Dissertation Abstract

論文の内容の要旨

Spatially resolved microspectroscopy of flavin-based magnetosensitive photochemistry

（フラビンに基づく磁気感受性光化学の空間分解顕微分光）

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An important scientific question derives from epidemiological studies that show a weak correlation between the development of cancer and teratological effects and exposure to 50-60 Hz extremely low frequency (ELF) magnetic fields (MFs). In 2002, IARC classified ELF MF as a 2B carcinogen based on studies of the association between exposure to ELF MFs and childhood leukaemia. Due to the lack of experimental laboratory evidence and plausible mechanisms, ELF MFs are classed as 2B or *"possibly carcinogenic to humans"*. The radical pair mechanism (RPM) is considered to be the most plausible mechanism for explaining the biological effects of ELF MFs, if they do indeed exist.

Evolution has given numerous species of animals the ability to perceive magnetic fields, known as magnetoreception. The extent to which a magnetic field influences an animal and the degree of magnetic sensing varies between species. In the simplest case, a species can detect only the presence of the geomagnetic field (~50 μT). At a more sophisticated level, the magnetic information is used as an inclination compass, which is the case in migratory birds. While this higher-level phenomenon has been known for almost half a century, the primary biophysical sensory mechanism behind this astonishing ability remains poorly understood.

To date, research related to animal magnetoreception has been conducted in a wide range of scientific areas, from observational and experimental biology to quantum physics. The results from these different scientific pursuits have established the following key findings: 1. Migratory birds cannot use their magnetic compass at night. 2. During the

day, the magnetic compass appears to be wavelength dependent (between ~400 and ~565 nm). The proposed magnetoreceptor is the protein cryptochrome, with a flavin adenine dinucleotide (FAD) chromophore. 3. Radiofrequency radiation appears to be able to disrupt the compass sensing ability. There is currently one hypothesis that can in principle, explain these observations, the chemical (RPM) magnetoreception hypothesis.

To fully understand the biological and chemical processes involved in animal magnetoreception, one needs to make observations at the cellular level. This requires spatially resolved spectroscopic investigations of magnetic field effects on biological reactions in living cells. The aim of the work described in this thesis was to develop a spatially resolved microspectroscope to allow the study of magnetosensitive photochemistry of flavins not only in solution, but also in biomimetic and cellular environments. The primary objective was to unravel the mechanisms of the photochemical reactions involved in cryptochromes and ultimately, develop key connections between the fields of spin chemistry and behavioural biology.

The work described in this thesis can be broken down into 4 main sections, each of which proceeds logically from the former. First is the optimisation, testing, and enhancement of the microspectroscope itself, along with an evaluation of its utility in studying spatially localised RP reactions (Chapter 2). Next is the exploitation of the instrument to study basic model chemical RP reactions, exploiting its high sensitivity and the low sample volume capabilities (Chapter 3). The third main objective of this work was to develop spatially localised RP reaction systems that mimic critical features of biology (Chapter 4). The final section of the thesis describes the first attempts to use the microspectroscope to study RP reactions and their magnetosensitivity in actual biological samples (Chapter 5).

The technique utilises a pump-probe methodology where one laser (pump) generates RPs in a photochemical reaction and one (probe) monitors the transient RPs by their optical absorption. Two kinds of measurement are possible: transient optical absorption detection (TOAD) imaging and magnetic intensity modulation (MIM) imaging. The former technique allows the photochemical reactions taking place in flavin-based systems to be monitored inside a tiny sample volume region (<4 fL) with a beam waist of ca. 240 nm. This allows selective photoexcitation of molecules within individual organelles inside cells (for example the nucleus) and any RPs produced can be monitored. Furthermore, any magnetic field response to the photochemistry within these regions can then also be recorded. In the latter MIM technique, direct detection of magnetic field sensitive photochemistry, which is used as the signal to create the image of a sample, only allows regions that contain magnetosensitive photochemical processes to materialise in the image.

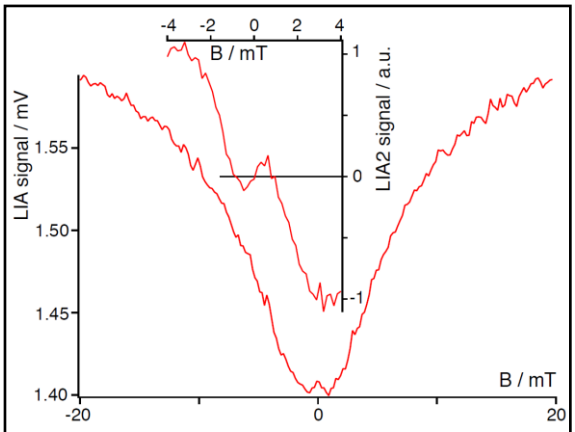

Figure 1: Single (main) and double modulation (inset) MARY spectra for the intramolecular photochemical reaction of FAD, revealing and confirming the presence of a LFE.

The starting point for this work was the successful construction of the microspectroscope and so, work began with its comprehensive testing and optimisation. To demonstrate the capabilities of the instrument, a model chemical system, believed to responsible for the magnetosensitivity of cryptochromes, was used. Flavin adenine dinucleotide (FAD) is a blue-light absorbing molecule which undergoes intramolecular electron transfer to form a RP, which is magnetic field sensitive. TOAD and MIM images of ~2.5 µm polymer beads surrounded by a sea of FAD in acidic solution were recorded. TOAD images are created with the signal from green light absorbing flavin semiquinone radicals which are created by blue light photoexcitation, therefore a greater RP concentration gives rise to a brighter pixel. For MIM images, the signal used to create the images is proportional to the magnetic sensitivity of the photo-generated FAD RPs, which means that greater magnetic sensitivity gives rise to brighter pixels. For both TOAD and MIM imaging, a low field effect (LFE) on the photochemistry of FAD was resolved for the first time (see Figure 1). This observation is an important step forward in addressing the potential role of FAD in biological magnetoreception.

At present, studies have only examined the MFE on FAD at pH values of <3.6, where the adenine moiety is protonated and FAD assumes an ‘open’ conformation. The reasoning behind this was that at higher pH it is believed that the adenine moiety is not protonated and that FAD adopts a ‘closed’ conformation, in which rapid electron transfer between the flavin and adenine moieties results in a considerable decline in fluorescence quantum yield and terminates RP formation. Exploiting the high sensitivity of our instrument, we studied reaction kinetics and MF dependence at pH values up to pH 8 and captured the time-resolved transient absorption signal with and without magnetic fields at each pH value. Furthermore, by measuring MARY spectra at each pH value, the pH dependence of the $B_{1/2}$ value for FAD was recorded. Measurements clearly demonstrate that at physiological pH and higher, FAD is capable of producing RPs that are magnetosensitive. The observed magnitude of the MFE under these conditions (~2 %, corresponding to $\Delta\Delta A \sim 2 \times 10^{-7}$) exemplifies the sensitivity of the instrument and its potential capability for observing MFEs in living tissues and cells.

Immobilisation of flavins is desirable to simulate the orientationally structured protein environment of cryptochrome. Our approach was to study the photochemistry and magnetosensitivity of flavins in different biomimetic systems, which included small and giant unilamellar vesicles. DOPC giant unilamellar vesicles (GUVs) were synthesised, using the water-in-oil centrifugation method, with various flavins-based systems, such as flavin mononucleotide (FMN) and hen egg-white lysozyme (HEWL), encapsulated within them (see Figure 2). Such systems are a means of mimicking cellular environments and exist on length scales which could be readily observed using our microspectroscope.

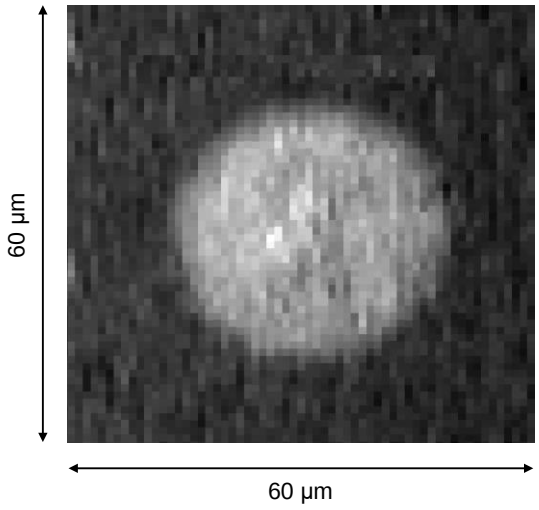

Figure 2: TOAD image of FAD semiquinone radicals created in the photochemical reactions between FMN and HEWL encapsulated in DOPC GUVs.

One of the key longer-term goals of this work was to enable direct spectroscopic measurements of RPs in real biological systems both '*in vitro*' purified protein samples, and '*in vivo*' by imaging photochemical RP reactions localised in living cells. Experiments on isolated *D. melanogaster* cryptochrome protein (*Dm*Cry) were conducted under pseudo-continuous illumination, similar to the continuous exposure to light that animals navigating in the geomagnetic field experience, rather than the series of short nanosecond pulses typical in photochemical kinetic measurements. No MFE was observed at room temperature, which is consistent with previous research, however, a clear reproducible light induced reaction cycle was observed where previous studies have suggested that substantial time must pass after photoexcitation before the sample returns to its initial state. Initial '*in vivo'* experiments were conducted on buccal mucosa squamous epithelial cells. The 'cheek' cells were incubated with FAD, which exhibited a TOAD image of flavin semiquinone radicals within the cell. However, for unaltered 'cheek' cells no TOAD image was resolved. Human cervical carcinoma (HeLa) cells are known to exhibit auto-fluorescence due to the presence of FAD. We have developed this as a model system to investigate the magnetosensitivity of FAD photochemistry at the cellular level.

This work demonstrated for the first time the techniques of TOAD microscopy, which allows direct imaging of photochemically generated radicals, and MIM microscopy, which can selectively image regions containing magnetically sensitive RPs. Both techniques possess sub-micron spatial resolution, high sensitivity, and high information content due to the ability to resolve spatial, kinetic, and magnetic information on flavin-based photoreactions in a wide range of reaction environments, from isotropic solutions to living cells.