitting fast exponential decays are significant limiting factors in the CRDS measurements. Indeed, for the substantial per-pass losses encountered in these studies for working solutions, the dynamic range of the CEAS technique is better than that of CRDS for the reasons discussed above (detector noise and uncertainties in fitting fast exponential decays).

The change in radical absorbance upon application of a $B_0 = 10$ mT field to the same solution was also measured by all three cavity-based techniques. In this case, CRDS is unable to discern the additional signal due to the applied field but the effect of introducing the field modulation to the CEAS measurements is to increase the SNR by an additional factor of *ca.* 5.

While modulated CEAS benefits from the gains in signal to noise described above due to both its CW nature and the well-known advantages of phase-sensitive detection, TA retains the advantage that its measurements are exclusively sensitive to $\text{TH}^\bullet$ decay and insensitive to scattering losses. By comparison, modulated CEAS probes the steady state $\text{TH}^\bullet$ absorbance change that results from both $\text{TH}^\bullet$ decay and the (magnetic field insensitive) $\text{TH}^\bullet$ generation process.

6.3 Conclusions and outlook

The main objective of this work was to test the applicability of cavity-based optical techniques for the study of magnetic field effects in solution. In the application described here, CEAS results in a clear improvement in terms of sensitivity compared with conventional TA based methods. The apparatus used here is a first prototype only, constructed from components available within the groups involved for the purpose of these proof-of-principle studies. Most experimental parameters had yet to be optimized. The number of passes of light within the cavity and, therefore, the enhancement in sensitivity, is limited primarily by the presence of the intra-cavity sample cell and losses arising from scattering and absorption in the micelles

themselves. It is likely that major gains in sensitivity could be achieved with the use of homogeneous solution and anti-reflection coated, custom-made optical cells (see Section 1.6). It should, however, also be recognized that the thionine system was carefully chosen for these initial experiments. It has two advantages for this purpose. Firstly, the system exhibits long radical lifetimes and, therefore, comparatively large nascent radical concentrations. Transient absorbance measurements also permit the study of short-lived species to which the CEAS techniques, as implemented here, would be less ideally suited. However, this problem can be overcome using a pump-probe variant of CRDS, as described in Chapter 7. Secondly, in direct absorption experiments, it is a major advantage that the ground state of thionine has negligible absorption at the detection wavelength. Overlap of absorption bands of radical and precursor species, is, however, quite common. As modulated CEAS is currently limited to regions in which a suitable light source is available, the number of systems which might benefit from the sensitivity gains demonstrated may be somewhat restricted. However, this problem can be overcome using the ultra broadband supercontinuum-source-based variants of CEAS discussed in other Chapters (in particular Chapter 8).

One promising aspect of the sensitivity gains CEAS offers is in the possibilities it affords for miniaturization. In the present modulated CEAS cavity, the sample volume probed is estimated to be *ca.* 5 μL (neglecting the sample flow). Proteins such as cryptochromes and photolyases can often only be produced in microliter volumes and nano to (few) micromolar concentrations which, according to this study, may be accessible to CEAS techniques. This eliminates the need for bulk production as required by TA methodology. The MFE experiments on the TA apparatus require milliliter volumes of solutions containing around 100 micromolar (or more) of photoactive compound, a demand often beyond the reasonable capability of synthetic labs. Cavity-based techniques, therefore, offer new possibilities in the investigation of biological samples in this field and these ideas are developed further in Chapters 7 and 8.

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Chapter 7

Following radical pair reactions in solution: a step change in sensitivity using cavity ring-down detection

Work reported in this chapter demonstrates the powerful capabilities of cavity ring-down spectroscopy in monitoring radical recombination reactions and associated magnetic field effects. These include sub-microsecond time-resolution, high sensitivity (baseline noise on the order of 10^{-6} Absorbance Units) and small (μL) sample volumes. Combined, these represent significant advantages over the single-pass flash-photolysis techniques conventionally applied. The studies described here focus on photo-induced radical pair reactions involving the protein lysozyme and one of two possible photosensitizers: anthraquinone-2,6 disulphonate and flavin mononucleotide. CRDS-measured MFEs are observed in pump-probe experiments and discussed in terms of the sensitivity gains and sample-volume minimization afforded by CRDS when compared with flash photolysis methods. Finally, CRDS is applied to an *in vitro* MFE study of intramolecular electron transfer in the DNA-repair enzyme, *Escherichia coli* photolyase, a protein closely related to cryptochrome which has been proposed to mediate animal magnetoreception. This work was performed in close collaboration with Dr Kiminori Maeda (Timmel group Oxford). The results described in this chapter have been reported previously and are reprinted (adapted) with permission from Maeda & Neil *et al. J. Am. Chem. Soc.*, **2011**, 133, 17807.¹ Copyright 2011 American Chemical Society.

7.1 Introduction

Chapter 6 describes highly sensitive MFE measurements using the combination of an optical cavity and magnetic field modulation *via* phase-sensitive detection.² A minimum detectable

absorbance on the order of 10^{-5} per pass was demonstrated for the transient radical analyte and, when coupled with phase-sensitive detection, MFE-induced absorbance changes of *ca.* 10^{-6} could be detected. However, as discussed in Section 6.3, such modulation-based approaches preclude acquisition of any temporal information on the transient species, such as their reaction kinetics.

Relatively few CRDS studies of solution reaction kinetics have been reported^{3,4} and, of these, only Alexander, in a kinetic study of nitrate radical reactions with terpenes, has demonstrated sufficient temporal resolution to study transient radical species in solution using a flash-photolysis-based approach.³ In this case the kinetics were extracted from individual ring-down decay profiles.

This chapter describes time-resolved CRDS measurements of photo-induced reactions, and their MFEs, in solution. Two model systems are studied and the results compared with those of conventional transient absorption measurements. Finally, the applicability of this approach to an *in vitro* MFE study of *E. coli* DNA photolyase is demonstrated. The technique, a pump-probe method with CRDS detection, uses varying pump-probe delay times to build up a temporal profile of the absorbing molecules and is particularly useful in detecting relatively short-lived species. It is thus complementary to other optical approaches for studying MFEs.

7.2 Experimental

7.2.1 Materials

Hen egg white lysozyme (HEWL) and the sodium salts of flavin mononucleotide (riboflavin-5'-mono-phosphate, referred to as FMN) and anthraquinone-2,6-disulphonate (referred to as AQDS) were used as supplied (Sigma-Aldrich). A mutant of *E. coli* photolyase (EcPL) that does not bind the methenyltetrahydrofolate (MTHF) cofactor was provided by

Stefan Weber and co-workers (University of Freiburg); the non MTHF-binding mutant was used to minimize sample heterogeneity and photodegradation⁵—the expression and purification methods are described elsewhere.⁶ The sample was diluted using a 50/50 (vol/vol) glycerol/water mixture, to give an EcPL concentration of *ca.* 60 μM (*vide infra*). All solutions were made up using ultra-pure water (Milli-Q, resistivity 18.2 $\text{M}\Omega\text{ cm}$, pH 6.3). The concentrations used for individual experiments are detailed later.

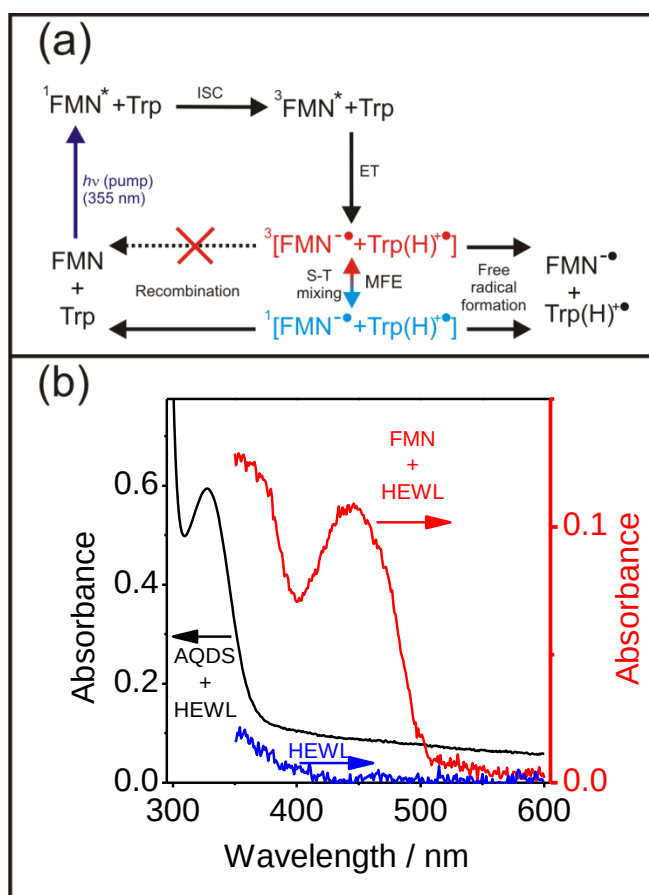


Figure 7.1 (a) Photochemical reaction scheme for the FMN, HEWL system; the reaction scheme for AQDS, HEWL is analogous (replace FMN with AQDS in the scheme). (b) UV/Vis absorption spectra of 10 μM FMN & 0.5 mM HEWL (red line), 0.1 mM AQDS & 0.1 mM HEWL (black line) and 0.5 mM HEWL only (blue line), in aqueous solution.

7.2.2 Photochemical systems

7.2.2.1 FMN and AQDS systems

Two closely related photochemical systems comprising lysozyme with one of two possible photosensitizers (FMN or AQDS) were investigated in aqueous solution. The common photochemical scheme is shown in Figure 7.1(a).

Irradiation of the solution with UV light (355 nm, close to the ground-state absorption maxima of both FMN and AQDS, Figure 7.1(b)) generates the electronically excited substrate $^1\text{FMN}^*$ ($^1\text{AQDS}^*$). This then undergoes fast intersystem crossing (ISC) on the timescale of a few nanoseconds, to yield the excited triplet state, $^3\text{FMN}^*$ ($^3\text{AQDS}^*$). Subsequent electron transfer (ET), from a tryptophan (Trp) residue in lysozyme (most likely Trp123,⁷ Trp62,⁸ or both) to FMN/AQDS with conservation of total spin angular momentum, generates a triplet state radical pair, $^3[\text{FMN}^{\bullet-} \text{Trp}(\text{H})^{+\bullet}]$ or $^3[\text{AQDS}^{\bullet-} \text{Trp}(\text{H})^{+\bullet}]$, respectively.

In both systems, which conform to the radical pair mechanism (see Section 5.4), the triplet-born RP can interconvert to the singlet state *via* singlet–triplet mixing induced by interaction of the electron spins with surrounding magnetic nuclei (Figure 7.1(a)). Importantly for these studies, the efficiency of this singlet–triplet mixing process may be affected significantly by applied magnetic fields. Upon radical re-encounter, only the singlet RP may recombine to form the molecular precursor; the triplet RP is unreactive. As a result, the total radical yield is magnetic field-sensitive (see also Chapter 5). In the experiments reported here, both CRDS and TA were used to monitor the radical concentration as a function of i) applied magnetic field and ii) pump–probe delay. A broad absorption band peaking around 520 nm and assigned to the photosensitizer radical, allows selective detection of the radicals present using a 532 nm Nd:YAG laser, whilst minimizing overlap with the ground state species.

7.2.2.2 EcPL system

A photochemical scheme devised by Henbest *et al.*,⁵ describing radical pair formation and decay in EcPL, is shown in Figure 7.2. Photoexcitation of the flavin adenine dinucleotide (FAD) cofactor in EcPL in its fully oxidised redox state, FAD^{ox} , produces a singlet excited state ($^1\text{FAD}^*$). $^1\text{FAD}^*$ is then rapidly reduced (on a picosecond timescale), *via* cascade electron transfer along a chain of three Trp residues,^{9–11} generating a radical pair, which, due to conservation of spin

angular momentum, must be formed with the same spin multiplicity as its precursor. This singlet-born RP, $^1[\text{FAD}^{\bullet-} \text{Trp(H)}^{\bullet+}]$ may either recombine to form its ground state precursor, interconvert to the triplet state RP, $^3[\text{FAD}^{\bullet-} \text{Trp(H)}^{\bullet+}]$, or react with external electron acceptors/donors. Singlet-triplet interconversion, together with competition between RP recombination and Trp cation radical deprotonation, confers magnetic field sensitivity on the radical yield.

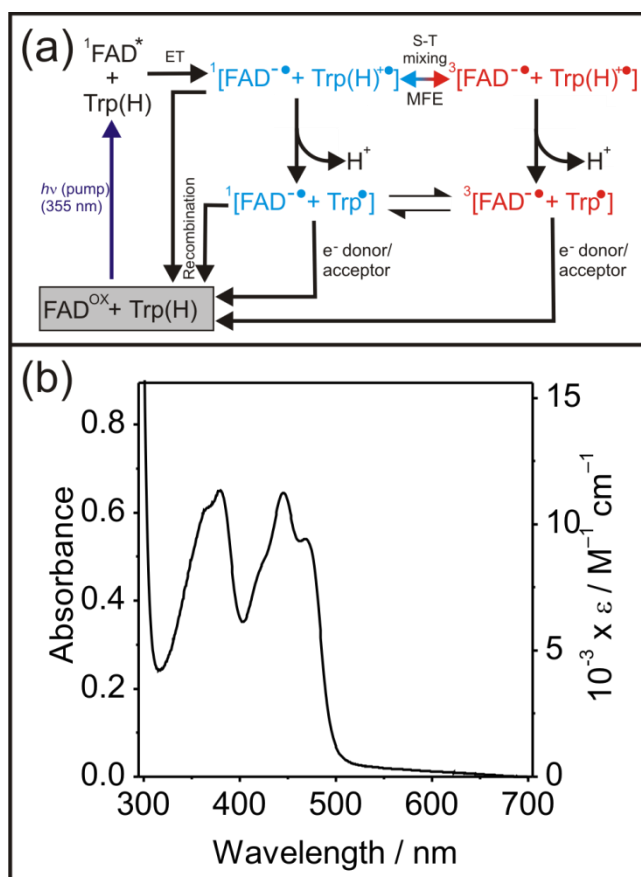


Figure 7.2 (a) Photochemical reaction scheme for the EcPL system (see text for details). (b) UV/Vis absorption spectrum of EcPL: molar extinction coefficients, ϵ , are estimated by scaling the measured absorbance to the known FAD extinction coefficient at 443 nm ($11200 \text{ M}^{-1} \text{cm}^{-1}$).¹² The EcPL concentration is thus estimated to be $60 \mu\text{M}$.

7.2.3 CRDS

The CRDS experiment is shown schematically in Figure 7.3(a). A simple optical cavity was formed by two high-reflectivity mirrors, M1 and M2 (mirror set 2, see Section 2.4 and Figure 2.3(b), 1 m radius of curvature) mounted 60 cm apart. Two different intra-cavity sample cells were used (Starna 45/Q, or Hellma 165-1.0-40) both of which have sample path lengths $l = 1$

mm. The Starna cell was used for the FMN (AQDS), HEWL experiments in which the (room temperature) sample flowed at 1 mL hr^{-1} . The Hellma sample cell (see Figure 7.4) was specifically designed for sample temperature control, *via* a glass jacket contacting the sample, through which coolant flowed. This cell was used for the EcPL studies, which are performed on a static sample at a temperature of -5°C .

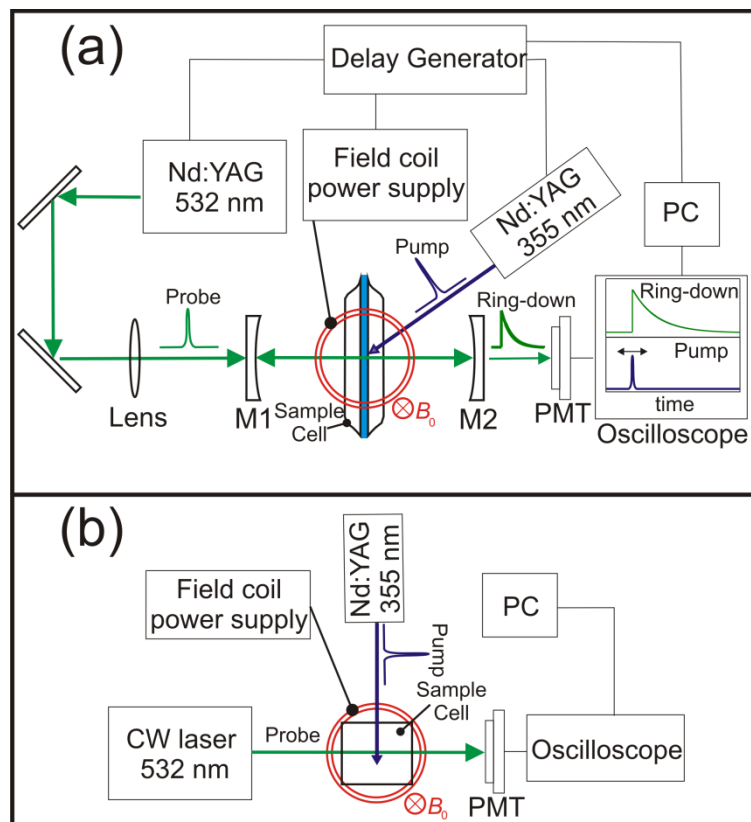


Figure 7.3 Schematics of the experimental CRDS (a) and TA (b) apparatus used to study radical concentration as a function of time and applied magnetic field strength. Helmholtz coils, indicated by concentric circles generate a static field, B_0 , at the sample cell.

In all cases, the sample cell was oriented with its windows normal to the cavity axis using a goniometer stage. This geometry proved preferable to a Brewster's angle arrangement,² provided the cell had good optical surfaces. The sample cell was positioned at the centre of the cavity between a pair of Helmholtz coils which provided the magnetic field. The photoexcitation (pump) pulse (355 nm, *ca.* 7 ns, 10 Hz, Continuum Surelite I Nd:YAG laser) was directed through

the sample as shown in Figure 7.3(a). The CRDS (probe) pulse at 532 nm (5 ns, 10 Hz) was provided by a Continuum Minilite I Nd:YAG laser. Light exiting the cavity *via* the rear mirror (M2) was detected by a PMT (Hamamatsu, R928, see also Section 2.5), recorded on a digital oscilloscope (LeCroy Wavesurfer 42Xs, 2.5 GS s^{-1} , 400 MHz) and the data transferred to a personal computer (PC). The ring-down time, τ , was determined by a non-linear least-squares curve-fitting routine in IGOR Pro[®] (Wavemetric). From the ring-down times recorded with, τ , and without, τ_0 , photoexcitation, the change in the per-pass absorbance, ΔA (on the conventional $\log_{10}$ scale), was calculated using Equation 1.13.

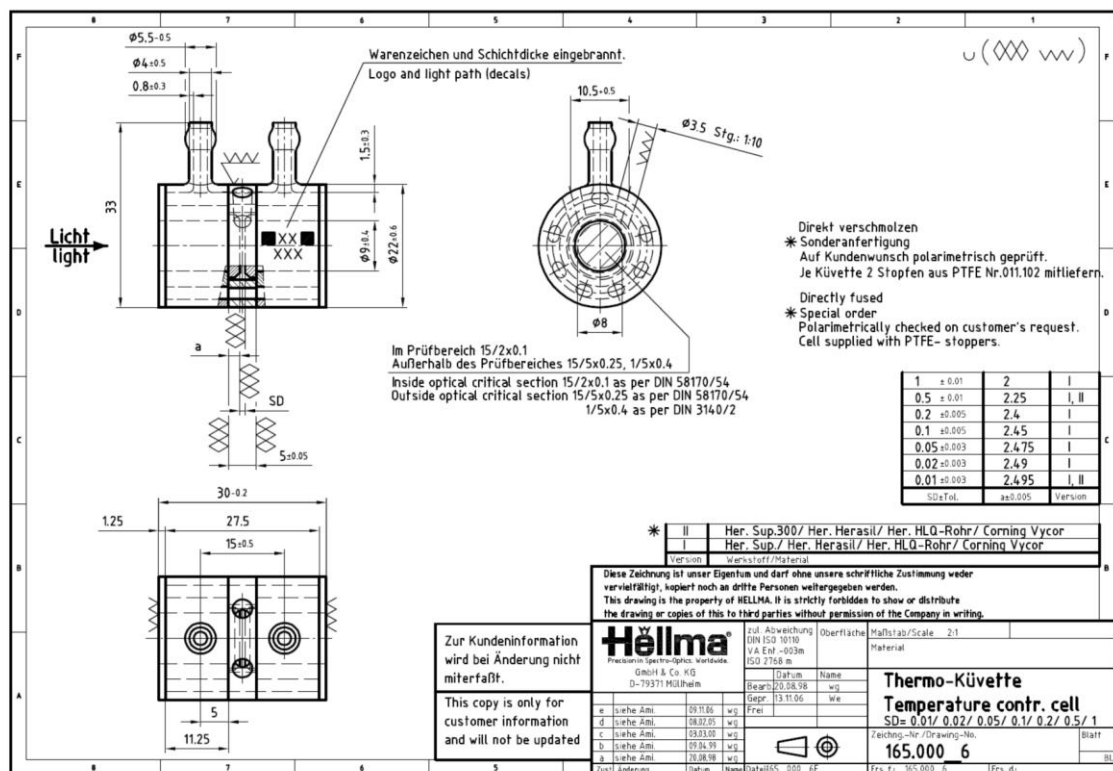


Figure 7.4 Schematic of the temperature control cell, provided by the manufacturer (Hellma).

Alignment of the pump beam path was achieved trivially *via* optimization of the CRDS-measured absorbance change within the sample cell. In order to build up the temporal evolution of the intracavity absorbance, a PC-controlled delay generator (SRS, DG535) was used to vary—in random order, between specified limits—the delay time (t_d) between pump and

probe pulses. Variation of the magnetic field strength was achieved in a similar random fashion, *via* the power supply of the Helmholtz coils. All measurements were made at 10 Hz, with the exception of the EcPL studies, in which the pump and probe pulses were operated at 1 Hz to allow for reoxidation of the flavin cofactor by dissolved dioxygen between laser shots.

7.2.4 Transient absorption measurements

The experimental setup for TA measurements is shown schematically in Figure 7.3(b). The sample flowed through a Hellma flow cell mounted between Helmholtz coils. The photoexcitation beam (Continuum Surelite I Nd:YAG, 355 nm output, 10 ns pulse at 10 Hz) and the CW probe beam (Laser Quantum, Torus, 532 nm) were directed through the sample cell at right angles to one another, with path lengths $l = 2$ mm and 10 mm, respectively. The probe beam intensity exiting the sample cell was monitored with a PMT (Hamamatsu R928) and used to determine the ($\log_{10}$ scale) absorbance.

7.3 Results and discussion

7.3.1 $^3\text{FMN}^*$ quenching kinetics

Later sections in this chapter demonstrate how CRDS may be used to monitor sensitively the effect of an applied magnetic field on the kinetics of photoinduced reactions. However, it is useful to provide first a more general illustration of CRDS's ability to probe chemical kinetics in solution. For this purpose, the quenching of the triplet excited state of FMN ($^3\text{FMN}^*$) has been studied. Photoexcitation of an aqueous solution of pure FMN results in the formation of $^3\text{FMN}^*$, whose absorption spectrum exhibits a peak around 670 nm.¹³

The inset of Figure 7.5 shows the CRDS-measured absorbance recorded at 532 nm—on the extreme blue tail of the $^3\text{FMN}^*$ absorption band—as a function of t_d . The time-base is built-up by varying the pump–probe delay time as described in Section 7.2.3. The decay in $^3\text{FMN}^*$

absorbance is dominated by a combination of i) non-radiative relaxation to the ground state, ii) quenching by molecular oxygen, iii) self-quenching with ground-state FMN and iv) triplet-triplet annihilation.¹⁴⁻¹⁹

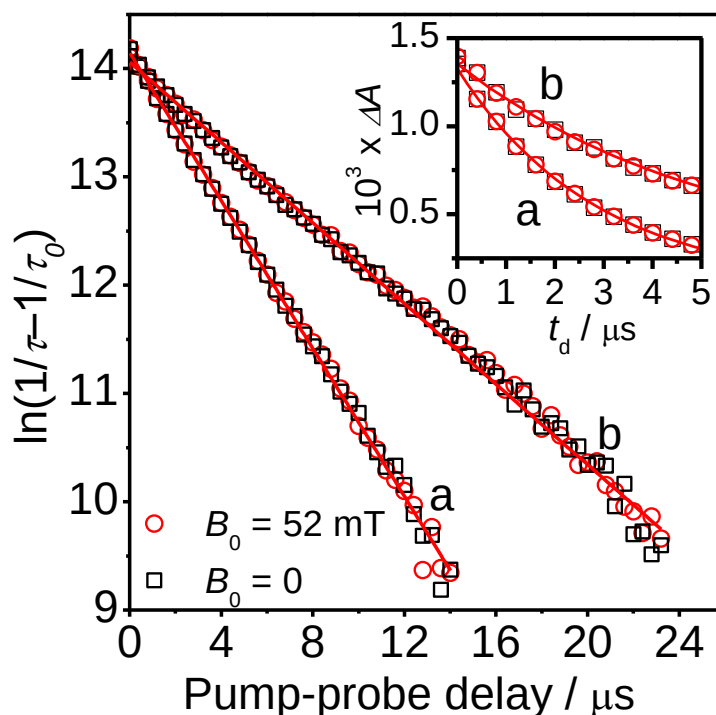


Figure 7.5 Kinetic study of the triplet excited state of FMN following photoexcitation (355 nm, pulse energy 0.6 mJ) of (a) un-purged (in ambient air) and (b) Ar-purged aqueous solution of 0.1 mM FMN. Points represent the experiments in the presence (red circles) and absence (black squares) of a 52 mT applied magnetic field, respectively. Linear fits are shown as solid red lines. The inset displays the decay in the absorbance change at short pump–probe delays, on a linear scale. Each data point represents a 100–shot average. Solid red lines signify fits of the decay profiles to single exponential decays.

In these experiments iii) and iv) are minimized by the use of low FMN concentrations (0.1 mM) and low laser pulse energies (0.6 mJ). As a result, the triplet decay may be treated as a first order process with the concentration of $^3\text{FMN}^*$ decaying exponentially from its initial value $[^3\text{FMN}^*]_0$. Equation 1.13 may then be used to derive the following expression for the measured ring–down time:²⁰

$$\ln\left(\frac{1}{\tau} - \frac{1}{\tau_0}\right) = \ln\left(\frac{cl\varepsilon[{}^3\text{FMN}^*]_0}{L}\right) - kt_d \quad (7.1)$$

in which k is the pseudo-first order rate constant for the decay of ${}^3\text{FMN}^*$. In all experiments, a small constant offset was observed resulting from production of some unidentified, persistent photoproduct. This contribution was removed from the $\ln(1/\tau - 1/\tau_0)$ data prior to fitting.

Plots of $\ln(1/\tau - 1/\tau_0)$ versus the pump-probe delay time, are shown in Figure 7.5. As expected, these plots are linear ($R^2 = 0.995\text{--}0.998$) and, *via* Equation 7.1, allow determination of k for both Ar-purged and un-purged systems: $(5.38 \pm 0.05) \mu\text{s}$ and $(2.93 \pm 0.02) \mu\text{s}$, respectively. These lifetimes are consistent with those determined elsewhere (*ca.* $5 \mu\text{s}$ in an Ar-purged system, see Figure 5(a) in Maeda *et al.*, and $2.3 \mu\text{s}$ in the presence of oxygen—Lasser *et al.*),^{14,21} and permit an estimation of the rate constant for oxygen quenching of $(1.57 \pm 0.02) \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$, (assuming $[\text{O}_2] = 2.60 \times 10^{-4} \text{ M}$) in the un-purged solution; this agrees well with the previously reported value due to Lasser *et al.* $(1.7 \pm 0.4) \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$.¹⁴ Measurements were performed in the presence and absence of an applied magnetic field as a negative control; the lack of magnetic field dependence is clear from the data in Figure 7.5.

The intercepts of the plots in Figure 7.5 are used to calculate $[{}^3\text{FMN}^*]_0 = 2.2 \times 10^{-5} \text{ M}$ assuming $\varepsilon({}^3\text{FMN}^*) = 1.25 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$, estimated from Grodowski *et al.*¹³ These triplet yields ($\approx 20\%$) are sensible in view of the values observed and calculated previously and justify the pseudo-1st order treatment above.¹³

Importantly, Figure 7.5 and its analysis demonstrate the application of CRDS to a kinetic study in the liquid phase, by varying the pump-probe delay time to build up a temporal absorbance profile. The temporal resolution of this approach is, in principle, limited only by the electronics used to create the delay and the pulse lengths of the light sources used. However, this method requires that the absorbance remains effectively constant over the ring-down times employed. As such, the lower limit of the temporal dynamic range may be taken as τ .²⁰ In these FMN

studies, the range of τ is around 300 – 600 ns, which is still considerably shorter than the chemical lifetimes measured (tens of μs). Kinetic information may be obtained from individual ring-down decays, but the extraction of the absolute absorbance requires fitting of the data to more complex decay profiles.^{3,22} Since the purpose of this chapter is to illustrate the use of CRDS in measuring MFEs, and to compare its performance with that of TA, this type of deconvolution is only touched upon briefly, in Section 7.3.1.1. In this regard it is worth noting that i) the user can to some extent control the ring-down time, without sacrificing the effective number of passes, *via* the length of the cavity, and ii) radical pairs need to live for at least several hundred nanoseconds²³ to exhibit any sensitivity to the relatively weak magnetic fields (millitesla and below) of interest for the biological systems investigated here (see also Chapter 5).

Under the experimental conditions employed, the upper limit of the temporal dynamic range is determined only by the time taken for the chemical decay to reach the minimum detectable change in absorbance, $\Delta A_{\min}$, which may be calculated using Equation 1.14. By way of example, in the $^3\text{FMN}^*$ decay measurements (in Ar-purged solution), $\Delta A_{\min}$ is determined as 2.8×10^{-6} —when $\Delta\tau_{\min} = 0.8$ ns is the standard deviation of the mean value of τ_0 over a 1 s period, during measurements on long time scales (not shown here). The upper limit of the temporal dynamic range may be subsequently estimated as 32 μs —as determined from the time taken for ΔA to reach $\Delta A_{\min}$. It should be noted that, when identifying the upper temporal dynamic range limit, the time taken for the absorber to flow out of the detection volume must also be considered. In the example given above this time is far longer than the $^3\text{FMN}^*$ lifetime and therefore, in this particular case, the temporal dynamic range is limited by the detection sensitivity and not the flow rate.

7.3.1.1 Accounting for concentration changes during the ring-down decay

The ring-down times in this work were extracted simply by fitting the signal decay trace to the functional form $A1\exp(-t/\tau) + y0$. However, as discussed above, it is important to note that this fitting procedure assumes the $^3\text{FMN}^*$ concentration remains constant during the ring-down signal decay. Of course, as already seen from Figure 7.5, this is not the case; the $^3\text{FMN}^*$ concentration decays exponentially in time, during its pseudo first-order loss.

Figure 7.6 shows semi-log plots of two ring-down decay traces, recorded for the unpurged FMN solution, at $B_0 = 52$ mT, in the absence and presence of photoexcitation (and used to calculate τ_0 and $\tau_{t_d=0.41 \mu\text{s}}$, respectively, which, in turn, are used to calculate ΔA at $t_d = 0.41 \mu\text{s}$, shown in Figure 7.5). As expected, in the absence of photoexcitation the decay trace is linear. However, in the presence of photoexcitation (and therefore a decaying $^3\text{FMN}^*$ concentration), the signal trace deviates from linearity. The importance of including this concentration change in the decay fitting is assessed below, using the approach developed by Brown *et al.*²²

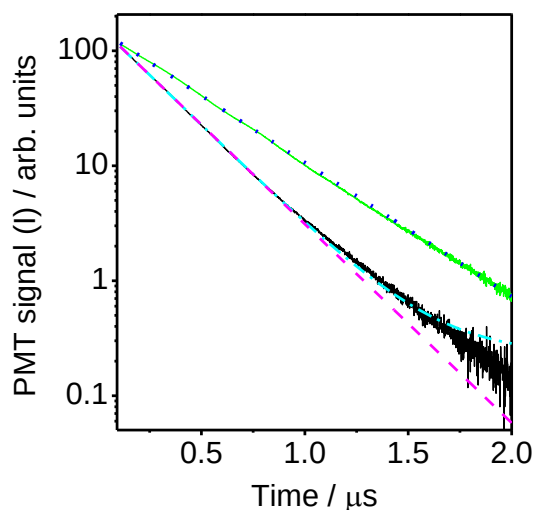


Figure 7.6 The fitted ring-down decay data used to calculate the absorbance, shown in Figure 7.5. Signal decays recorded in the absence (green line) and presence (black line) of photoexcitation are shown. The baseline offset (present in all decay traces and determined from measurement at long timescales, $> 10 \times \tau_0$) was removed before plotting. Blue (dotted) and magenta (dashed) lines represent fits of the signal decay traces to $A1\exp(-t/\tau) + y0$, where $y0 = 0$, giving $\tau_0 = 371$ ns and $\tau_{t_d=0.41 \mu\text{s}} = 251$ ns respectively (used to calculate $\Delta A_{t_d=0.41 \mu\text{s}} = 1.15 \times 10^{-3}$, shown in figure 7.5). Cyan (dot dashed) line: the decay data recorded, at $t_d = 410$ ns, after the pump pulse, is fit to the expression in Equation 7.5, where c , L and τ_0 were fixed (at $2.998 \times 10^8 \text{ ms}^{-1}$, 0.6 m and 371 ns, respectively), whilst $\Delta A(0)$ and k were allowed to vary.

The instantaneous rate of loss of light intensity, I , within the cavity during beam propagation along the cavity- (x -) axis can be described by:

$$\frac{dI}{dx} = -\frac{2.3026A}{L}I - \frac{1-R}{L}I \quad (7.2)$$

substitution of $dx = c dt$ and $\tau_0 = \frac{L}{c(1-R)}$ (Equation 1.12) into Equation 7.2 gives:

$$\frac{dI}{I} = -\left(c \frac{2.3026A}{L} + \frac{1}{\tau_0}\right) dt \quad (7.3)$$

If the concentration (and therefore absorbance) is assumed to remain constant then Equation 7.3 yields:

$$I(t) = I(0)e^{\left(-c \frac{2.3026A}{L}t - \frac{t}{\tau_0}\right)} \quad (7.4)$$

which is a result that has already been derived (albeit using a slightly different approach) in Chapter 1 (Equation 1.9).

However, for an absorber with pseudo first-order loss Equation 7.3 yields:

$$I(t) = I(0)\exp\left[-\frac{c}{k} \frac{2.3026A(0)}{L}(1 - e^{-kt}) - \frac{t}{\tau_0}\right] \quad (7.5)$$

where $A(0)$ is the initial absorbance, at $t_d = 0$

Therefore, one approach to determining absorbance (or, as is the case in these pump-probe experiments, the absorbance change) is to fit ring-down decay profiles directly to Equation 7.5. One such fit, for the ring-down decay recorded with $t_d = 410$ ns, is shown in Figure 7.6 (cyan line). The fit, which gives $\Delta A(0) = 1.31 \times 10^{-3}$, is clearly better than that achieved using a fit of the ring-down decay trace to $A1\exp(-t/\tau) + y0$. As expected $\Delta A(0)$, the initial absorbance, is larger than the absorbance (1.15×10^{-3}) calculated using Equation 1.13 (with τ obtained from fitting the decay to $A1\exp(-t/\tau) + y0$, as discussed above).

This fitting approach was repeated for all pump-probe delay times, giving $\Delta A(0)$ as a function of t_d , shown in Figure 7.7 (which also includes the ΔA data shown previously in Figure 7.5, for comparison).

Finally, as illustrated in Figure 7.8, using the same approach as that shown in Figure 7.5, a plot of $\ln\left(\frac{1}{\tau} - \frac{1}{\tau_0}\right)$ versus t_d (calculated from $\Delta A(0)$ in Figure 7.7), yields $1/k$ ($= 2.94 \pm 0.8$ μ s).

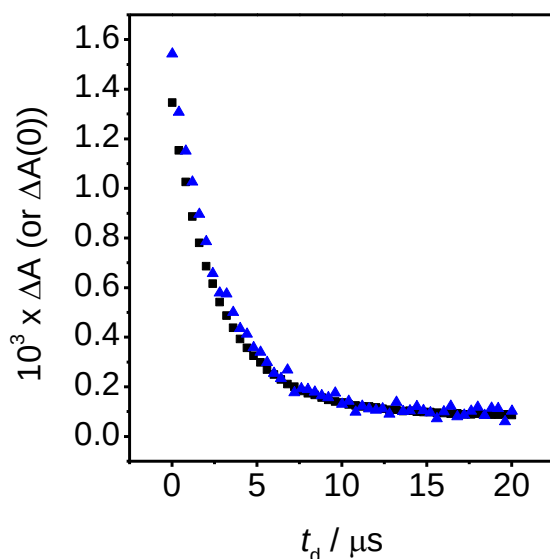


Figure 7.7 comparison of absorbance changes (for the un-purged FMN solution at $B_0 = 52$ mT) as a function of pump-probe delay time: ΔA (black squares) taken from the inset of Figure 7.5 (expanded to a longer timescale) and $\Delta A(0)$ determined using fits of ring-down decays to Equation 7.5 (blue triangles).

From the results shown in Figure 7.8—giving a $1/k$ value (2.94 ± 0.09 μs), determined from $\Delta A(0)$, that is the same (within error) as that determined from Figure 7.5—it is concluded that, for the relatively long chemical lifetimes and short ring-down decay times studied here, fitting ring-down decay profiles to expressions that account for changes in concentration does not appear to be a crucial procedure when monitoring decay kinetics with the pump-probe CRDS instrument described here. However, from Figure 7.7, it is noted that ignoring these concentration changes during fitting does result in absorbance values which are systematically lower than they should be at any given time.

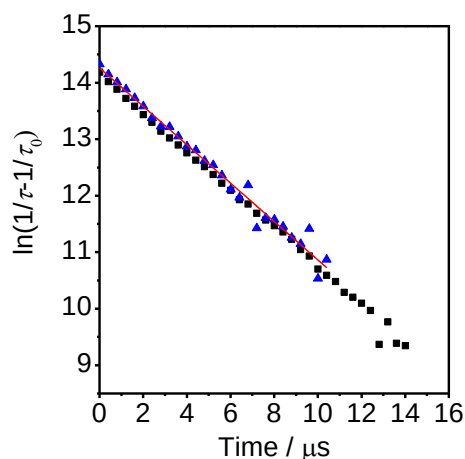


Figure 7.8 Comparison of data from Figure 7.5 (black squares) with that calculated from $\Delta A(0)$ data shown in Figure 7.7 (blue triangles) and used to determine a value of $1/k$ (2.94 ± 0.09 μs , *via* a linear fit, red line), which is the same (within error) as that determined from Figure 7.5, (see text for details).

7.3.2 MFE on the AQDS, HEWL system

Having demonstrated the capabilities of the CRDS apparatus in studying simple solution-phase kinetics the current section proceeds to illustrate the technique's applicability in the investigation of MFEs.

Figure 7.9 displays the field-sensitive % MFE (Equation 5.19) of the AQDS, HEWL system detected *via* CRDS at 532 nm in the absence, $\Delta A(B_0 = 0)$, and presence, $\Delta A(B_0)$, of a magnetic field.

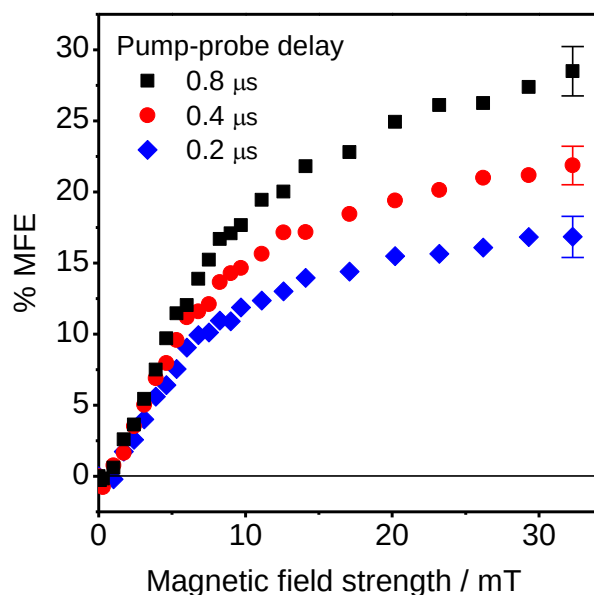


Figure 7.9 Percentage magnetic field effects (Equation 5.19) as a function of magnetic field strength for a photo-excited aqueous solution of 0.1 mM AQDS and 0.1 mM HEWL measured by CRDS for various pump-probe delay times. Data points represent 500-shot averages. MFE traces are acquired with a 50 shot average per point: each MFE graph shown is the average of 10 such traces, the standard deviation of which is displayed at one data point, as a representative uncertainty. The field effects and their time dependence are consistent with those observed by Maeda *et al.*²⁴

In order to facilitate comparison with a previously conducted TA-based study of this system, MFE graphs are plotted both as a function of pump-probe delay and magnetic field. As expected for a triplet-born radical pair, an increasing magnetic field produces a rise in the radical concentration (and hence % MFE) as the $T_{\pm}$ spin levels become energetically more and more isolated from the S/T_0 manifold (see Section 5.5). The resulting drop in singlet-triplet interconversion efficiency leaves more radicals in the triplet state, unable to recombine hence contributing strongly to the absorbance at 532 nm.

In satisfying agreement with previous reports,²⁴ the MFE for the early times rises most sharply between 2 and 10 mT, whilst the MFE for the 0.8 μ s pump-probe delay time is still not saturated at the highest field applied here. For the 0.2 μ s delay dataset, the magnetic field at which the MFE reaches half its maximum value $B_{1/2} = 8$ mT, exceeds the theoretically predicted value²⁵ (4.5 mT) by a factor of nearly 2, indicating slow spin dynamics and the involvement of relaxation processes. Such behavior is typical for long-lived, triplet-born radical pairs in micelles or biradicals. As in previous TA studies,²⁴ the MFE graphs in Figure 7.9 contain contributions from radical pairs present throughout the ring-down time (250 – 350 ns for the AQDS, HEWL system). The uncertainty in the % MFE, determined from the standard deviation in 15 data points at 52 mT (1000 shot average per data-point, data not shown), was found to be 0.8%, equivalent to a standard deviation in ΔA of 8×10^{-6} . This excellent SNR achieved in the MFE results from the large field effect observed in the AQDS system combined with the high sensitivity of the CRDS. The SNR is probably limited by two factors. Firstly, the ring-down times correspond to only 60 – 90 cavity round trips per ring-down time, limited by scattering losses at the surface of, and within, the sample cell itself. Secondly, the scattering within the sample introduces measurement instability *via* fluctuations in the ring-down time. The ultimate sensitivity of the technique is discussed in more detail later.

The % MFE traces are useful in determining the initial spin state of the radical pair. In addition, the $B_{1/2}$ value permits identification of the radical pair and allows the investigation of exchange processes as a function of the concentration of the radical pair precursors.²⁶ Moreover, the % MFE traces establish if a given system exhibits significant Low Field Effects.²⁷ However, crucial parameters regarding the radical pair recombination and relaxation kinetics and their dependence on the magnetic field can only be obtained *via* time-resolved studies. Figure 7.10(A) demonstrates how CRDS can be used to monitor both the kinetics of RP decay and the effect of an external magnetic field on RP yield and decay kinetics. The figure shows the

temporal profile of AQDS radical absorbance (in the AQDS, HEWL system), as a function of pump–probe delay, in the presence and absence of a 30 mT field. Each data point represents a 500-shot average. In both $B_0 = 0$ and $B_0 = 30$ mT cases, an initial rise in ΔA at pump–probe delay = 0, is followed by a non-exponential decay in absorbance. The rise in ΔA , due to RP generation, occurs within a few nanoseconds following photoexcitation²⁴ and is temporally unresolved. The decay of the absorbance comprises two components: a fast, strongly field-sensitive decay component corresponding to geminate recombination of the RP and a slower component resulting from free radical decay (bulk recombination).

Maeda *et al.*²⁴ calculated the zero-field RP recombination rate constant, as $1.1 \times 10^6 \text{ s}^{-1}$ (a 0.9 μs lifetime) and estimated the free radical decay lifetime between 5 μs and 16 μs . The relatively short time-base in Figure 7.10(A) prohibits fitting an unconstrained double-exponential decay. Fixing the time constants (0.9 μs and 5 μs) in a double-exponential fit produces the curve shown in the figure, with an acceptable root-mean-squared error of 3.8×10^{-5} , although this treatment should be viewed with caution, due to the second-order nature of free radical recombination kinetics. This is also a situation in which a more refined kinetic analysis would require a full deconvolution of ring-down time and radical pair kinetics.³

The real advantage of CRDS detection over TA becomes apparent in the measurement of MFEs themselves. Upon application of a magnetic field ($B_0 = 30$ mT), there is a clear change in ΔA , which evolves in time as shown in Figure 7.10(B). Following the initial increase in ΔA , due to the field effect on RP recombination, the influence of (field-dependent) spin relaxation processes on recombination is observed, as ΔA decays in time.

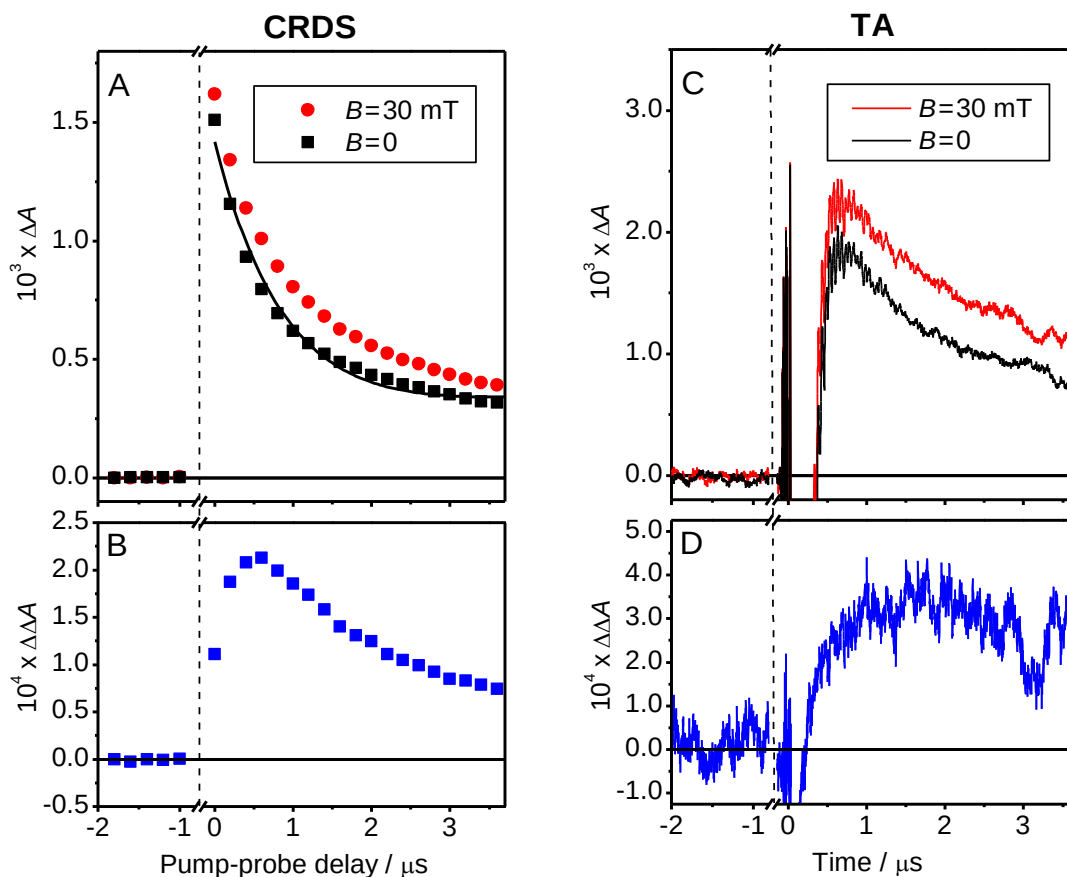


Figure 7.10 (A) and (C): Decay-time profiles of a photo-excited aqueous solution of 0.1 mM AQDS and 0.1 mM HEWL in the presence ($B_0 = 30$ mT, red) and absence ($B = 0$, black) of an applied magnetic field as measured by (A) CRDS (1 mJ photoexcitation pulse energy and 1 mm path length) and (C) TA (1 mJ photoexcitation pulse energy and 10 mm path length). The black line in (A) is a constrained double exponential fit to the $B_0 = 0$ data (see text for details). (B) and (D): MFE time profiles $\Delta\Delta A(B_0 = 30 \text{ mT}, t_d)$, determined from the decay profiles, (A) and (C), respectively. Note the difference in signal-to-noise between the CRDS and TA profiles. Note also the breaks in the horizontal axes: CRDS data in the excluded range is corrupted by temporal overlap between the photoexcitation pulse and ring-down decay trace.

The kinetics of this system are described in detail elsewhere: Maeda *et al.*²⁴ used the kinetic model summarized in Figure 7.11 in a global fit to $\Delta\Delta A(B_0, t_d)$ data. (incorporating rate constants for hyperfine coupling, relaxation, escape (to free radicals), RP recombination and free radical decay).

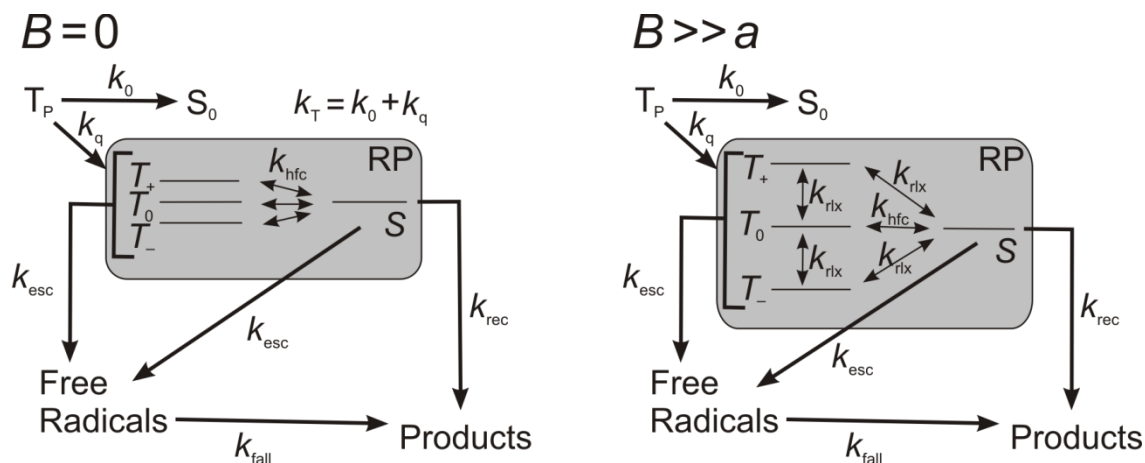


Figure 7.11 Kinetic model of Maeda *et al.* used for fitting of $\Delta\Delta A$ time profiles. S_0 and T_P are the ground state precursor and triplet excited state of AQDS, respectively. k_q is the rate constant for quenching of the excited state. k_0 represents radiative and non-radiative decay from the triplet excited state. S-T mixing (between the spin substates: T_+ , T_- , T_0 and S) is characterized by a hyperfine coupling rate constant, k_{hfc} , and (where the field B_0 exceeds the hyperfine coupling, a) a rate constant for relaxation, k_{rlx} . k_{esc} , k_{fall} and k_{rec} are the rate constants for escape to free radicals, free radical decay and geminate recombination, respectively.

Temporal profiles analogous to the CRDS measurements in Figures 7.10(A) and 7.10(B) have been recorded by TA and are shown in Figures 7.10(C) and 7.10(D). Each profile in Figure 7.10(C) is a 2000-shot average. The noise on ΔA (and thus $\Delta\Delta A$) on the TA data (*ca.* 10^{-4}) is two orders of magnitude larger than in the CRDS experiments (*ca.* 10^{-6}). This reflects directly the increased optical path-length through the sample in CRDS and the inherent insensitivity of CRDS to shot-to-shot intensity fluctuations in and the probe beam. An additional advantage of CRDS is exemplified by the comparison of Figures 7.10(A) and 7.10(C) at early times ($\leq 0.75 \mu s$). During this time the TA detector is blinded by light scattered from the pump beam and/or fluorescence of the sample. CRDS, by contrast, is insensitive to such problems. Knowledge of the temporal evolution of the decay profile at early times is important when determining fitting parameters and hence CRDS has a distinct advantage over TA in this respect. Of course, a gated PMT (not available in this study) or suitable optics could go some way to removing the extent of interference seen in Figure 7.10(C).

It is clear from the above that the sensitivity gain achieved in CRDS represents a major advantage over conventional TA. In turn, this sensitivity gain allows sample miniaturization: the sample volume probed in CRDS is estimated to be *ca.* 4 μL , a factor of 15 smaller than that in TA (*ca.* 60 μL). Alternatively, CRDS permits the use of lower photoexcitation pulse energies than in TA. These attributes suggest CRDS will be well-suited to the study of biological samples, which can typically be produced only in microliter volumes, nano- to (few) micro-molar concentrations and which readily photodegrade. To demonstrate further the potential of CRDS in this field of interest, the biologically relevant model system, FMN, HEWL (described in Section 7.2.2.1), was studied, as described in the next section.

7.3.3 MFE on the FMN, HEWL system

Figure 7.12 shows the % MFE as a function of field strength for the FMN, HEWL system. As for AQDS, HEWL, the field effect arises due to a decrease in S-T mixing efficiency and concomitant increase in the transient radical concentration. The MFE is, however, considerably smaller than that for AQDS, HEWL. In the latter, Coulombic interactions of the triply negatively charged AQDS radical with HEWL, which is positively charged at neutral pH (isoelectric point 11.4), extends the lifetime of the geminate radical pair, promoting the evolution of significant magnetic field effects. By contrast, the relatively weak negative charge on the FMN radical results in a shorter geminate phase and hence a less pronounced field effect.

As a result, the field effect curve for the FMN, HEWL system is noisier than that shown in Figure 7.9, for AQDS. The MFE is still not saturated at the maximum field strength employed (52 mT) and the $B_{1/2}$ value (*ca.* 20 mT) is much larger than that predicted by theory (3 mT).²⁵ Again, this indicates slow spin dynamics in the system and the characteristic MFE can, therefore, be attributed to a combination of the hyperfine and spin relaxation mechanisms.

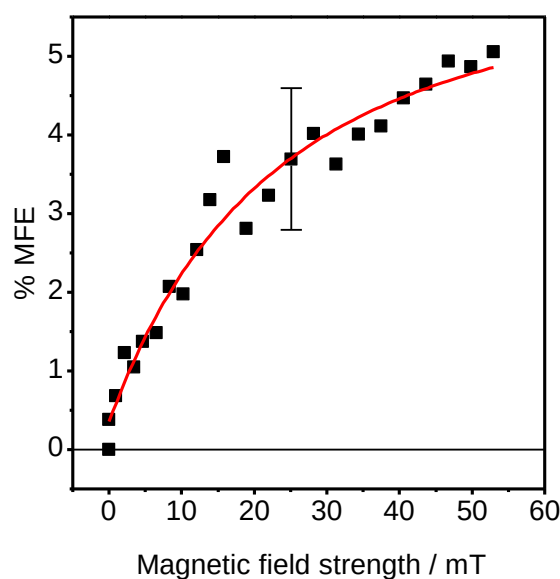


Figure 7.12 Percentage magnetic field effect (Equation 5.19) as a function of magnetic field strength for an aqueous solution of 10 μM FMN and 0.5 mM HEWL using CRDS detection. The pump–probe delay time was held constant at 6 μs . MFE traces are acquired with a 200 shot average per point: the graph shown is the average of 10 such traces. The experimental uncertainty shown represents one standard deviation for an individual data point, and is representative of all points. The red line is included to guide the eye.

The decay kinetics in the FMN, HEWL system, measured by CRDS, in the presence and absence of an applied field, are shown in Figure 7.13(A). As for AQDS, the FMN radical decay is observed as a function of time and comprises fast (geminate recombination and excited triplet decay) and slow (free radical decay) components, in this case with respective time constants (0.60 ± 0.09) μs and (11 ± 7) μs , obtained from a double exponential fit to the $B_0 = 0$ data in Figure 7.13(A). The effect of applying the magnetic field is clear in Figure 7.13(A) and the difference profile (Figure 7.13(B)), even at the very low radical concentrations generated here: $\Delta\Delta A$ values of around 4×10^{-6} in Figure 7.13(B) correspond to changes in $[\text{FMN}^{\bullet-}]$ of 13 nM (assuming $\epsilon_{532 \text{ nm}} = 3160 \text{ M}^{-1} \text{ cm}^{-1}$).²⁸ The MFE at a pump–probe delay of 6.4 μs is calculated as 4.5%, close to the 5% effect shown in Figure 7.12 at the same field strength. The TA decay profiles, shown for comparison in Figure 7.13(C), are qualitatively similar to those for CRDS. However, as illustrated in Figure 7.13(D), under the conditions employed here, the effect of applying an external magnetic field is undetectable in the TA experiment.

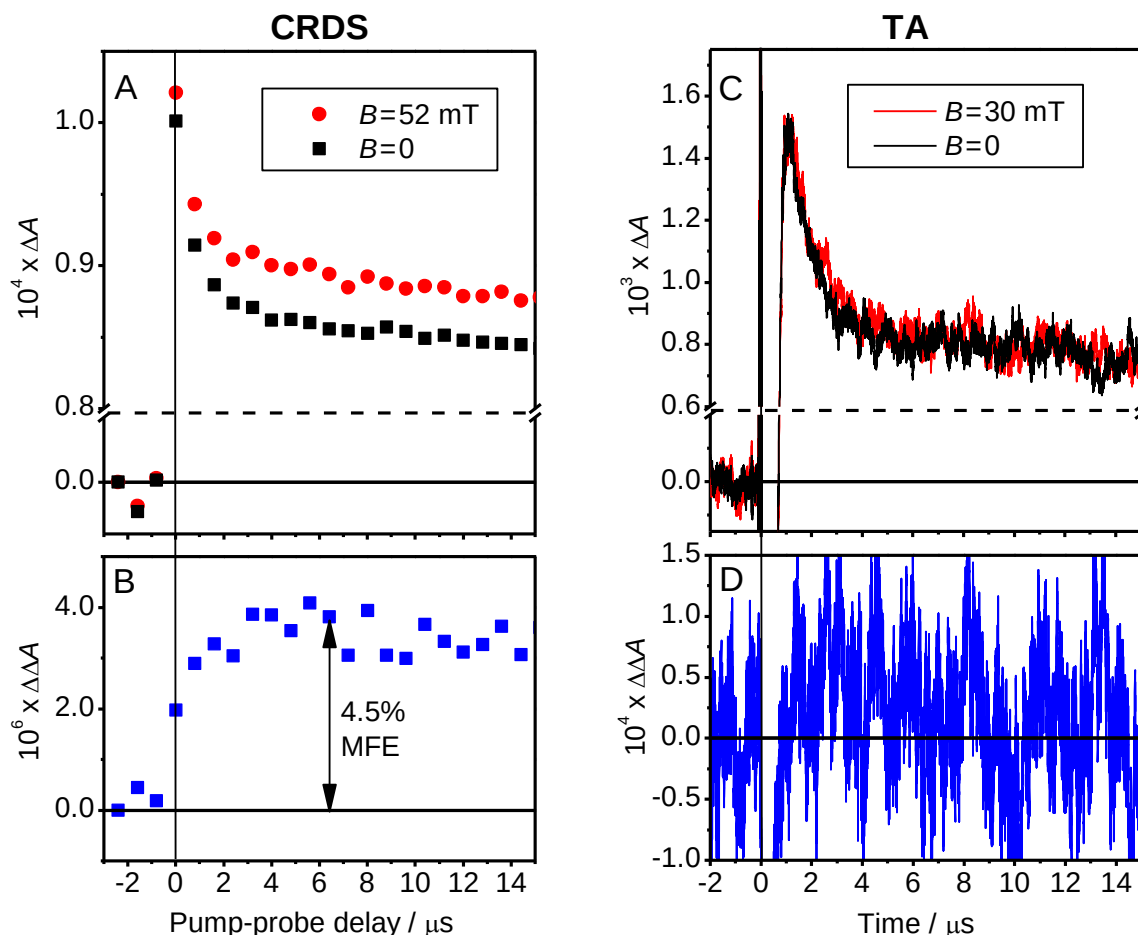


Figure 7.13 (A) and (C) Decay-time profiles of a photo-excited aqueous solution of $10 \mu\text{M}$ FMN and 0.5 mM HEWL, measured by (A) CRDS (0.75 mJ pump pulse energy, 1 mm path length) and (C) TA (1 mJ pump pulse energy, 10 mm path length), in the presence and absence of an applied magnetic field (52 mT for CRDS and 30 mT for TA). (B) and (D) MFE time profiles $\Delta \Delta A(t_d)$, determined from decay profiles (A) and (C), respectively. Note the breaks in the vertical axes and the difference in $\Delta \Delta A(t_d)$ between CRDS and TA of roughly two orders of magnitude. All data-points are 2000-shot averages.

Note that the difference in applied field strength between TA and CRDS measurements (as necessitated by the coil geometries) is expected to produce slightly different % MFEs (4% MFE at 30 mT , compared with 5% at 52 mT). However, despite this and the associated effect on $\Delta \Delta A$, the superior SNR in CRDS is clear from a comparison of Figures 7.13(B) and 7.13(D). The minimum detectable change in ΔA in TA (estimated from the standard deviation in $\Delta \Delta A$ over 100 data points around $4 \mu\text{s}$, in Figure 7.13(D)) is *ca.* 4.5×10^{-5} , from which the minimum detectable % MFE may be estimated as 5.3% . By contrast, 4.5% MFEs are easily observed using

CRDS (minimum detectable % MFE *ca.* 1 %), even though the ΔA values measured are significantly smaller than in TA. As in the AQDS study, PMT blinding in the TA experiment prevents any useful data being extracted during the first microsecond after the pump pulse. From Figure 7.13 and the analysis above it has been demonstrated that the data obtained from CRDS are superior to that of TA.

7.3.4 MFEs in a biological sample: EcPL

The decay kinetics in the EcPL system, measured by CRDS, are shown in Figure 7.14(A). The advantages of CRDS are clear: despite the small sample volume and low photoexcitation pulse energies used, changes in radical absorbance on the order of 10^{-5} are clearly observed. In contrast to the systems discussed above, the RP in EcPL is born in the singlet state⁵ and hence application of a magnetic field leads to an *increased* recombination efficiency and a net decrease in the transient radical concentration. A detailed analysis of the decay kinetics has not been performed here, but over the pump–probe delay range shown in Figure 7.14(A) the changes in absorbance are probably dominated by radical recombination from the primary radical pair (see Figure 7.2) with concomitant loss of $\text{FAD}^{\bullet-}$ and $\text{Trp}(\text{H}^+)^{\bullet}$ radical absorption (with broad maxima at *ca.* 500 nm and 600 nm, respectively). A small additional contribution from conversion of $\text{Trp}(\text{H}^+)^{\bullet}$ to $\text{Trp}^{\bullet}$ ($\lambda_{\text{max}} \approx 500$ nm) in the secondary radical pair is also likely.⁵

By contrast with the earlier work of Henbest *et al.*,⁵ in which ferricyanide was added to ensure rapid re-oxidation of $\text{FAD}^{\bullet-}$ back to the FAD^{ox} precursor, in this work no external electron acceptor was added to the sample prior to the experiments. Additionally, only a minor fraction of $\text{Trp}^{\bullet}$ is thought to be reduced by an unknown external electron donor. Therefore, a very long re-oxidation lifetime (17 ms) is expected in the secondary radical pair.¹⁰ Although such a long decay component is not apparent in Figure 7.14(A), the observation of a MFE, clear in Figure 7.14(B), suggests that rapid re-oxidation from the secondary RP is not necessary, as under the mild irradiation conditions used there is minimal conversion of the protein into its radical state.

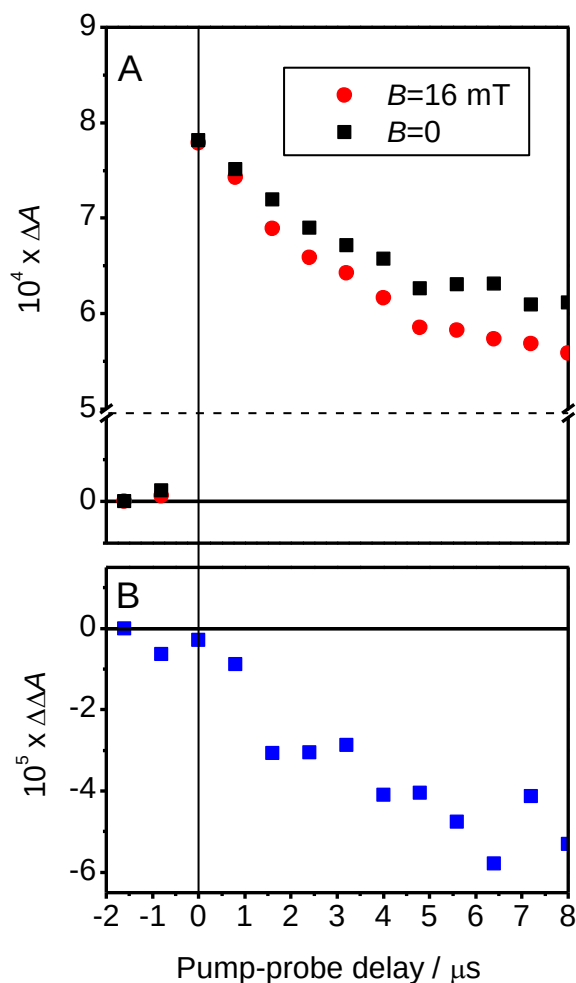


Figure 7.14 (A) Decay-time profiles for EcPL, in the presence and absence of a 16 mT applied magnetic field as measured by CRDS (1 mJ pump pulse energy, 1 mm sample optical path length). Note the break in the vertical axis in (A). (B) MFE time profile, $\Delta\Delta A(B_0 = 16 \text{ mT}, t_d)$, determined from the decay-time graph in (A). All data show 50-shot averages to minimize photodegradation.

This is probably a direct result of the weak photoexcitation powers required using CRDS detection, again demonstrating the advantage of this technique over TA. The noise in $\Delta\Delta A$ shown in Figure 7.14(B), for a 100 s measurement, is on the order of 10^{-5} and is currently limited by scattering within the EcPL sample (due to the EcPL itself, and possibly bubbles within the glycerol). Thus, it is feasible to believe that by carefully adjusting the EcPL concentration and sample-cell path length, additional sensitivity gains may be achieved, permitting lower

photoexcitation powers and/or measurement repetition-rates to be employed, thereby further reducing the risk of sample photodegradation.

7.4 Conclusions and Outlook

In summary, a pump–probe variant of CRDS allows the time-resolved study of photo-induced reactions and their MFEs in solution, with a detection sensitivity on the order of 10^{-6} , around two orders of magnitude smaller than that achieved with conventional TA. The sensitivity gains in CRDS allow a reduction in the probed sample volume (down to 4 μL —around an order of magnitude lower than in TA), facilitating low-volume biological-sample experiments. The significant advantages of CRDS have been demonstrated in proof-of-principle studies, using AQDS- and FMN-based systems and have been successfully applied to study MFEs in EcPL, a biomolecule with a close similarity to cryptochrome.

The pump-probe variant of CRDS is complementary to other optical approaches for studying MFEs, including modulated CEAS (described in Chapter 6) and a BBCEAS variant—using a supercontinuum light source to monitor radical absorption and MFEs over wide wavelength ranges—described in Chapter 8.

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Chapter 8

BBCEAS MFE studies

This chapter describes initial proof-of-principle experiments using SC-BBCEAS to monitor photoinduced reactions and their MFEs, in solution, over wide wavelength ranges (hundreds of nanometers). First, by way of proof-of-principle, the FMN, HEWL system described previously in Section 7.2.2.1 is investigated and used to optimize the technique's capabilities. The applicability of this approach to *in vitro* MFE studies of *Escherichia coli* photolyase (EcPL) and *Drosophila melanogaster* cryptochrome (DmCry) is then demonstrated. The results of the DmCry experiment mark the first *in vitro* observation of a magnetic field response in an animal cryptochrome, a key result supporting the hypothesis that cryptochromes are involved in the magnetic sense in animals. This chapter concludes with a discussion of ongoing and future BBCEAS experiments used to monitor radical pair reactions and associated magnetic field effects .

8.1 Introduction

The cavity-based MFE studies described in Chapters 6 and 7 demonstrate the powerful capabilities of optical cavity-enhanced techniques, such as CRDS and CEAS, in monitoring radical recombination reactions and associated MFEs. However, the techniques discussed previously are limited to spectral regions in which suitable light sources are available and where there is negligible overlap between the absorption bands of the radical and precursor species. Furthermore, the techniques described in Chapters 6 and 7 do not permit the acquisition of optical absorption spectra as a function of wavelength and in this context may be

considered somewhat one-dimensional. Thus, the number of systems which might benefit from the sensitivity gains demonstrated in Chapters 6 and 7 may be rather restricted. This chapter illustrates how these problems may be circumvented by the application of broadband variants of CEAS (see Section 1.2.3) utilizing, for example, white light SC sources and broadband mirrors to dramatically increase the number of magneto-sensitive systems benefiting from the application of cavity-based techniques.

8.2 Experimental

8.2.1 Materials and Photochemical Systems

8.2.1.1 FMN and EcPL systems

The materials and solution concentrations used were the same as those described in Chapter 7: FMN, HEWL experiments used 10 μM FMN & 0.5 mM HEWL solutions, whilst the EcPL sample was diluted using a 50/50 (vol/vol) glycerol/water mixture, to give a concentration *ca.* 60 μM . The photochemical reaction schemes for both FMN-HEWL and EcPL systems are described in Sections 7.2.2.1 and 7.2.2.2 respectively.

8.2.1.2 DmCry system

Drosophila melanogaster (insect) cryptochrome (in HEPES buffer, pH 7) was provided by Stefan Weber and co-workers (University of Freiburg). Unlike EcPL—which uses a pteridine derived co-factor (e.g. MTHF) as an antenna, sensitizing the flavin cofactor to blue light—chemical, spectral and crystallographic analyses by Zoltowski *et al.* find no evidence for DmCry binding MTHF, or, indeed, any cofactor other than FAD.¹ From a UV/Vis measurement of the DmCry sample absorbance,² and the known FAD extinction coefficient (Section 7.2.2.2), the DmCry concentration was estimated as *ca.* 30 μM . For BBCEAS, this sample was diluted by a factor of 5, using a 50/50 (vol/vol) glycerol/water mixture.

The cryptochrome hypothesis for magnetoreception and evidence in support of this hypothesis has already been outlined in Section 5.6.1.1. A previous behavioral study has demonstrated that DmCry is required for magnetosensitive responses in *Drosophila melanogaster*.^{3,4} The study, by Gegebar *et al.*,³ used a binary-choice behavioral assay for magnetosensitivity, to show significant responses of wild-type flies to a magnetic field ($B_0 < 0.5$ mT) under full-spectrum light (~ 300 - 700 nm, in both a naïve state and after training, by pairing the magnetic field with a sucrose reward), and a lack of response when light in cryptochrome-sensitive spectral regions ($\lambda < 420$ nm) was blocked. Furthermore, under full-spectrum light, cryptochrome-deficient flies exhibited no response to the magnetic field.

Despite the genetic and behavioral evidence for cryptochrome-based magnetosensitivity in flies, described above, very little is known about the photochemical reaction scheme of DmCry, or its biophysical mechanisms. Crucially, there exist no previous *in vitro* observations of a magnetic field response in DmCry (or any other animal cryptochrome).

In previous studies, it has been established that irradiation of DmCry with blue light results in conversion of the internally bound, fully oxidised FAD (see also Section 5.6.1.1) to the anionic semiquinone radical, $\text{FAD}^{\bullet-}$, which subsequently decays back to fully oxidised FAD in an oxygen-dependent manner.^{1,5-8} However, whether $\text{FAD}^{\bullet-}$ represents the signaling state, or is just a short-lived intermediate with insignificant functionality, is currently under debate.⁷⁻⁹

Until very recently, the only member of the cryptochrome/photolyase family known to show a magnetic response was EcPL.¹⁰ However, very recently, Maeda *et al.* reported the observation of an MFE in *Arabidopsis thaliana* (plant) cryptochrome (AtCry) and proposed a photochemical reaction scheme (similar to that for EcPL) for the protein.¹¹ Given that EcPL and AtCry have very different biological functions (DNA repair and entraining circadian rhythms, respectively), Maeda *et al.*¹¹ suggest that magnetosensitivity is a general feature of the

cryptochrome/photolyase family and could, therefore, be present in DmCry, as well as avian- and even human-cryptochromes.

8.2.2 Experimental setup and operation

The experimental apparatus is shown schematically in Figure 8.1 and comprised four main components: (i) an optical cavity, (ii) an SC source, spectrograph and CCD for radical detection by direct absorbance, (iii) a 405 nm excitation source for radical generation and (iv) a Helmholtz coil arrangement for applying magnetic fields.

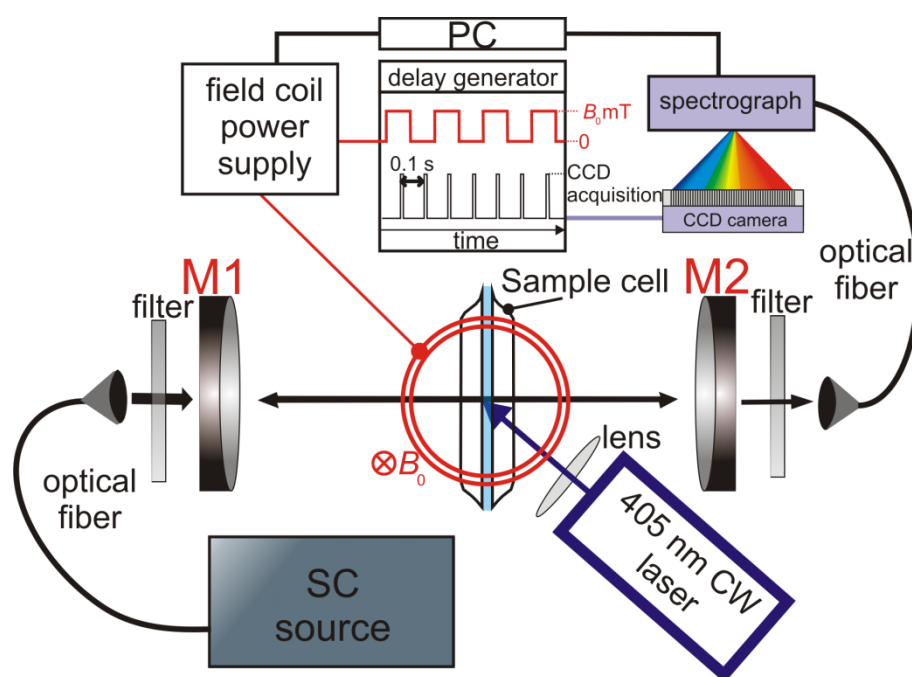


Figure 8.1 Schematic of the experimental apparatus for BBCEAS. Helmholtz coils, indicated by concentric circles, generate a pulsed magnetic field, B_0 , at the sample cell.

A simple linear optical cavity was formed using two highly reflective mirrors, M1 and M2 (mirror set 2, see Figure 2.3(b), radius of curvature = 1 m) mounted 20 cm apart. The mirrors surrounded an intracavity sample cell—Starna 45/Q (See Figure 6.2, inset), or Hellma 165-1.0-40 (see Figure 7.4), both with a sample path length $l = 1$ mm—located at the centre of the cavity and oriented with its windows normal to the optical cavity axis by mounting it on a goniometer-rotation stage arrangement.

The cavity light source was an SC radiation source (NKT, Koheras SuperK Power Blue, see Table 2.2). Light from the SC source was spectrally filtered, before entering the cavity, to yield a bandwidth 425-800 nm (see also Section 2.3). Light exiting the cavity was focused (using an achromatic doublet pair) into an optical fiber (Thorlabs, 400 μ m core, NA 0.39), coupled to a grating spectrograph (Andor Technology, SR-303i) that dispersed its component wavelengths onto a CCD camera (Andor Technology, Newton, see also Section 2.5). All spectra were acquired at a repetition rate of 10 Hz, with a 40 ms acquisition time, which was chosen on the basis of Allan deviation calculations described in Section 8.2.2.1.1. Each acquisition was performed using full vertical (spatial) binning of the charge on the CCD array before readout.

A CW diode laser (Power Technology 1Q1H350, 405 nm, see Section 2.2) was used as the photoexcitation source and was focused (through a 1 m focal-length lens) into the sample cell as shown in Figure 8.1. Various laser powers, specified for individual experiments, were used.

The intracavity sample cell was positioned between a pair of Helmholtz coils, used to generate a magnetic field. To monitor magnetic field effects, the light intensity exiting the cavity was recorded whilst the sample was simultaneously subjected to continuous photoexcitation at 405 nm and a pulsed magnetic field: the power supply of the Helmholtz coils was controlled by an external pulse generator (TTi, TGP 110), so that the field at the sample stepped between 0 and B_0 mT (specified for individual experiments) at a rate of 10 Hz, with a 50 % duty cycle. The pulse generator was also used to delay the field pulse with respect to CCD acquisition, so that CCD acquisitions alternated between measurements of light intensity in the absence ($I_{B_0=0}$) and presence (I_{B_0}) of the B_0 mT field.

8.2.2.1 FMN, HEWL experiments

At the beginning of each FMN, HEWL experiment, the BBCEAS light intensity spectrum was recorded, first with water ($I_0(\lambda)$) and then with FMN and HEWL ($I_{GS}(\lambda)$) in the intracavity cell

(Starna 45/Q). The light intensity was then recorded during FMN, HEWL photoexcitation (at 405 nm), to give $I_{B_0=0}(\lambda)$.

MFEs were monitored using the method described in Section 8.2.2, with $B_0 = 14.4$ mT (generated by the Helmholtz coils pictured in Figure 6.2).

For all measurements the FMN, HEWL sample flows (to waste) through the cell at a rate of 0.25 mL min⁻¹, chosen using the Allan deviation measurements described in the next section. The flow rate is maintained by means of a syringe pump (Chemyx, Fusion 200).

8.2.2.1.1 Allan deviation experiments

During preliminary FMN, HEWL experiments it rapidly became clear that sample flow was required to prevent photodegradation by the 405 nm laser. However, it was equally clear that this flow introduced additional noise to the measurements (possibly through turbulent flow in the cell itself). Therefore, Allan deviation measurements were performed to ascertain how the instrument sensitivity could be optimized during sample flow.

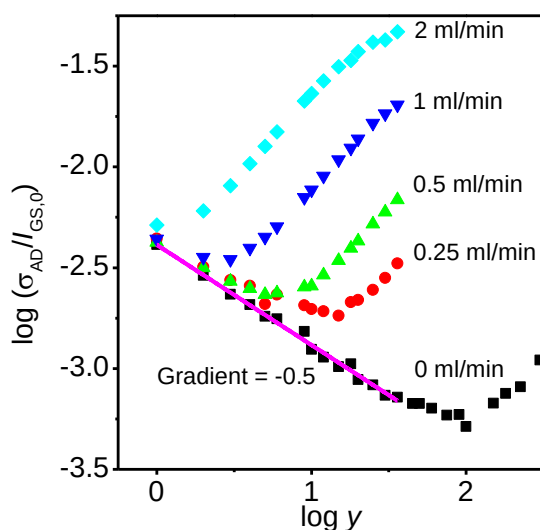


Figure 8.2 Instrument sensitivity (displayed as $\log \frac{\sigma_{AD}}{I_{GS,0}}$) versus measurement averaging number (displayed as $\log_{10} y$), for various sample flow rates. $I_{GS,0}$ is the average of 100 I_{GS} measurements recorded before the main measurement series used to calculate σ_{AD} . Solid magenta line: linear fit of the Allan deviation data between $\log y = 0$ and 1.5

The Allan deviation was calculated using the approach described previously in Section 2.7.1: a consecutive series of ($N = 4500$) individual 3.66 ms I_{GS} spectral acquisitions were processed to form M subgroups containing y individual measurements (so that $M = N/y$). $\sigma_{AD}(y)$, at $\lambda =$

600 nm, close to the absorption maximum of the radicals to be detected, was then calculated using Equation 2.3, and used to generate Figure 8.2.

For a static sample (0 mL/min flow), the Allan deviation plot shown in Figure 8.2 agrees with the results described in Chapter 2; both studies indicate that the instrument sensitivity improves with signal averaging up to $y \approx 100$ (i.e. an effective integration time at the CCD of *ca.* 366 ms). During preliminary experiments it became obvious that continuous photoexcitation of a static FMN sample resulted in a slow, yet significant deterioration in the cavity's transmitted light intensity, indicating a reduction in the CEF. The rate of this deterioration was particularly noticeable at higher photoexcitation powers. Additionally, following deterioration, the light intensity did not recover when the photoexcitation was turned off (in this case recovery of light intensity could only be achieved by cleaning the cell, using, for example, a Hellmanex II surfactant solution). This behavior suggests the formation of a persistent photoproduct on the inside of the cell windows. Therefore, sample flow during experiments was deemed necessary.

However, as shown in Figure 8.2, sample flow has a significant, detrimental influence on instrument sensitivity. As the sample flow is increased, the Allan deviation departs more rapidly from the white noise ideal, moving into a drift dominated regime at shorter and shorter effective integration times. At a flow rate of 0.25 mL/min the instrument enters a drift dominated regime at $y \approx 15$ (*ca.* 55 ms). This timescale is much faster than that on which the probed solution volume is refreshed; from calculations using the cell's geometry, a 0.25 mL/min flow is expected to be laminar and to fully refresh the solution within the beam path in *ca.* 1 minute. One potential source of long term drift could be unexpected turbulent flow within the cell, which is not designed for this purpose, however this needs to be investigated further. It was felt that the noise levels observed were satisfactory for these initial experiments and this was deferred.

As a result of the study described above, all subsequent FMN, HEWL experiments were performed using CCD acquisition times of 40 ms and a 0.25 mL/min flow rate, which was chosen as a compromise, to try and minimize both sample photodegradation (which became noticeable at slower flow rates) and flow-rate-dependent long-term drift. It should, however, be noted that the effect of flow rate on photodegradation was only estimated in this case and a more rigorous optimization of the sample flow rate (for individual photoexcitation powers) would probably result in better instrument sensitivity (by allowing longer signal averaging).

The repetition rate for spectral acquisition (10 Hz) was deliberately chosen to ensure that the field had sufficient time to ramp up or down, as appropriate, before each (40 ms) CCD measurement, as well as to ensure that the photochemical system had time to respond to the change in B_0 . However, more recent experiments, using a time-resolved Gaussmeter, suggest that the 10 Hz acquisition rate may be excessively slow and could potentially be increased to benefit the instrument sensitivity and improve data acquisition rates.¹²

8.2.2.2 EcPL and DmCry experiments

At the beginning of each EcPL (or DmCry) experiment, the BBCEAS light intensity spectrum was recorded with the EcPL (or DmCry) sample in the intracavity cell (Hellma 165-1.0-40, Figure 7.4), giving $I_{GS}(\lambda)$. The Hellma cell is specifically designed for temperature control measurements (see also Section 7.2.3) and all EcPL and DmCry measurements were made on (static, *i.e.*, un-flowed) samples cooled to *ca.* -5 °C. Following $I_{GS}(\lambda)$ measurement, the light intensity was then recorded during EcPL (or DmCry) photoexcitation at 405 nm (powers specified later, for individual experiments), to give $I_{B_0=0}(\lambda)$.

MFEs were monitored using the method described in Section 8.2.2, with $B_0 = 25$ mT. The field was generated using a pair of water-cooled Helmholtz coils (80 mm diameter, see Figure 8.3) that had been specially constructed to accommodate the Hellma cell, which would not fit between the coils used for the room temperature FMN, HEWL measurements (Section 8.2.2.1).

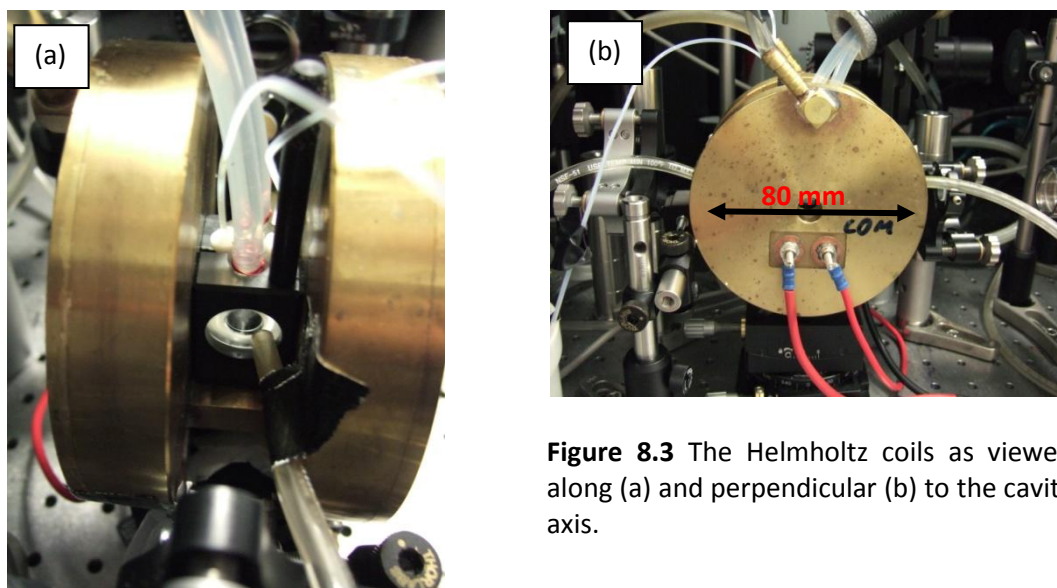


Figure 8.3 The Helmholtz coils as viewed along (a) and perpendicular (b) to the cavity axis.

Due to the low sample volumes of the proteins available (the total volume of sample was *ca.* 200 μL), sample flow was not practicable. Therefore, the sample was periodically agitated using a pipette, to encourage reoxidation of the flavin cofactor by dissolved dioxygen. Occasionally the sample was also centrifuged to remove small amounts of precipitate that formed during the experiment (probably due to sample photodegradation).

For these initial proof-of-principle experiments, no optimization of the CCD acquisition time/rate was performed for EcPL or DmCry, the sample being considered too precious to “waste” on such studies. All spectra were thus acquired using the same CCD settings as for the FMN, HEWL experiments (described in Section 8.2.2.1), at 10 Hz, with an integration time of 40 ms.

8.3 Results and discussion

8.3.1 FMN, HEWL

Figure 8.4(a) shows the raw BBCEAS spectra ($\propto A(\lambda)$, see Equation 1.23) of an FMN, HEWL sample recorded in the absence, $\frac{I_0(\lambda)}{I_{GS}(\lambda)} - 1$, and presence, $\frac{I_0(\lambda)}{I_{B_0=0}(\lambda)} - 1$, of photoexcitation at 405 nm. The difference spectrum, $\Delta A_r(\lambda) \left(= \frac{I_0(\lambda)}{I_{B_0=0}(\lambda)} - \frac{I_0(\lambda)}{I_{GS}(\lambda)} \right)$, is shown in Figure 8.4(b).

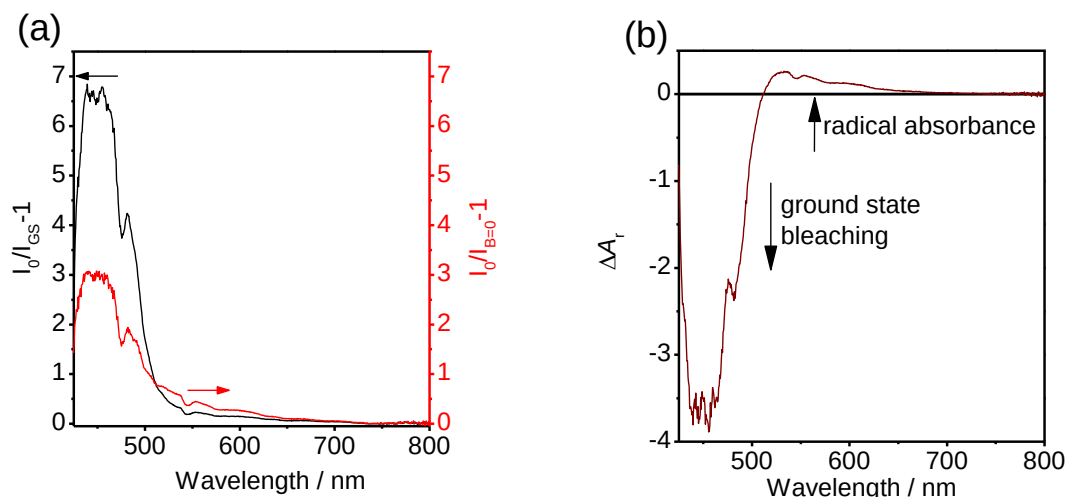


Figure 8.4 (a) BBCEAS spectra of 10 μM FMN, 0.5 mM HEWL (average of 100 individual spectra), in the absence (black line) and presence (red line) of 405 nm photoexcitation (249 mW laser power). (b) difference spectrum, $\Delta A_r(\lambda)$, calculated using the data in (a). Ground state bleaching and radical absorbance contributions to the spectrum are indicated.

The results of these FMN, HEWL measurements are discussed later, in Section 8.3.1.2. However, it is informative to discuss first the general format in which the results are presented. The majority of BBCEAS spectra in this chapter are displayed in the same ‘raw’ state as the results shown in Figure 8.4. As a result, each spectrum provides only a relative (as opposed to absolute) measure of absorbance as a function of wavelength. Additionally, due to the significant amount of structure present in the cavity mirrors’ reflectivity profile (Figure 2.3(b)), the spectra also exhibit some false structure. The use of relative absorbance (raw BBCEAS) spectra is sufficient for the purposes of the preliminary studies described in this chapter, and the presence of false structure can be tolerated. However, it is useful to illustrate how absolute absorbance spectra, $A(\lambda)$ and $\Delta A(\lambda)$, can be generated, from raw BBCEAS spectra, if desired.

8.3.1.1 Absolute absorbance spectra

As shown by Equation 1.23, absorbance spectra, $A(\lambda)$, can be calculated by multiplying raw BBCEAS spectra, $\frac{I_0(\lambda)}{I(\lambda)} - 1$, by $1 - R(\lambda)$ (the reciprocal of the CEF as a function of wavelength).

The discussion below provides an example of a calibration procedure that can be used to determine $CEF(\lambda)$ and thereby calculate BBCEAS-measured absorbance spectra.

Immediately before the FMN measurements described in Section 8.3.1, the raw BBCEAS spectrum of an aqueous, 45 μM K_2IrCl_6 solution (Ir(IV)) was recorded (see Figure 8.5(a)), which, when compared with a single-pass absorbance spectrum of Ir(IV) scaled for differences in concentration and per-pass optical path length (Figure 8.5(b)), yields $CEF(\lambda)$ *via*:

$$CEF(\lambda) = \frac{\frac{I_0(\lambda)}{I(\lambda)} - 1}{2.3026 A(\lambda) \text{ (single pass)}} \quad (8.1)$$

(see also Equation 1.23).

The CEF as a function of wavelength, determined using the above method, is shown in Figure 8.6. The variation in the CEF as a function of wavelength, which causes the false structure seen previously in Figure 8.4, is clearly observed in Figure 8.6. A precipitous drop in the CEF is observed at wavelengths to the blue and red of *ca.* 425 nm and 610 nm, respectively. In the blue, the drop is caused by a spurious drop in $\frac{I_0(\lambda)}{I(\lambda)} - 1$, as the light intensity transmitted through the cavity reduces beyond the CCD's dynamic range (see also Chapter 2). At red wavelengths, beyond *ca.* 610 nm, the weak Ir(IV) absorption makes CEF determination using the calibration method described above particularly challenging. From Figure 8.5(b) it appears there may be a small baseline offset present in the single pass spectrum, which has a dramatic affect on the calculated CEF at longer wavelengths (> 610 nm, where $\frac{I_0(\lambda)}{I(\lambda)} - 1$ becomes particularly small); a calibrant with significant absorption at longer wavelengths would be required to allow reliable CEF determination at $\lambda > 610$ nm. The results in Figures 8.5 and 8.6 also illustrate the importance of using calibrants with well known concentrations and absorption cross sections, as any error in calibration will add in quadrature to the error in the

light intensity measurements during BBCEAS and ultimately limit the instrument's sensitivity (see also Equation 3.3).

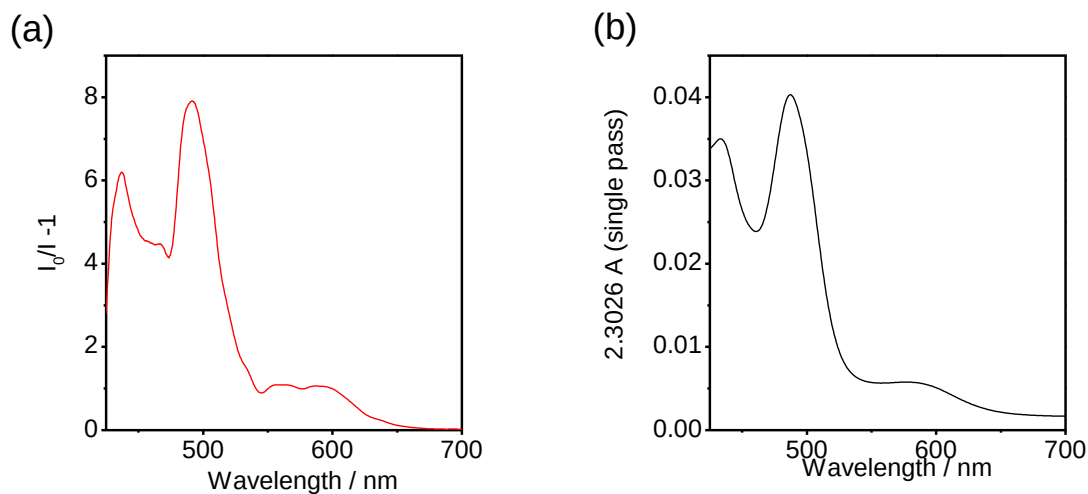


Figure 8.5 (a) BBCEAS spectrum of a 45 μM Ir(IV) solution (I_0 recorded with water only in the cell), smoothed using 10 point adjacent averaging. (b) UV/Vis (single pass) spectrum of a 2mM Ir(IV) solution, scaled for C and l used in BBCEAS.

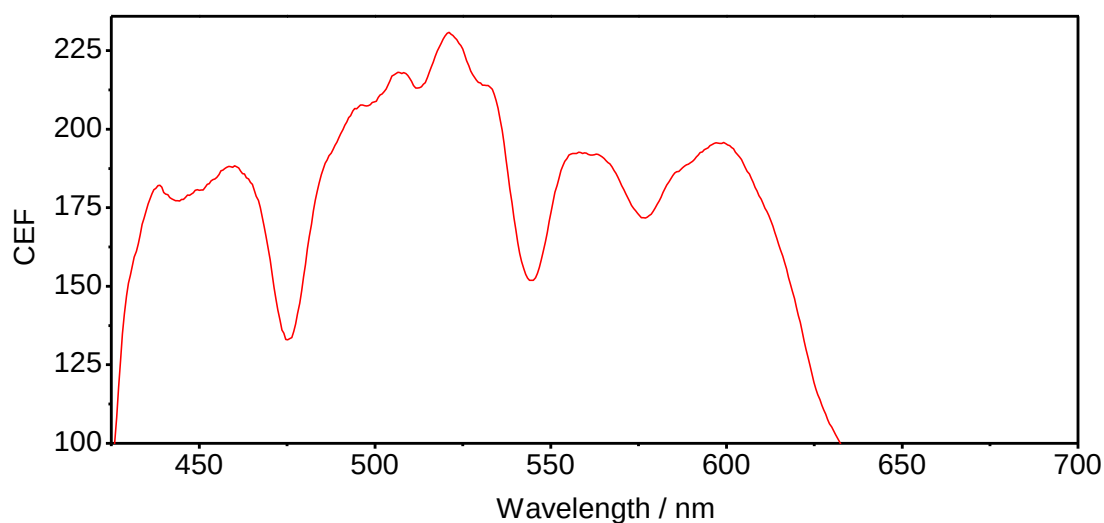


Figure 8.6 CEF as a function of wavelength, calculated using Equation 8.1 and the data shown in Figure 8.5, see text for details.

Having determined $CEF(\lambda)$ using the method described above, $A(\lambda)$ and $\Delta A(\lambda)$ for the FMN, HEWL sample in the absence and presence of photoexcitation at 405 nm can be generated—as shown in Figure 8.7—from the raw BBCEAS spectra in Figure 8.4 and using Equation 1.23. The features observed in Figures 8.7 and 8.4 are discussed in the next section.

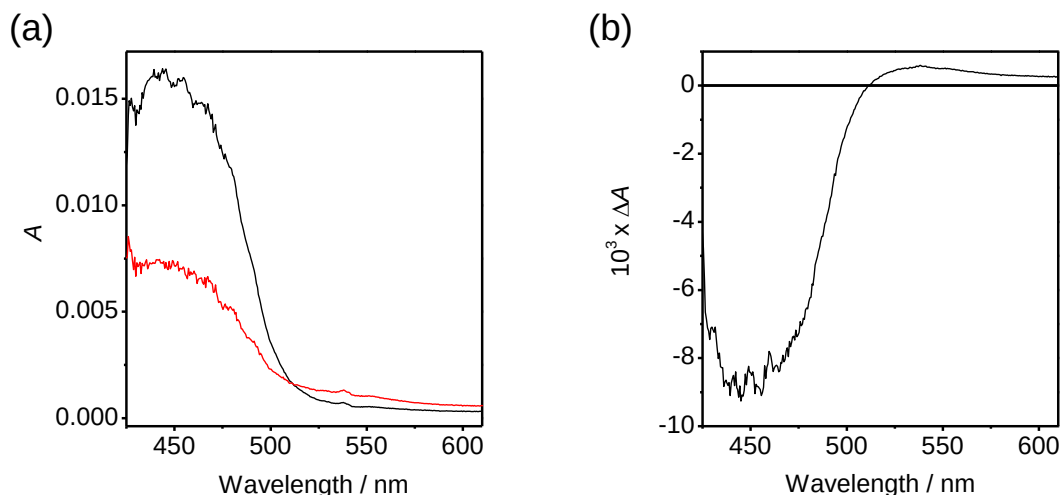


Figure 8.7 (a) A and (b) ΔA spectra, calculated from data shown in Figure 8.4, using Equation 1.23 and $CEF(\lambda)$ shown in Figure 8.6, see text for details. The false spectral structure (seen in Figure 8.4) is no longer apparent, as it is accounted for in the CEF calibration

8.3.1.2 FMN, HEWL photoexcitation—discussion

HEWL is weakly absorbing in the visible region¹³ and in the absence of photoexcitation, the main contribution to the spectrum in Figure 8.4 (and Figure 8.7) is from ground state FMN absorption (see the UV/Vis absorption spectrum of FMN-HEWL, shown in Figure 7.1).

Upon 405 nm photoexcitation, a large reduction in absorbance, attributed to FMN ground state bleaching, is observed in the spectral region 430–510 nm. Additionally, the difference spectrum, $\Delta A_r(\lambda)$, shown in Figure 8.4(b), as well as the $\Delta A(\lambda)$ spectrum in Figure 8.7(b), clearly show an *increased* absorbance at wavelengths longer than *ca.* 515 nm, which is attributed to flavin and tryptophan radical absorption. It seems likely that $FMNH^{\bullet}$, as well as $Trp(H^+)^{\bullet}$ radicals are observed, with broad maxima at *ca.* 525 nm and 600 nm, respectively: $FMNH^{\bullet}$ may be produced from $FMN^{\bullet}$ by proton-transfer from a water molecule.¹⁴⁻¹⁶

When differences in molar absorptivities are taken into account ($\epsilon_{FMN}(450\text{ nm}) \approx 10500\text{ M}^{-1}\text{ cm}^{-1}$,¹⁷ $\epsilon_{FMNH^{\bullet}}(584\text{ nm}) \approx 4700\text{ M}^{-1}\text{ cm}^{-1}$),¹⁸ the large difference between ΔA_r in the ground state (*ca.* 425 – 510 nm) and radical (above *ca.* 510 nm) absorption regions strongly suggests

that $\text{FMN}^{\bullet-}$ is not the exclusive product of ground state bleaching. Photoexcitation also appears to generate some other, unknown product(s) from ground state FMN: it might be that FMN is photolysed, producing lumichrome.^{19,20}

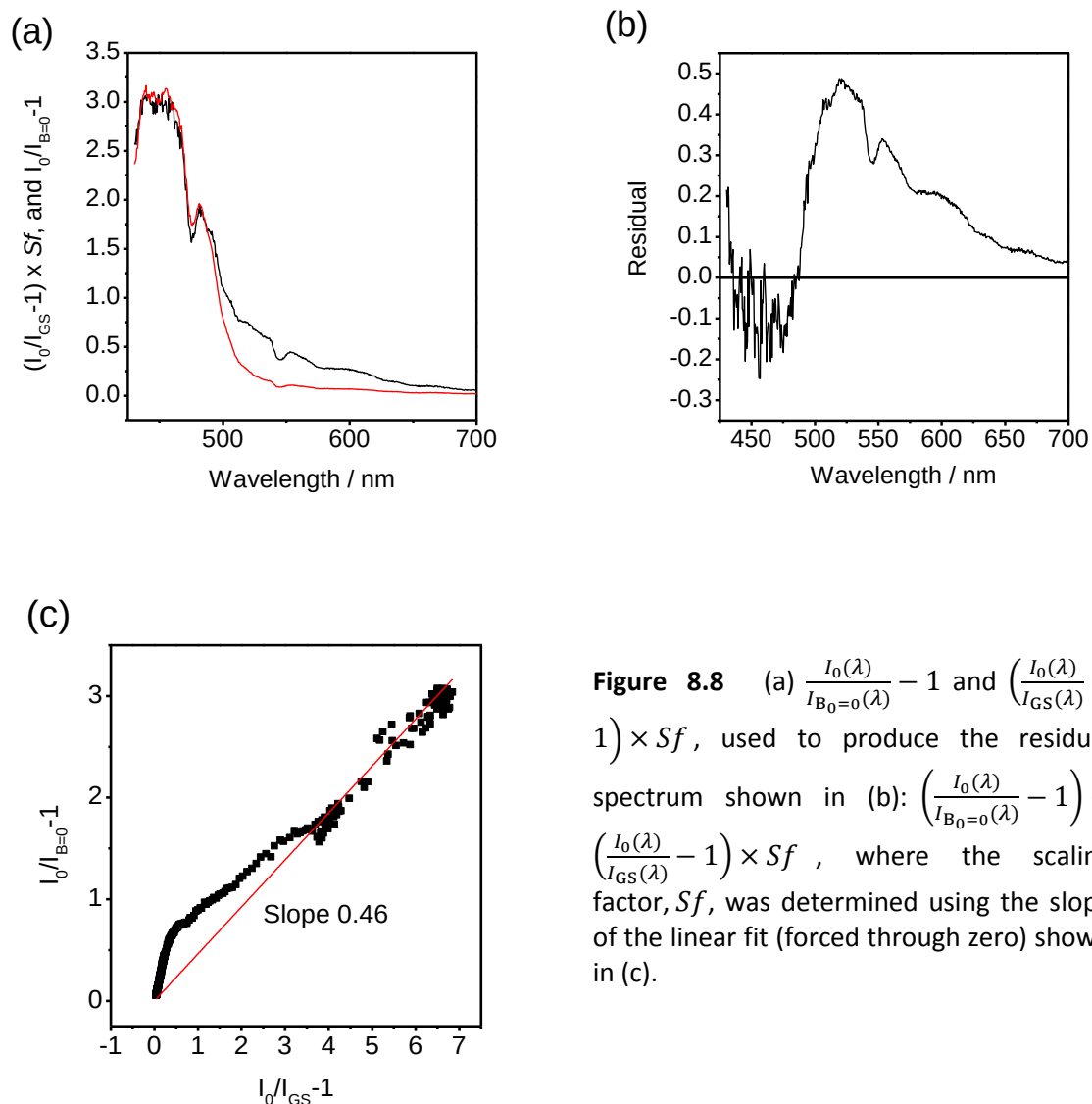


Figure 8.8 (a) $\frac{I_0(\lambda)}{I_{B=0}(\lambda)} - 1$ and $\left(\frac{I_0(\lambda)}{I_{GS}(\lambda)} - 1\right) \times Sf$, used to produce the residual spectrum shown in (b): $\left(\frac{I_0(\lambda)}{I_{B=0}(\lambda)} - 1\right) - \left(\frac{I_0(\lambda)}{I_{GS}(\lambda)} - 1\right) \times Sf$, where the scaling factor, Sf , was determined using the slope of the linear fit (forced through zero) shown in (c).

An alternative approach to spectral analysis might be to remove the ground state bleaching contribution to the spectra, leaving only the radical absorbance profile. To this end, the BBCEAS measured radical spectrum can be observed in the residual: $\left(\frac{I_0(\lambda)}{I_{B=0}(\lambda)} - 1\right) - \left(\frac{I_0(\lambda)}{I_{GS}(\lambda)} - 1\right) \times Sf$, shown in Figure 8.8. Where Sf is a scaling factor (accounting for the reduction in ground state absorbance upon photoexcitation) determined from the linear fit

shown in Figure 8.8(c). As shown in Figure 8.9(a), the same procedure can be applied to the absolute absorbance spectra in Figure 8.7(a), to give the radical absorbance spectrum shown in Figure 8.9(b). The feature at around 532 nm is attributed to instability in the light source (when a relatively large period of time—ca. 2 minutes—was left between signal and reference measurements the spectrum of the SC source was typically seen to change at around 532 nm, causing artifacts in BBCEAS spectra such as that shown in Figure 8.9(b)).

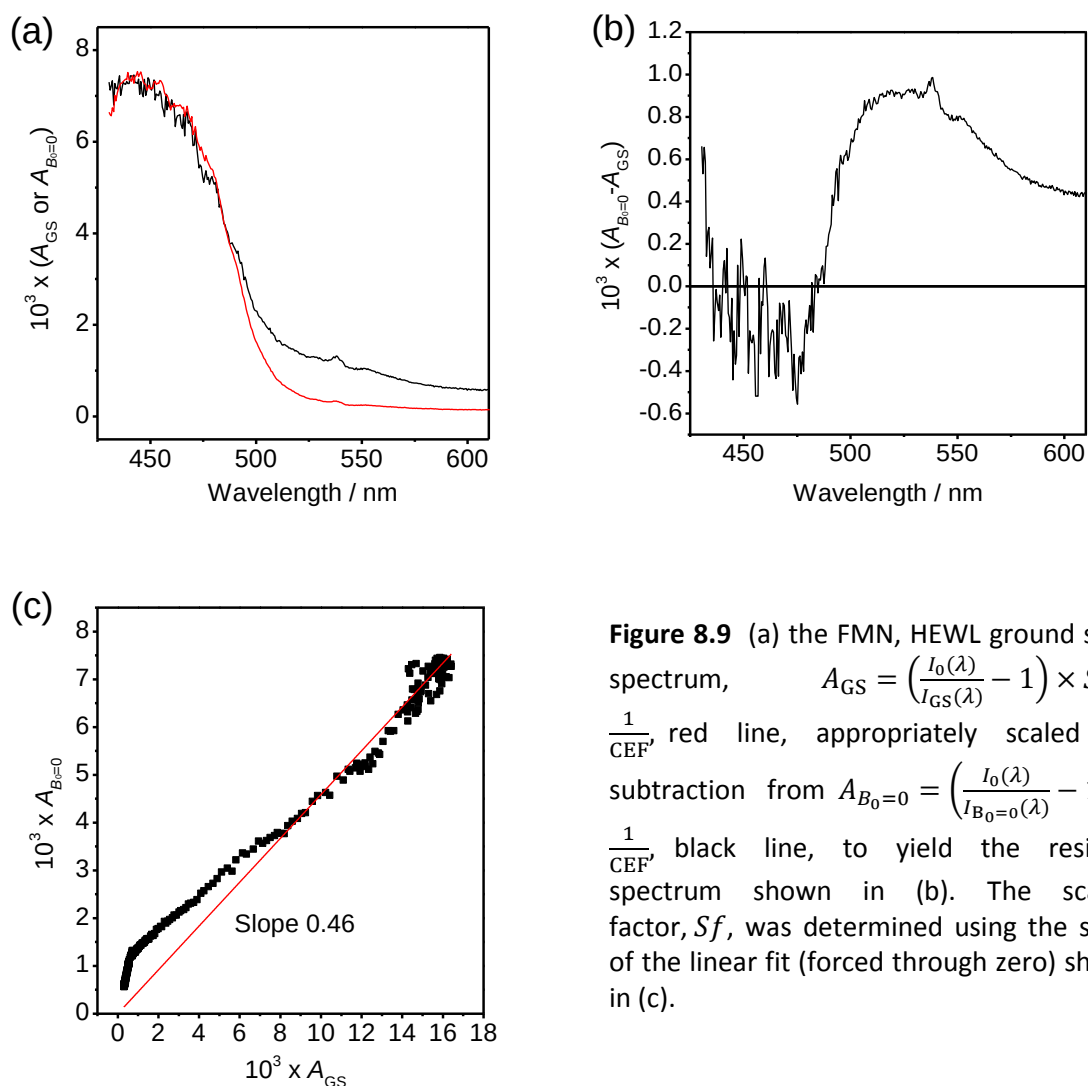


Figure 8.9 (a) the FMN, HEWL ground state spectrum, $A_{GS} = \left(\frac{I_0(\lambda)}{I_{GS}(\lambda)} - 1 \right) \times Sf \times \frac{1}{\text{CEF}}$, red line, appropriately scaled for subtraction from $A_{B=0} = \left(\frac{I_0(\lambda)}{I_{B=0}(\lambda)} - 1 \right) \times \frac{1}{\text{CEF}}$, black line, to yield the residual spectrum shown in (b). The scaling factor, Sf , was determined using the slope of the linear fit (forced through zero) shown in (c).

Having removed the ground state bleaching component, the individual contributions of FMNH[•] and Trp(H⁺)[•] radicals to the spectrum shown in Figure 8.9(b) could, in principle, be determined. However, this deconvolution of overlapping absorbance spectra is not attempted here, as it would require a well known absorption cross section for each of the absorbers (which was not

available in this case) and more confidence in the CEF calibration and absolute absorbance determination.

8.3.1.2.1 Varying the photoexcitation power

Figure 8.10 shows FMN, HEWL ΔA_r spectra acquired at various photoexcitation laser powers: the spectrum shown previously in Figure 8.4(b) is also included here (the 249 mW spectrum). As expected, ΔA_r increases with photoexcitation laser power in the region above *ca.* 515 nm and decreases in the region below this wavelength, due to the dominance of radical and ground state absorption in these respective regions (discussed in the Sections above).

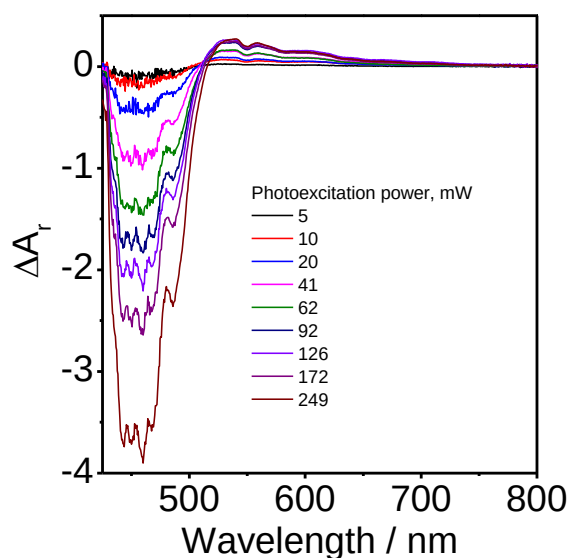


Figure 8.10 Difference spectra, $\Delta A_r(\lambda)$, (each an average of 100 individual spectra) acquired at various photoexcitation laser powers.

However, as demonstrated by Figure 8.11(a), the response of ΔA_r —particularly in the radical absorption region, *ca.* 515 nm—is non-linear with respect to photoexcitation power. The apparent onset of saturation might be due to rapid disproportionation of flavin radicals, which is known to generate fully oxidized (FMN_{ox}) and fully reduced (FMNH_2) species: $2\text{FMN}(\text{H})^\bullet \rightarrow \text{FMN}_{\text{ox}} + \text{FMNH}_2$. FMN_{ox} is simply the ground state FMN, but FMNH_2 has negligible absorption to the red of 500 nm.^{17,21} The response of ΔA_r at 536 nm, 600 nm and 650 nm is compared in

Figure 8.11(b), which suggests that all spectral features grow in at the same rate with laser power. The apparent onset of saturation should be investigated further. To this end, the SNR in the data must first be improved, possibly by careful adjustment of the solution flow rate and CCD acquisition times (see also the caption of Figure 8.11).

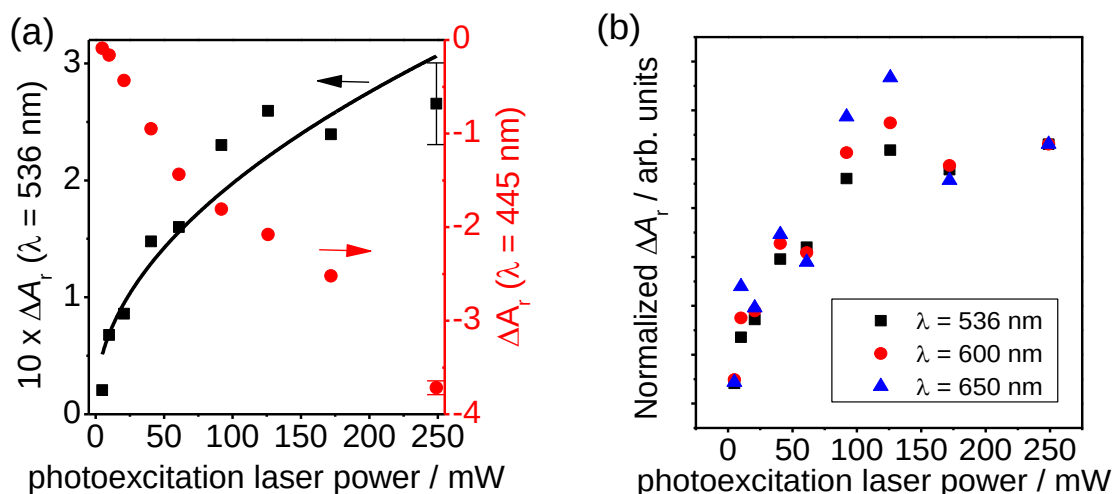


Figure 8.11 (a) ΔA_T at 536 nm (black squares) and 445 nm (red circles), as a function of photoexcitation laser power. Each data point is determined from the average of one hundred spectral acquisitions, the standard deviation of which is displayed for a single data point as a representative uncertainty. The black line is included as a guide for the eye. (b) ΔA_T versus photoexcitation power, measured at 536 nm, 600 nm and 650 nm (each data point is determined from the average of one hundred spectral acquisitions and normalized, using ΔA_T at 250 mW). N.B the relatively large total acquisition times (4 seconds per data point)) used here are not beneficial to instrument sensitivity (see Section 8.2.2.1.1) and it is likely that the uncertainty in ΔA_T could be reduced by using shorter acquisition times to minimize the effect of long-term drift on measurement sensitivity.

8.3.1.2.2 Aside: photoexcitation of an FMN only solution

Interestingly, when FMN is excited in the absence of HEWL, a positive ΔA_T signal is still observed at around 520 nm (See Figure 8.12). However, in this case the absorption band is much narrower than that observed in the presence of HEWL and is attributed to the anion and/or cation radical of the flavin semiquinone, which may be formed by the reaction of excited triplet state FMN with ground state FMN: ${}^3\text{FMN}^* + \text{FMN} \rightarrow \text{FMN}^{\cdot-} + \text{FMN}^{+\cdot}$.²²

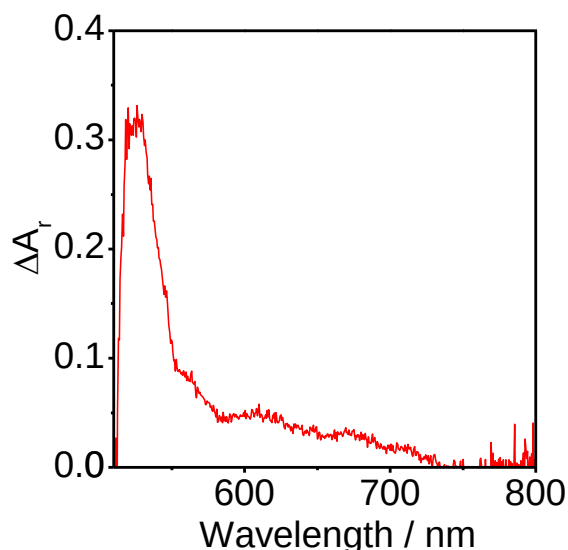


Figure 8.12. Normalized $\Delta A_r \left(= \frac{I_0}{I_{B_0=0}} - \frac{I_0}{I_{GS}} \right)$ spectrum (average of 100 individual spectra) of a 60 μM FMN-only sample (i.e., no HEWL present), where $I_{B_0=0}$ was recorded using a 405 nm photoexcitation laser power of 9 mW.

8.3.1.3 Magnetic field effects in FMN, HEWL

Figure 8.13(a) shows the FMN, HEWL ΔA_r spectrum (from 510-700 nm) generated upon 405 nm photoexcitation (laser power = 61 mW). Following this, the light intensity exiting the cavity was subsequently recorded during magnetic field pulsing (as described in Sections 8.2.2 and 8.2.2.1) and used to calculate the $\Delta\Delta A_r \left(= \frac{I_0}{I_{B_0}} - \frac{I_0}{I_{B_0=0}} \right)$ spectrum shown in Figure 8.13(b). The percentage MFE spectrum $\left(= 100 \times \frac{\Delta\Delta A_r(\lambda)}{\Delta A_r(\lambda)} \right)$ is shown in Figure 8.13(c). During the course of $\Delta\Delta A_r(\lambda)$ measurements, a small number of random scattering events caused large fluctuations in light intensity and, therefore, in the baseline of $\Delta\Delta A_r(\lambda)$. To militate against this effect of scattering, spectra with measurements of $\Delta\Delta A_r(\lambda)$ (at 600 nm) that fell outside three standard deviations from the mean were routinely removed before further data analysis (see Figure 8.14).

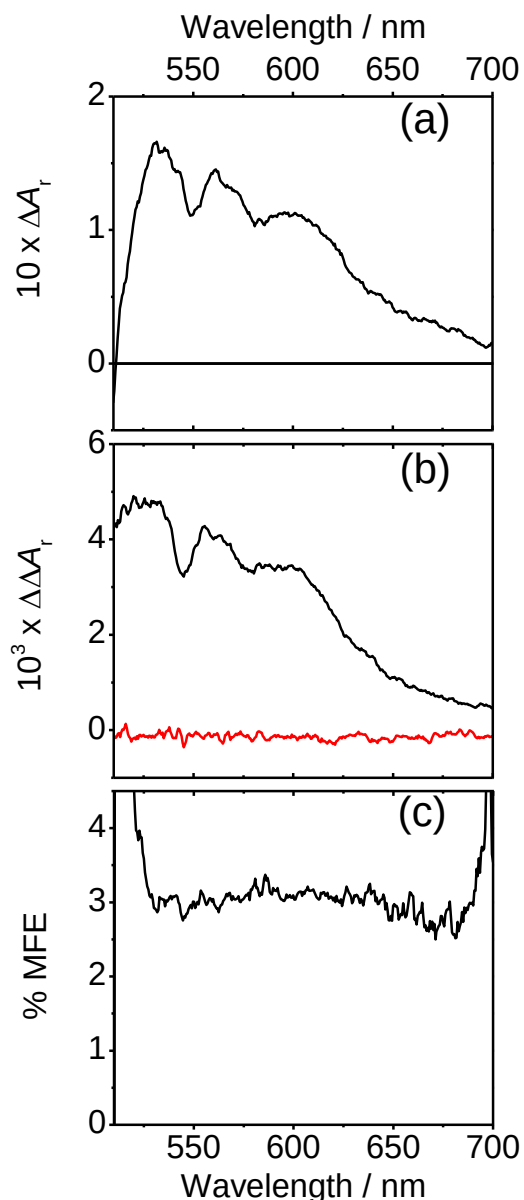


Figure 8.13 (a) the average of one hundred $\Delta A_r \left(= \frac{I_0}{I_{B_0=0}} - \frac{I_0}{I_{GS}} \right)$ spectra and (b) the average of one thousand $\Delta \Delta A_r \left(= \frac{I_0}{I_{B_0}} - \frac{I_0}{I_{B_0=0}} \right)$ spectra (black line). All I_{B_0} acquired with $B_0 = 14.4$ mT. All I_{B_0} and $I_{B_0=0}$ acquired during 61 mW (405 nm) photoexcitation. Each $\Delta \Delta A_r$ spectrum determined using a unique $I_{B_0}(\lambda)$ measurement and the average of the two surrounding $I_{B_0=0}(\lambda)$ measurements. Red line: $\Delta \Delta A_{r,0} \left(= \frac{I_0}{I_{B_0=0}} - \frac{I_0}{I_{B_0=0}} \right)$ spectrum, recorded with zero applied magnetic field throughout the experiment. Spectra with measurements of $\Delta \Delta A_r$ (and $\Delta \Delta A_{r,0}$) at 600 nm that fell outside three standard deviations from the mean have been excluded from the plot. (c) % MFE(λ) $\left(= 100 \times \frac{\Delta \Delta A_r(\lambda)}{\Delta A_r(\lambda)} \right)$. All lines smoothed with 5 point adjacent averaging.

As demonstrated by Figure 8.13(b), changes in $\Delta A_r(\lambda)$, due solely to the application of a (14.4 mT) magnetic field, are clearly visible using BBCEAS. As expected from the system's photochemistry, upon magnetic field application, a positive change in $\Delta A_r(\lambda)$ arises due to a decrease in S-T mixing efficiency and concomitant increase in the transient radical concentration (see also Chapter 7). The size of these changes ($\Delta \Delta A_r(\lambda)$ on the order of 10^{-3} , corresponding to absorbance changes on the order of 10^{-6}), are roughly an order of magnitude smaller than the uncertainty in $\Delta A_r(\lambda)$ measurements (exemplified by the error bars in Figure 8.11(a)): by repeatedly, rapidly and alternately acquiring light intensity spectra in the presence

and absence of the applied field, large gains in the $\Delta\Delta A_r(\lambda)$ SNR are achieved through signal averaging. As discussed in previous sections, it is important to note that the ΔA_r and $\Delta\Delta A_r$ spectra shown in Figure 8.13 exhibit some false structure, which must be removed (using CEF calibration) if any detailed spectral analysis is required.

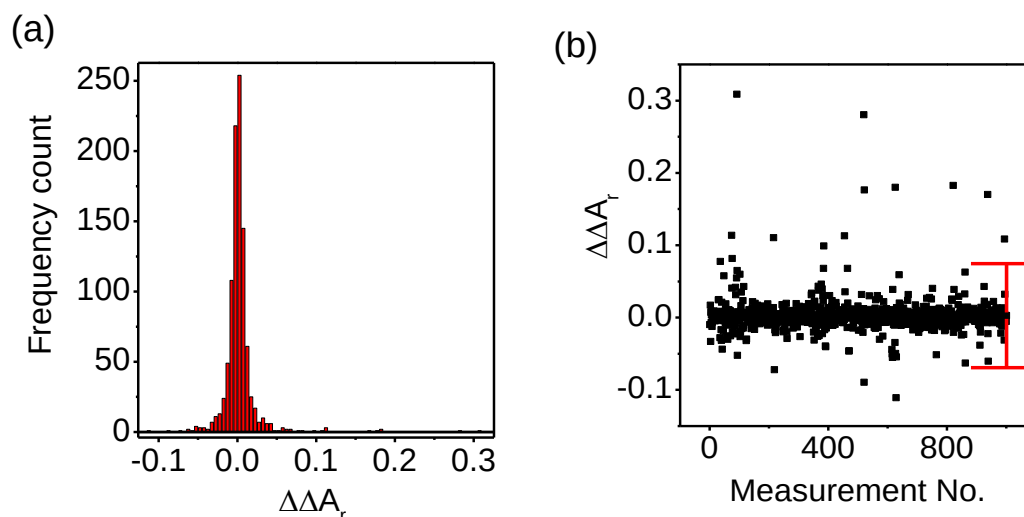


Figure 8.14 (a) Histogram showing a Gaussian distribution in the frequency count of $\Delta\Delta A_r$ (at 600 nm) during FMN, HEWL measurements (b) $\Delta\Delta A_r$ re-plotted as a function of measurement number: the small number of very large signals, which fall outside 3 standard deviations from the mean (red bars), are due to random scattering events, and should be removed.

As confirmed by the flat, featureless % MFE(λ) plot shown in Figure 8.13(c), the spectral shapes of $\Delta A_r(\lambda)$ and $\Delta\Delta A_r(\lambda)$ are very similar, indicating that the concentrations of all (FMN and Trp radical) species observed during photoexcitation are affected by the application of an external magnetic field. The percentage MFE in Figure 8.13(c) is gratifyingly close that shown previously in Figure 7.12, as determined by CRDS. The apparent increase in % MFE at wavelengths below *ca.* 525 nm appears to be a spurious effect caused by the unusually large ΔA_r values in this spectral region (possibly due to photodegradation, discussed above). Additionally, at wavelengths below *ca.* 500 nm (not shown) the $\Delta\Delta A_r$ (and % MFE) spectra become extremely noisy due to the high level of ground state FMN absorption, with the result that the cavity essentially appears black to the CCD in this region. Although the CCD could have been adjusted

to increase its sensitivity and detect light below ca. 500 nm, this would have resulted in its saturation at longer wavelengths. In future, this problem could be easily solved using suitable filters, to ensure light intensity across the entire visible region fell within the dynamic range of the CCD and could be monitored at all desired wavelengths simultaneously. Alternatively, the concentration of FMN, or the reflectivity of the cavity mirrors, could be reduced, allowing the cavity to transmit more light in the ground state absorption region: very recently, this approach has been used by Jing Li in the Mackenzie Group, to successfully observe a negative ΔA_T signal in the ground state spectral region.

8.3.2 EcPL

Figure 8.15(a) shows the BBCEAS spectrum acquired during illumination of the EcPL sample with 405 nm laser light. The sample's photoexcitation results in a broad positive absorption spectrum in the region 500 – 700 nm (peaking at around 530 nm). Figure 8.15(b) shows the change in the BBCEAS spectrum upon magnetic field application; its spectral pattern appears to mirror that shown in Figure 8.15(a) (when taking into account a possible baseline offset due to instrument instability—see Figure 8.15(b)). In contrast to the FMN, HEWL system discussed above, the radical pair in EcPL is born in the singlet state (see Section 7.2.2.2) and hence application of a magnetic field leads to an *increased* recombination efficiency and a net *decrease* in the transient radical concentration and absorbance above ca. 500 nm.

As in the FMN, HEWL studies described in Section 8.3.1, the dynamic range of the CCD did not permit simultaneous acquisition of spectral information across the full visible wavelength range; only the spectral region (above ca. 500 nm) with minimal contribution from ground state absorption is shown in Figure 8.15.

Despite the exclusion of spectra with measurements of $\frac{I_{GS}}{I_{B_0=0}} - \frac{I_{GS}}{I_{B_0=0}}$ that fell outside three standard deviations from the mean, the remaining spectra are still rather noisy (see error bar

in Figure 8.15(b)). This noise—attributed to bubbles in the glycerol—causes the $\frac{I_{GS}}{I_{B_0=0}} - \frac{I_{GS}}{I_{B_0=0}}$ spectrum in figure 8.15(b) to appear offset from zero. Further averaging of the spectra and allowing adequate time for the sample to settle once introduced into the cavity should help reduce this noise.

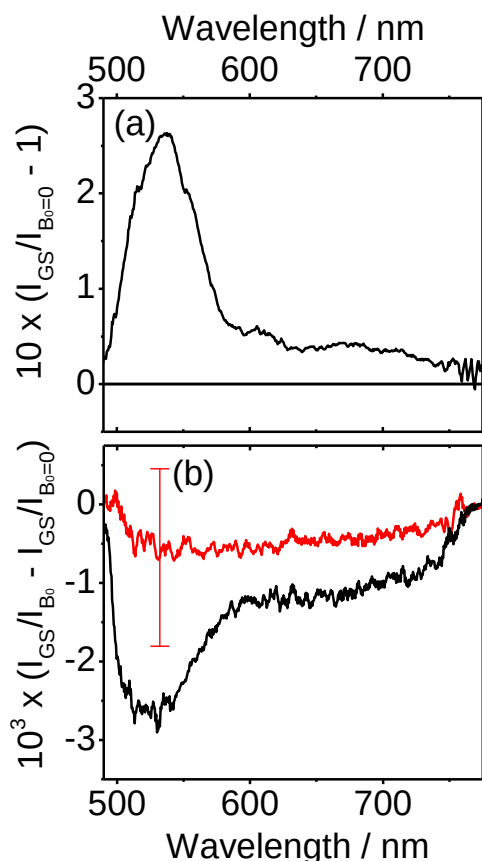


Figure 8.15 (a) the average of one hundred $\frac{I_{GS}}{I_{B_0=0}} - 1$ spectra and (b) the average of two thousand $\frac{I_{GS}}{I_{B_0}} - \frac{I_{GS}}{I_{B_0=0}}$ spectra (black line). All $I_{B_0}(\lambda)$ acquired in the presence of a 25 mT field. All $I_{B_0}(\lambda)$ and $I_{B_0=0}(\lambda)$ acquired during 0.6 mW (405 nm) photoexcitation. Each $\frac{I_{GS}}{I_{B_0}} - \frac{I_{GS}}{I_{B_0=0}}$ spectrum has been determined using a unique $I_{B_0}(\lambda)$ measurement and the average of the two surrounding $I_{B_0=0}(\lambda)$ measurements. Red line: $\frac{I_{GS}}{I_{B_0=0}} - \frac{I_{GS}}{I_{B_0=0}}$ spectrum, recorded with zero applied magnetic field throughout the experiment. Spectra with measurements of $\frac{I_{GS}}{I_{B_0}} - \frac{I_{GS}}{I_{B_0=0}}$ (and $\frac{I_{GS}}{I_{B_0=0}} - \frac{I_{GS}}{I_{B_0=0}}$) at 600 nm that fell outside three standard deviations from the mean have been excluded from the plot. All lines smoothed with 5 point adjacent averaging. Error bar represents three standard errors of the mean.

On the basis of previous (transient absorption) measurements^{10,23} (and as described in Section 7.3.4) the signals shown in Figure 8.15 are expected to contain contributions from $\text{FAD}^{\bullet-}$ and $\text{Trp}(\text{H}^+)^{\bullet}$ radical absorption (with broad maxima at *ca.* 500 nm and 600 nm, respectively). A small additional contribution from conversion of $\text{Trp}(\text{H}^+)^{\bullet}$ to $\text{Trp}^{\bullet}$ ($\lambda_{\text{max}} \approx 500$ nm) in the secondary radical pair is also likely (see Figure 7.2). However, as discussed below, in future a more rigorous treatment will be required to assign confidently the spectral features.

Unlike the FMN, HEWL spectra described in Section 8.3.1, which are generated using water reference measurements (I_0), the spectra shown in Figure 8.15 use a measurement of ground state EcPL (in the absence of 405 nm photoexcitation) as a reference (I_{GS} , see left hand axes in Figure 8.15), simply because water measurements were not possible at the sub-zero temperatures employed (see Section 8.2.2.2). However, this method must be applied with care, as the significant ground state absorption of EcPL (below *ca.* 500 nm, see Figure 7.2) acts to distort (lower) the effective mirror reflectivity (and the cavity enhancement factor) and, therefore, the spectral pattern of $\frac{I_{GS}}{I_{B_0=0}} - 1$ and $\frac{I_{GS}}{I_{B_0}} - 1$, at shorter wavelengths. This undesirable spectral distortion is minimal at the wavelengths shown in Figure 8.15 (> 500 nm), however, future experiments should be performed using a water glycerol reference, which (like water) has no significant absorption features within the wavelength range of interest, or, preferably, using a calibration to determine the CEF and, in turn, absolute ΔA and $\Delta\Delta A$ spectra.

8.3.3 DmCry

The BBCEAS spectrum of photoexcited DmCry is shown in Figure 8.16(a). The spectrum bears a close resemblance to that of EcPL. From Figure 8.16(b), a field effect is clearly observed in DmCry, manifest by a negative change in absorbance upon magnetic field application. Interestingly this negative change in absorbance, although considerably weaker, remains in the absence of 405 nm photoexcitation. This seems reasonable, given that blue (SC source) light within the cavity is estimated (from the SC power per nm, the cavity's finesse and free spectral range) to have a circulating power *ca.* 0.2 mW (one third of the 405 nm laser photoexcitation power). By spectrally filtering the SC source input (using a 518 nm long pass filter) to remove all blue light incident on the sample, the negative absorbance change was removed (magenta line, Figure 8.16(b)). Thus, in the case of DmCry, the SC source itself can be used simultaneously as an MFE detection- and (on-cavity-axis) photoexcitation-source. In future applications this may

have considerable advantages, as it ensures optimal overlap between excitation and probe beams.

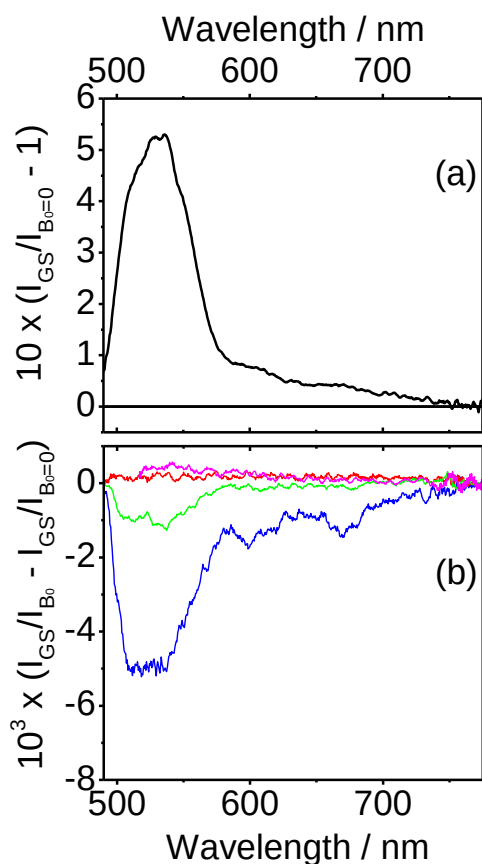


Figure 8.16 BBCEAS spectra demonstrating absorbance changes during DmCry photoexcitation and magnetic field application. (a) $\frac{I_{GS}}{I_{B_0=0}} - 1$ spectra, all $I_{B_0=0}(\lambda)$ recorded in the presence of 0.6 mW, 405 nm photoexcitation. (b) Blue line: the average of one thousand $\frac{I_{GS}}{I_{B_0}} - \frac{I_{GS}}{I_{B_0=0}}$ spectra, all $I_{B_0}(\lambda)$ and $I_{B_0=0}(\lambda)$ recorded in the presence of 0.6 mW, 405 nm photoexcitation. Green line: the average of two thousand $\frac{I_{GS}}{I_{B_0}} - \frac{I_{GS}}{I_{B_0=0}}$, all $I_{B_0}(\lambda)$ and $I_{B_0=0}(\lambda)$ recorded in the *absence* of 405 nm photoexcitation (on-axis photoexcitation from SC source only, see text). Red line: the average of three thousand $\frac{I_{GS}}{I_{B_0=0}} - \frac{I_{GS}}{I_{B_0=0}}$ spectra, all $I_{B_0}(\lambda)$ and $I_{B_0=0}(\lambda)$ recorded in the presence of 0.6 mW, 405 nm photoexcitation. Magenta line: the average of two thousand $\frac{I_{GS}}{I_{B_0}} - \frac{I_{GS}}{I_{B_0=0}}$ spectra. All $I_{B_0}(\lambda)$ and $I_{B_0=0}(\lambda)$ recorded in the absence of all blue photoexcitation (both 405 nm diode laser and blue SC light blocked). In all cases, each $\frac{I_{GS}}{I_{B_0}} - \frac{I_{GS}}{I_{B_0=0}}$ spectrum was determined using a unique $I_{B_0}(\lambda)$ measurement, made with $B_0 = 25$ mT, and the average of the two surrounding $I_{B_0=0}(\lambda)$ measurements. All lines smoothed with 7 point adjacent averaging.

The spectral pattern shown in Figure 8.16(b) mirrors that shown in Figure 8.16(a). These results, along with a comparison of those for EcPL described in Section 8.3.2, the existing knowledge of DmCry's photochemical reaction scheme⁸ and the results *Arabidopsis thaliana* cryptochrome studies by Maeda *et al.*,¹¹ strongly suggest that (as in EcPL) illumination of DmCry with blue light results in a photoinduced, singlet-born, radical pair, probably comprising $FAD^{\bullet-}$ and $Trp(H^+)^{\bullet}$ (see also Section 8.3.2), with an associated magnetic field effect. It is also likely that a secondary radical pair (probably comprising $FAD^{\bullet-}$ and $Trp^{\bullet}$) is also involved in the reaction scheme (see Section 8.3.2, Berndt *et al.*⁸ and Maeda *et al.*¹¹). These results mark the

first *in vitro* observation of a magnetic field response in an animal cryptochrome, a key result supporting the hypothesis that cryptochromes are involved in the magnetic sense in animals.

8.4 Conclusions and future direction

The work reported in this chapter demonstrates the first application of BBCEAS to the study of magnetic field effects in the condensed phase. The use of an SC source, in combination with a sensitive, fast CCD detector and field pulsing, has allowed the recording, with good signal-to-noise, of full visible spectra of RPs and their associated MFEs in three different systems. Furthermore, a magnetic field effect has been observed for the first time in an animal cryptochrome, DmCry—its system can now be added to the two other members of the photolyase-cryptochrome family (EcPL and *Arabidopsis thaliana* cryptochrome) known to exhibit field effects.

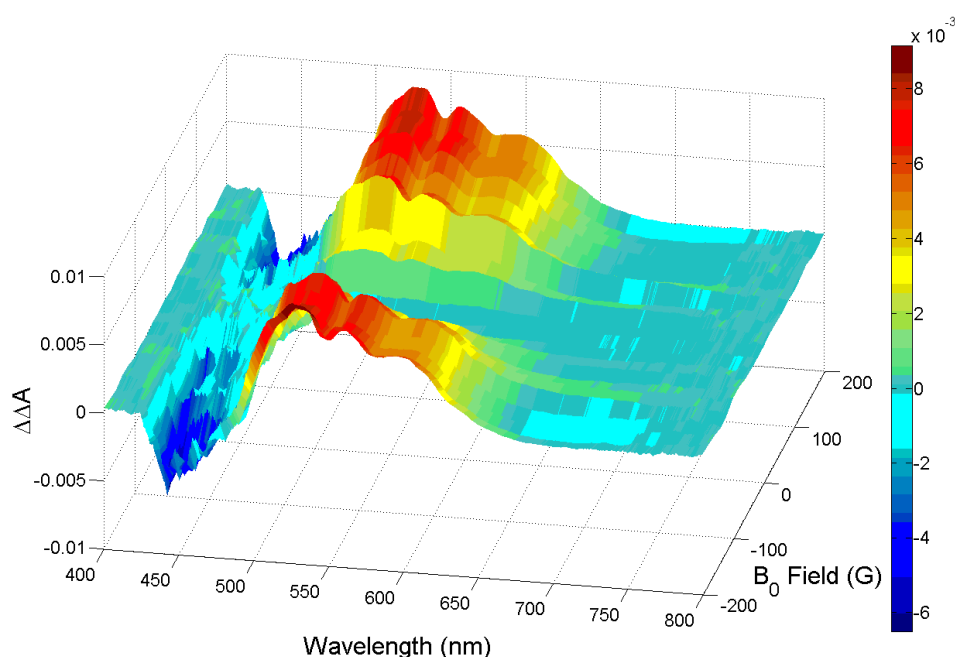


Figure 8.17 A surface plot, comprising multiple $\Delta\Delta A_r$ FMN, HEWL spectra acquired (by Dean Sheppard and Jonathan Storey) at multiple field strengths (between +200 and -200 Gauss (+20 to -20 mT))

By rapidly switching between measurements of light intensity in the absence ($I_{B_0=0}$) and presence (I_{B_0}) of a pulsed field, the BBCEAS instrument militates against the effects of long term drift, and absorbance changes on the order of *ca.* 10^{-6} can easily be detected. This sensitivity is at least as good as that achieved using the modulated CEAS and pump-probe CRDS variants described in Chapters 6 and 7, with the additional benefit of spectral resolution. However to take full advantage of the instrument's broadband capability, future experiments must fully address the issue of false structure in the BBCEAS spectra (*via* a rigorous calibration method, to determine $CEF(\lambda)$). Only then can a full analysis of the absorbance spectra be undertaken (including any deconvolution of overlapping absorbance spectra).

Building directly on the work reported here, the BBCEAS instrument has already been developed further and has now been fully automated (by Jonathan Storey, Timmel Group), to allow rapid acquisition of spectra at multiple field strengths, as shown in Figures 8.17 and 8.18. Cut-throughs in the field direction of such plots will ultimately provide further information on the photochemical system studied, which will be particularly useful in the case of cryptochromes.

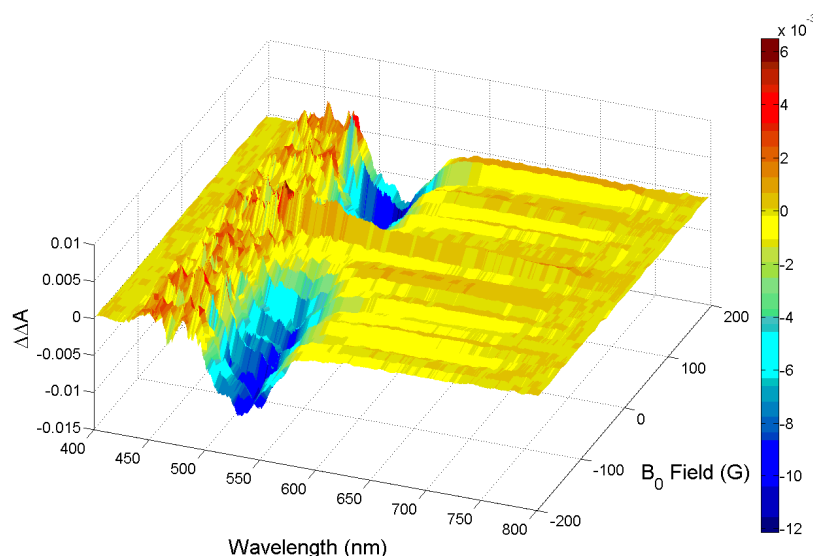


Figure 8.18 A surface plot, comprising multiple $\Delta\Delta A_r$ DmCry spectra acquired (by Dean Sheppard) at multiple field strengths (between +200 and -200 Gauss (+20 to -20 mT))

In the immediate future, BBCEAS will be used to search for a low field effect (see Section 5.4.1.4) in DmCry. An avian (garden warbler) cryptochrome may also be tested for the presence of a field effect, using BBCEAS. Another important future step will be to undertake an in-depth quantitative analysis of the BBCEAS results, in particular those of DmCry, to identify the individual absorbing species present and gain information on the photochemical reaction scheme. Some future BBCEAS instrument designs are also discussed in the Chapter 9.

8.5 Bibliography

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Chapter 9

Conclusions and future directions

This thesis has focused on the development of novel cavity-based spectroscopic techniques (namely CRDS, CEAS and BBCEAS) for the applications in the condensed-phase.

The basic principles of CRDS and (BB)CEAS have been outlined, and the previous applications of these techniques to condensed-phase systems discussed, in Chapter 1. Cavity-based spectroscopic techniques take advantage of the long optical path lengths achieved within optical resonators to achieve an enhancement in the sensitivity to sample absorption when compared with conventional single-pass absorbance measurements. Although condensed-phase variants of CRDS and (BB)CEAS must contend with significant background losses, a variety of novel cavity designs have been developed, which are typically capable of achieving high per-pass absorbance sensitivities that surpass those of single-pass techniques by 2-3 orders of magnitude (usually whilst probing smaller sample volumes).

As summarized in Chapter 2, CRDS, CEAS and (SC-based) BBCEAS instruments, employing various intracavity optical elements, have been designed and applied to study: processes within evanescent fields generated at solid|liquid and solid|air interfaces, microfluidic droplets, and magnetic field effects in the radical recombination reactions of chemical and biological systems. The use of SC sources in condensed-phase BBCEAS, demonstrated in this project, is now becoming increasingly popular. Some important considerations for SC-BBCEAS experiments are described in Chapter 2. In particular the SC source stability and its implications for instrument sensitivity have been explored using Allan deviation measurements. The results of these experiments illustrate the inherent instability of SC sources and demonstrate that, for the SC source tested, signal averaging above *ca.* 0.4 seconds is of no benefit to measurement

sensitivity. However, even when SC-based instruments are operated at the short timescales that are required for measurements to remain within a white-noise dominated regime, the high spectral irradiance of these light sources allows excellent SNRs to be achieved. Therefore, SC sources are particularly applicable to measurements which require high temporal resolution. Where short measurement times and high repetition rates are not required, LEDs should be considered as an alternative light source.

During the past decade, evanescent wave variants of CRDS and CEAS have been widely and successfully used to study condensed-phase species and interfacial phenomena. The underlying principles of EW generation (*via* TIR within an optical cavity) are introduced in Chapter 1 and expanded upon in Chapter 3. The inherent interfacial layer sensitivity of these techniques has been applied to various systems, using a variety of different cavity geometries (reviewed in Chapter 3). In this project, the comparatively new technique of EW-BBCEAS has been used to detect Ir(IV) complexes, electrogenerated in a thin-layer electrochemical cell. The use of an SC radiation source and sensitive CCD detector allowed EW-BBCEAS measurements with a high absorbance sensitivity ($A_{\min} = 3.9 \times 10^{-5}$, comparable to that of previous narrowband EW-CRDS/-CEAS studies), over a very wide spectral range (*ca.* 400–700 nm) and at repetition rates (>600 Hz) that are easily fast enough to follow solution kinetics and interfacial processes in real time.

The extension of EW cavity-based techniques beyond the silica|air interface has also been demonstrated for the first time, in this project. A Langmuir-Blodgett trough, modified to support an optical cavity and intracavity TIR at a water|air interface, has been used to demonstrate EW-CRDS, EW-CEAS and EW-BBCEAS detection of a Langmuir film's absorbance during its compression and expansion. τ_0 measurements suggest that these techniques enhance measurement sensitivity by a factor of *ca.* 100. However, a proper assessment of the techniques' sensitivity has not been performed here. It is clear that by reducing the per-pass

optical path length through the water subphase and by stabilising the instrument, to reduce vibrations in the water, the sensitivity could be significantly improved, however these challenging considerations will almost certainly require a full re-design of the Langmuir trough. Nevertheless, the optical cavity used here represents a significant step forward in the sensitive *in situ* absorbance detection of Langmuir films.

EW cavity-based techniques will undoubtedly find many more applications in the study of molecular adsorption; nanostructured materials; thin films and spectroelectrochemistry. One potential application, particularly pertinent to this project, would use an EW cavity to study MFEs in the radical recombination reactions of surface-immobilized proteins. This project has involved the development of highly sensitive cavity-based techniques for solution-phase MFE measurements (Chapters 6-8), which may ultimately aid our understanding of the avian magnetocompass (see Chapter 5). However, for an RP reaction to act as a chemical compass, it must not only respond to an applied field's intensity, but also to its direction. Any such anisotropic response is likely to be averaged out in the solution-phase, by rapid molecular tumbling. Therefore, in order to preserve anisotropic interactions, radicals must be (at least partially) immobilized and oriented. EW-cavity based techniques would appear to be a particularly appropriate method of monitoring MFEs in immobilized RP reactions. In this case, the elegant TIR-photoexcitation prism design shown in Figure 3.17 might be used. Alternatively, if sample cooling is required, a monolithic prism design would be particularly appropriate, for easy use within a cryostat.

SC-BBCEAS has also been used to record, in real time, the absorption spectrum of microlitre volume aqueous-phase slugs within a microfluidic chip assembly. In preliminary studies, presented in Chapter 4, the full visible spectrum ($430\text{ nm} < \lambda < 700\text{ nm}$) of each passing slug was acquired *in situ* at high repetition rates (273 Hz) and high sensitivity ($\alpha_{\text{min}} < 10^{-2}\text{ cm}^{-1}$). These results were achieved with a standard microfluidic chip—without any precision optical

surfaces, or any care taken to ensure that the surfaces are precisely parallel to one another. However, it is clear even in these preliminary efforts, that the sensitivity of the BBCEAS technique surpasses that of previous absorption measurements in microfluidic chips. The chapter concludes with a discussion of chip designs that could improve the instrument sensitivity. It seems likely that improving the chip's optical quality would have a dramatic effect on the instrument sensitivity. To this end, there are a wide variety of commercially available chips (see, for example Dolomite.com), with good optical qualities. However, most off-the-shelf chips have channel dimensions around an order of magnitude smaller than those used in this project (tens to hundreds of micrometers) and their use might require some narrowing of the beam waist within the cavity using, for example, a near concentric cavity geometry ($r_1 = r_2 \rightarrow L/2$, giving the smallest waist spot size possible). Alternatively a custom-made chip could be designed and built, with the optical quality and channel dimensions specifically tailored to suit the cavity. It remains to be seen whether the relatively expensive (SC) variant of BBCEAS used in this project will find popularity in the microfluidics community, where cost is a major factor in driving instrument development. However, whilst SC-BBCEAS may only be adopted by a specific sub-set of the microfluidics community (probably by those particularly interested in temporal and spectral resolution), it is hoped that the wide variety of cavity-based techniques now available in the liquid-phase will encourage individual researchers in the wider community to embrace the use of optical cavities in one form or another; it is predicted that CRDS/CEAS using relatively inexpensive diode lasers will find wide popularity in the microfluidics community, along with the use of various cavity designs including wet-mirror cavities, with very small L , and—as their sensitivity improves further—fiber-based cavities. It is also useful to note that in future, due to their low sample volume requirements, high optical-quality microfluidic chips may prove extremely useful as intracavity elements for constraining biological samples during MFE measurements.

The concept of using optical cavities to study magnetic field effects on radical recombination reactions in solution has been developed and applied in this project, for the first time. Chapter 5 provides an introduction to MFEs and assesses their importance in biological systems. In particular, the chapter stresses the importance of MFE studies of cryptochrome, which will help develop an understanding of the processes controlling the avian magnetocompass. The chapter concludes that, to facilitate meaningful MFE studies of biologically relevant systems, a step change in the sensitivity of MFE measurement techniques is required. Chapters 6-8 demonstrate that this step change can be provided by a variety of optical cavity-based techniques.

Chapter 6 describes a proof-of-principle experiment designed as an initial test of the applicability of cavity-based optical techniques for the study of MFEs in solution. Narrowband CEAS, combined with phase-sensitive detection, was used to detect MFEs in a chemical system comprising the photochemically generated thionine semiquinone radical and a DABCO cationic radical. Phase-sensitive detection—which improved significantly the SNR of the MFE measurements—was achieved using a lock-in amplifier to specifically record absorbance oscillating only at the applied field's modulation frequency. CEAS results in a clear improvement in terms of MFE detection sensitivity compared with conventional transient absorption based methods. Using (un-modulated) CEAS, a minimum detectable absorbance on the order of 10^{-5} per pass was demonstrated for the radical analyte and, when coupled with phase-sensitive detection, MFE-induced absorbance changes of $\sim 10^{-6}$ could be detected. For comparison, the minimum detectable absorbance change in a conventional TA experiment was estimated to be 1.5×10^{-4} . Additionally, the probed sample volume in CEAS was *ca.* 5 μL , around an order of magnitude smaller than that in TA.

The modulation-based approaches demonstrated in Chapter 6 preclude acquisition of any temporal information on the transient species, such as their reaction kinetics. The work

described in Chapter 7 addresses this issue using a pump-probe variant of CRDS to monitor radical recombination reactions and associated MFEs with sub-microsecond time-resolution, whilst achieving sensitivities and sample volumes comparable with those of modulated CEAS. Pump-probe CRDS was applied to study RP reactions in FMN-HEWL and AQDS-HEWL systems, and (in a similar approach to that taken for modulated CEAS), to demonstrate the technique's sensitivity advantages, the results were compared to those of single-pass TA experiments. Finally, Chapter 7 describes an *in vitro* MFE study of intramolecular electron transfer in the cryptochrome-related DNA-repair enzyme, EcPI: despite the small sample volume and low photoexcitation pulse energies used, changes in radical absorbance on the order of 10^{-5} were clearly observed upon application of an external magnetic field.

The CEAS- and CRDS-based techniques discussed in Chapters 6 and 7, respectively, are somewhat one-dimensional, as they do not permit the acquisition of optical absorption spectra as a function of wavelength. Furthermore they are limited to spectral regions in which suitable light sources are available and where there is negligible overlap between the absorption bands of the radical and precursor species. These issues are addressed in Chapter 8, which describes the application of SC-BBCEAS to observe the full visible spectra of RPs and associated MFEs in FMN-HEWL, EcPI and, crucially, DmCry. The DmCry results represent the first ever observation a magnetic field effect in an animal cryptochrome and can be added to the growing body of evidence in support of the cryptochrome hypothesis for avian magnetoreception.

The Mackenzie and Timmel Groups are now focusing on increasing the dimensionality of the cavity-based techniques used to study MFEs. Jonathan Storey, Dean Sheppard and Kevin Henbest are currently developing further the SC-BBCEAS instrument used in this project, to allow acquisition of MARY spectra covering the full visible wavelength range, whilst Kiminori Maeda is seeking to improve the dimensionality of pump-probe CRDS using an OPO tunable laser to add spectral resolution to the technique's capabilities. Experiments in both these areas

are now well underway and already producing impressive results. An exciting range of SC-BBCEAS experimental concepts for time-resolved MFE measurements are also now being developed. A new generation of SC sources, with variable repetition rates achieved via pulse-picking, are now commercially-available. This opens up the possibility of using individual SC source pulses to make discrete broadband absorbance measurements as a function of time after photoexcitation, in a similar approach to the narrowband pump-probe CRDS experiments described in Chapter 7. Alternatively, time-resolved SC-BBCEAS measurements could also be achieved in an experiment more closely resembling TA, using a pseudo-CW SC source in combination with an electron multiplying CCD with sub-microsecond time resolution, to record the transient absorption of RPs.

Condensed-phase applications of cavity-based spectroscopic techniques seem set to become more numerous and varied in the near future; with ever-increasing popularity and scope, the next few years in this field will undoubtedly prove exciting.

Appendix

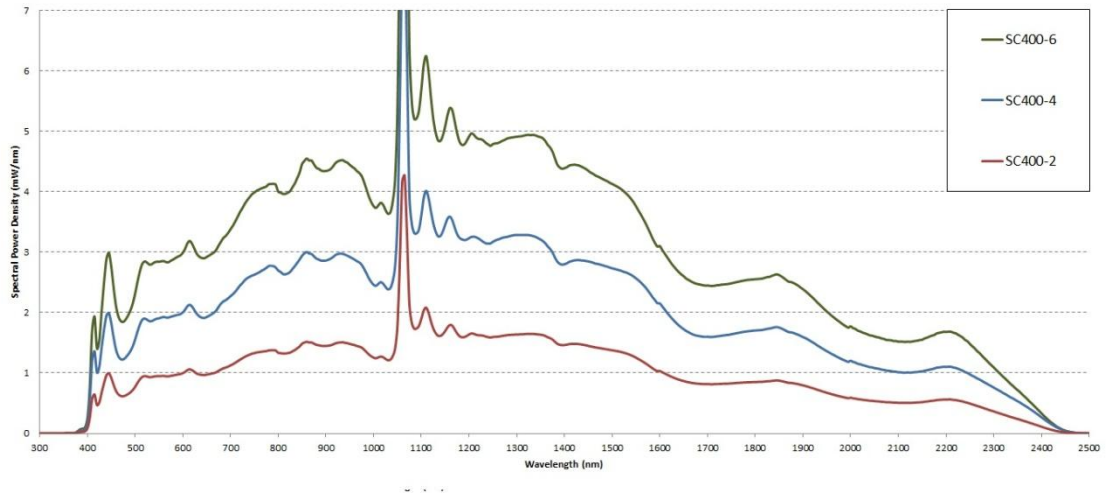


Figure A1 Emission spectrum of light exiting the SC400-2 SC source (red line, bottom spectrum in the graph). The emission spectrum of light exiting the SC450-4 is the same as that shown in the graph for the SC400-4 (blue line, middle spectrum), except for a cut off in the emission at 450 nm.