公開

研 究 終 了 報 告 書

「動物磁気感受のためのクリプトクロム時空間計測」

研究期間： 2019 年 10 月～2023 年 3 月

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1. 研究のねらい

This project aims to clarify the obscurities of animal magnetoreception by shedding light on the photochemical process of cryptochrome (the prime candidate protein) and the influence of the magnetic field thereon from the viewpoint of its most fundamental signal formation and propagation. The purpose of this study is to clarify the protein's reaction processes and structural changes using optical microspectroscopy, molecular biology, molecular dynamics, and spin dynamics. We hope to add insight into the above processes by investigating low field effects (LFEs), anisotropic magnetic field effects (MFEs), conformational changes, and oligomer formation in cryptochrome and cryptochrome models. This study is a model of quantum biology and applying this broad scope approach is an ambitious challenge.

2. 研究成果

Research utilising the unique fluorescence-based microspectroscopic technique revealed hidden interactions between proteins and flavins (not present in standard fluorescence intensity measurements) whilst controlling the rate of photodegradation with sample flow under continuous irradiation on 65 molecules in a 0.54 fL confocal volume (see below). The results of which can be used as a reference for future cellular measurements. A best presentation award and best poster presentation awards were awarded for myself and my student, respectively, at domestic and international conferences in recognition of this PRESTO project. Furthermore, we have developed a model cryptochrome protein-ligand complex for investigating magnetoreception (Section 5.1.3.). Investigation into *Er*Cry4a (E. rubecula) homo-oligomerisation incorporating native-mass spectrometry and proteomics experiments (with collaborators in Oxford and Oldenburg) (see below).

Additionally, the current development of an open-source spin chemistry toolbox in the Python language is nearly ready for publication. In recent times, the radical pair mechanism (RPM) has gained popularity with non-specialists through its proposed involvement in biological magnetoreception, quantum teleportation, and qubits. Traditionally, successful spin dynamics simulations have only been accessible by select research groups and the complexities of the quantum nature of the RPM often perturbs non-specialists, which can oftentimes lead to incorrect or over-simplified theoretical modelling of their experimental observations. With this in mind, we (collaborator Emil Vatai, RIKEN) aimed to democratise spin dynamics simulations by developing an intuitive open-source framework in the Python programming language, to which we name RadicalPy (https://pypi.org/project/radicalpy/). We hope that this can contribute to the future of the

“quantum native” movement. This work is nearly ready for submission, the author list is provided in Section 5.1.4.

Our approach to synthesising *Er*Cry4a has proven successful. Incorporating GST has enabled us to remove the affinity tag providing label free purified proteins. We are currently “scaling up” the protocol for mass production (in collaboration with Shin Hatakeyama (Saitama) and Alex R. Jones (NPL, UK).

I have provided detailed descriptions of the M-FFS microspectroscope and *Er*Cry4a homooligomerisation:

Design and construction of a new instrument with high sensitivity and high spatial resolution for investigating magnetosensitive photochemistry in biological environments.

（1）概要

Development of a unique fluorescence-based microspectroscopic technique for observing radical pair reactions in biological locales. The technique named magneto-fluorescence fluctuation spectroscopy (M-FFS) provides multifaceted information with, for example, Förster resonance energy transfer (FRET for distance measurements and binding), fluorescence (cross-) correlation spectroscopy (F(C)CS for diffusion and number of molecules), fluorescence anisotropy (binding studies), and magnetic field effects (radical pair dynamics and protein-ligand interactions). Magnetic field effects with single-photon avalanche diodes (SPADs) have been demonstrated for the first time, offering a novel approach for studying magnetosensitive photochemistry on protein-ligands at the single-molecular level. Inspired by flow cytometry techniques, we were able to monitor magnetic field sensitive photochemistry on protein-flavin interactions and diagnose the effect of photodegradation on protein-ligand binding. Such interactions were hidden by standard fluorescence-based measurements but were revealed by monitoring the coherent quantum mechanical processes between the flavin and protein. The combination of fluorescence intensity, fluorescence anisotropy, and MFE measurements paint a clearer picture of the nature of the protein-ligand dynamics demonstrated here.

（2）詳細

The current home-built microspectroscope (Fig. 1) arrangement includes a high numerical aperture objective lens in a confocal arrangement. The sample is excited at 450 nm by either a CW diode laser or a supercontinuum source-acousto-optic tuneable filter (SC-AOTF) combination. The fluorescence is spectrally filtered via dichroic mirror (DM) and band-pass filter (BP) arrangements, before being directed, via a piezo mirror, onto one or two single-photon avalanche diodes (SPADs). The SPADs are arranged to monitor unpolarised or polarised fluorescence, which is easily achieved by switching between a DM and a polarising beam splitter (PBS), respectively, with no realignment required.

For polarised fluorescence experiments, a motorised half-wave plate is used to rotate the angle of the polarised excitation beam before entering a polarisation-maintaining single mode optical fibre. An external magnetic field (10 mT, 20 ms rise time) is applied via water-cooled Helmholtz

coils situated around the sample. The sample is housed in a flow channel slide situated on a 3D stepper motor/piezo stage. Sample flow is achieved via a programmable microfluidic syringe pump. Acquisition software was written in LabVIEW. All data were processed in MATLAB or Python.

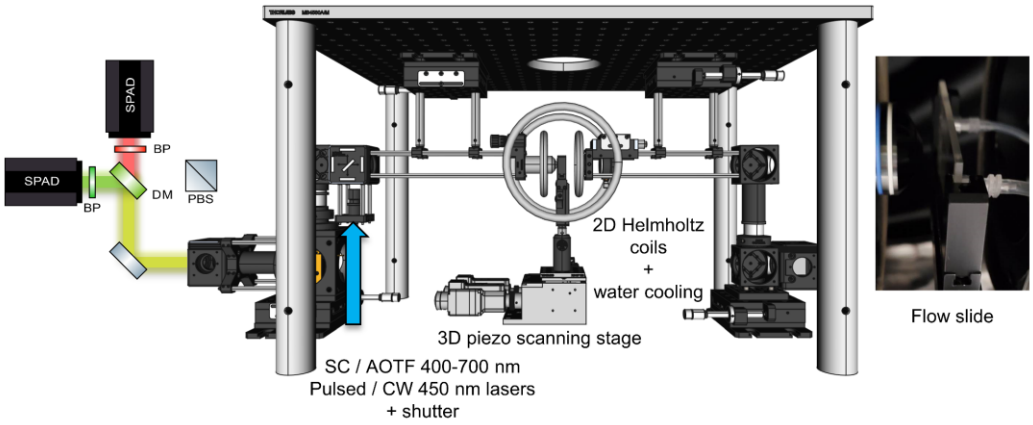

Figure 1. Schematic of the magneto-fluorescence fluctuation spectroscope.

It is known that most protein-flavin interactions proceed via the N_{10}-ribityl and phosphate side chains of FAD and FMN. Flavins are photosensitive compounds, which absorb strongly in the UV to blue region. Upon absorption the isoalloxazine ring experiences intramolecular photoreduction from the electron donor ribityl sidechain, which may lead to fragmentation of the molecule. During side chain oxidation several photoproducts are produced, predominantly 42 % lumiflavin (LF) and 58 % lumichrome (LC). The photoproducts retain typical flavin absorption and emission characteristics, however, LC possesses an alloxazine ring which causes a dramatic blue shift in the absorption spectra at 445 nm. One can glean from the above that 1) photodegradation can influence protein-flavin interactions and 2) this could have a dramatic effect on quantum mechanical radical pair dynamics.

Investigating radical pairs in biology requires a plethora of techniques to uncover the complex mechanisms involved. One approach is to amalgamate the power of fluorescence fluctuation spectroscopy and magnetic field effect methodologies. By monitoring variations in flavin fluorescence, the M-FFS technique provides a multitude of quantitative information, for example, distance measurements, binding, diffusion, number of molecules, radical pair dynamics, and protein- ligand and protein-protein interactions.

Two protein-flavin complexes were used to test the capabilities of the M-FFS technique. The first protein HEWL (hen egg-white lysozyme) is known to form radical pairs with FMN via electrostatic interactions on the surface of the protein. The kind of the binding interactions for the second protein complex BSA (bovine serum albumin)-FMN were unknown.

The nature of the protein-ligand interactions was investigated with various fluorescence techniques. Fluorescence spectra were recorded for FMN in the presence and absence of both proteins. FMN is quenched by 37 % and 80 % in the presence of HEWL and BSA, respectively.

The fluorescence lifetime of FMN (~5 ns) did not change in the presence of both proteins, characteristic of static quenching (ground state complex formation).

Concentration dependent fluorescence anisotropy measurements reveal the form of protein-ligand binding. Alignment-free quick change optical components allow one to utilise the two SPAD detectors for high-sensitivity fluorescence anisotropy measurements without the need to move the sample. The experimentally determined anisotropy is calculated by the following,

$$r = \frac{I_{\parallel} / I_{\perp} - 1}{I_{\parallel} / I_{\perp} + 2}. \tag{1}$$

Figure 2 shows a stark contrast between the two protein-FMN complexes. The data for BSA-FMN (green) are characteristic of specific binding. The Hill slope tells us how many binding sites the ligand resides, and the fit informs us that FMN is binding to 1.33 ± 0.06 sites with a relatively high equilibrium dissociation constant, Kd = 103 ± 4.99 μM. Conversely, HEWL-FMN (blue) displays a distinctive nonspecific binding model, which is consistent with a surface interaction, as previously reported.

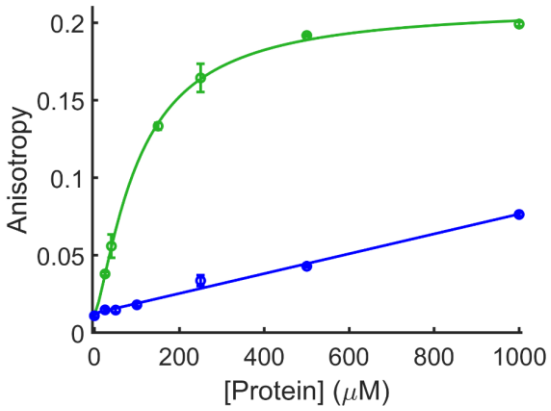

Figure 2. Fluorescence anisotropy for HEWL-FMN (blue) and BSA-FMN (green).

Magnetic field effects on the protein-flavin complexes can provide unique information on the character of the binding interactions. The MFE is calculated as follows,

$$MFE(\%) = \frac{I_{mean}(B \neq 0) - I_{mean}(B = 0)}{I_{mean}(B = 0)} \times 100. \tag{2}$$

Figure 3 displays quite distinct differences between the two complexes. The flow rate dependence on MFE for HEWL-FMN (blue) starts off at a relatively large value of -1 %, reducing by a half as we approach 50 μL/min, then recovering to over -1 % and finally trailing off as the flow rate increases. This trend can be explained as follows, the 50 μL/min boundary represents the change in concentration ratios between FMN and LF. Below this boundary, [LF] > [FMN] and the opposite is true above. At low flow rates, the dwell time of the molecule in the excitation beam is on the millisecond timescale, which is sufficient time for ample

photodegradation at our power density (91 kW/cm^2). As explained above, LF lacks the sidechains required for effective protein-ligand interactions, which leads to a lower re-encounter probability between the two, thus reducing the MFE. At higher flow rates, the opposite is true and the MFE recovers before reducing again at flow rates above 100 µL/min, as the dwell time is competing with the number of photocycles within the excitation beam. The fewer the number of cycles the smaller the MFE.

BSA-FMN (green) tells us a different story. At low flow rates we observe a negative MFE (triplet born RP), however, at high flow rates positive MFEs arise implying singlet born RPs. This can again be explained by the above arguments with the added facet of FMN located in a binding pocket of BSA. The MFE is considerably smaller than those observed for HEWL-FMN, which suggests a shorter radical pair lifetime. This is reasonable if the radical pair separation is small. A small fluorescence recovery is observed below 100 µL/min, suggesting [LF] is dominant, LF is more mobile and fluorescence quenching is reduced. Above 200 µL/min, [FMN] is greater than [LF] and a singlet born RP is observed, suggesting a relatively short distance between FMN and a protein residue.

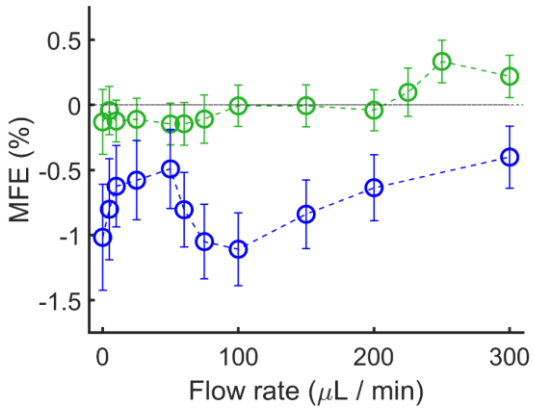

Figure 3. Flow rate dependence on magnetic field effects for HEWL-FMN (blue) and BSA-FMN (green).

Competitive binding experiments between NPS (naproxen) and FMN with BSA produce a complete FMN fluorescence recovery as the concentration of NPS increases. NPS is known to bind to three locations within the same BSA binding pocket (K_d = 5.8×10^{-7} M), confirming FMN binds within the same pocket. Supporting the anisotropy measurements and molecular docking simulations, which places FMN within 4 Å of W211. A theoretical maximum electron transfer rate of 5.8×10^{-12} s^{-1} far exceeds the ISC rate for FMN (2×10^{-8} s^{-1}). Therefore, the evidence suggests we are observing singlet born RPs to which we propose as a cheap, easy to handle model for cryptochrome and quantum biology.

The above results are now under revision for publication (Section 5.1.1). This work has also won a Best Presentation Award (Section 5.3.2) and published as an invited article in a science society magazine (Section 5.3.1).

Discovery of the binding nature of *Er*Cry4a homo-oligomers.

（1）概要

Formation of homodimers is important for the function of many proteins. Although dimeric forms of cryptochromes (Cry) have been seen by crystallography and were recently observed in vitro for Cry4a from the migratory European robin, little is known about the dimerisation of avian cryptochromes and the role it might play in the mechanism of magnetic sensing in migratory birds. Here we present a combined experimental and computational investigation of the dimerisation of European robin Cry4a resulting from covalent and non-covalent interactions. Experimental studies using native mass spectrometry, mass spectrometric analysis of disulphide bonds, chemical cross-linking and photometric measurements show that disulphide-linked dimers are routinely formed, the most likely cysteines being C317 and C412. Computational modelling and molecular dynamics simulations were used to generate and assess a number of possible dimer structures. The relevance of these findings to the proposed role of Cry4a in avian magnetoreception is discussed.

（2）詳細

To learn more about *Er*Cry4a dimerisation and its possible role in magnetoreception, we have explored a variety of candidate structures, including covalently and non-covalently linked forms of the full-length and truncated protein, using a combination of experimental and computational methods to identify potential *Er*Cry4a dimers. Native mass spectrometry (MS), mass photometry (MP), and gel electrophoresis were used to establish the presence of dimers, while chemical cross-linking followed by mass spectrometry (XL-MS) provided information about the relative orientation of the monomer units. To obtain plausible model structures that fit the experimental data, we combined molecular docking and molecular dynamics (MD) techniques. The atomistic structure of *Er*Cry4a, modelled on the crystal structure of pigeon *Cl*Cry4a in a previous study, was used to construct a large number of dimeric models *in silico*.

We show that *Er*Cry4a readily forms dimers and that they are covalently linked by disulphide bonds. Several cysteines seem to be involved, C317 and C412 being the most likely. Computational modelling supports this conclusion, provides insights into the relative orientation of the monomer units, and suggests that non-covalently bound dimers may also be relatively stable. The relevance of these findings to the proposed role of Cry4a in avian magnetoreception is unclear.

That *Er*Cry4a dimers are not readily disrupted in a reducing environment (10 mM DTT) suggests that they could persist *in vivo*. Despite the reducing environment of the cell, there are well-documented examples of biologically significant intra-cellular disulphide-linked proteins involved in redox processes. Moreover, little is known about the redox conditions in the avian photoreceptor cells that are thought to contain the magnetoreceptors. It therefore seems premature to exclude the possibility that *Er*Cry4a dimerisation could have a biological function.

If *Er*Cry4a does have a role in magnetic sensing and/or signalling, then an immediate question is why the protein has evolved to stabilise inter-monomer disulphide bonds to the extent that only a

proportion of the proteins dimerise under the conditions of the experiments reported here. One explanation is that these dimers are simply aggregates of unfolded monomers. However, we think this is unlikely, as the *Er*Cry4a dimers bind FAD and have native MS charge-state distributions that are similar to those of the monomers suggesting that the protein remains correctly folded and therefore potentially functional in the dimeric state. Partially unfolded proteins typically display higher charge states than the native states. An alternative explanation is that a monomer-dimer equilibrium could play a regulatory role in magnetic sensing or signal transduction.

An observation reported by Xu *et al.*, *Nature*, 594, 535–540 (2021) may be relevant here. Samples of *Er*Cry4a that had been purified without adding 10 mM BME to prevent dimerisation showed clear evidence for the formation of relatively long-lived (nanosecond to microsecond) photo-excited singlet (^{S}FAD*) and triplet (^{T}FAD*) states of FAD in addition to the normal photo‑induced electron transfer reactions that generate radical pairs. It was speculated that subtle differences in protein conformation, induced by dimerisation, might inhibit electron transfer from the tryptophan tetrad to ^{S}FAD*, thereby stabilising ^{S}FAD* and allowing formation of ^{T}FAD* by intersystem crossing. Samples of *Er*Cry4a purified with 10 mM BME, and therefore more likely to be monomeric, showed much weaker signals from ^{S}FAD* and ^{T}FAD*. Recognizing that the environment of *Er*Cry4a in these experiments, and those reported here, are very different from the interior of a cell, it nevertheless seems possible that a monomer-dimer equilibrium could in some way regulate the magnetic sensing functions of *Er*Cry4a. One could also imagine a regulatory role for dimerisation in the signal transduction pathway of *Er*Cry4a.

This work is now under review, the author list is provided in Section 5.1.2.

3. 今後の展開

*Er*Cry4a has six potential interacting partners, one of which is the voltage-gated potassium channel subunit Kv8.2 (KCNV2). A soluble fraction of this protein KCNV2-2 and *Er*Cry4a have been supplied by collaborator Shigeki Arai (QST) for FRET experiments to determine the interactions between the two proteins. In preparation for this project, a model FRET pair EGFP-Alexa Fluor 594 has been used to calibrate the home-built microspectroscope. The Förster radius (R_0) was determined to be 9.63 nm. SPAD detected FRET yielded a FRET efficiency (E) of 0.70, giving a fluorophore distance of 8.39 nm. This distance is consistent with previous measurements of collaborator Miho Suzuki (Saitama University) who provided the sample. Radostin Danev (UTokyo) is contributing to this project via CryoEM measurements to elucidate the 3-dimensional structure of the heterooligomer. This project is currently underway. The impact of this collaboration could shed light on potential signalling pathways involved in avian magnetoreception.

Cryptochrome expressed in HeLa cells is also currently underway. We are currently investigating homo-oligomerisation of *At*Cry2, and other cryptochromes in the future, with EMCCD and SPAD detection in the M-FFS apparatus. The aim is to elucidate the role of the radical pair and blue-light on the dimerisation processes and how its relationship to magnetoreception. Our long-term goal is

to compare and contrast various cryptochromes proteins from numerous species to provide a deeper understanding of the light-induced conformational changes and magnetoreception of the proteins. Finally, the human flavo-genome encodes 90 genes for flavin-dependent proteins, predominately FAD and FMN cofactors, which are involved in numerous metabolic pathways. The role of oxygen in magnetosensing is also open question to which I aim to explore with M-FFS. A major long-term goal is to explore these proteins for radical pair spin dynamics with the techniques and methodologies developed during my PRESTO tenure.

4. 自己評価

The aim of this project was an ambitious one to complete in 3½ years. That being said, I believe I have made significant progress in reaching these aims. This project is multidisciplinary in nature and requires expertise in various fields. My knowledge and experience in various fields from quantum mechanics to molecular biology has helped overcome certain hurdles one would face without said knowledge and expertise. Of course, progress would not have reached the stage it has without the work of my numerous collaborators, to which I am grateful. The PRESTO project has undoubtedly given me the opportunity to make my goals a reality. Establishing a high sensitivity microspectroscope and protein synthesis laboratory have certainly made this possible. The techniques developed here can help to uncover yet unknown quantum biological phenomenon and help to establish the quantum biology field in the scientific community.Overall, I believe this PRESTO project an answered and uncovered key questions towards the understanding of biological magnetoreception.

5. 主な研究成果リスト

(1)代表的な論文(原著論文)発表

研究期間累積件数: *under review & near submission.*.4 件

1. L. M. Antill*, M. Kohmura, C. Jimbo, and K. Maeda*, Introduction of magnetosensitive single-photon microspectroscopy for analysing protein-ligand interactions, *under review*.
Flow cytometry techniques are invaluable tools for clinical research, where areas such as cancer detection and drug discovery require detailed analysis of protein-ligand interactions. Here we present a novel approach for investigating protein-ligand binding dynamics in the form of magnetosensitive single-photon microspectroscopy. Monitoring magnetic field sensitive photochemistry on protein-flavin interactions revealed the influence of photodegradation on protein-ligand binding at the single-photon level. As flavin mononucleotide photoreduces to lumiflavin, the ribityl/phosphate side chain detaches, subsequently removing existing protein-flavin interactions dramatically altering coherent quantum mechanical processes between the flavin and native tryptophan residues in the protein. The methodologies reported here form a platform for enhancing current diagnostic research.
2. M. Hanić, L. M. Antill, A. S. Gehrckens, J. Schmidt, K. Görtemaker, R. Bartlöke, T. J.

El-Baba, J. Xu, K. W. Koch, H. Mouritsen, J. L. P. Benesch, P. J. Hore, and I. A. Solov'yov*, Dimerisation of European robin cryptochrome 4a, *under review.*

Formation of homodimers is important for the function of many proteins. Although dimeric forms of cryptochromes (Cry) have been seen by crystallography and were recently observed *in vitro* for Cry4a from the migratory European robin, little is known about the dimerisation of avian cryptochromes and the role it might play in the mechanism of magnetic sensing in migratory birds. Here we present a combined experimental and computational investigation of the dimerisation of European robin Cry4a resulting from covalent and non-covalent interactions. Experimental studies using native mass spectrometry, mass spectrometric analysis of disulphide bonds, chemical cross-linking and photometric measurements show that disulphide-linked dimers are routinely formed, the most likely cysteines being C317 and C412. Computational modelling and molecular dynamics simulations were used to generate and assess a number of possible dimer structures. The relevance of these findings to the proposed role of Cry4a in avian magnetoreception is discussed.

3. N. Iwata, L. M. Antill, S. Fukumoto, K. Maeda*, Dynamics of radical pairs in binding pockets of bovine serum albumin probed by static and pulsed magnetic field effects, *near submission.*

We observed clear time-resolved MARY spectra of a low field effect on the protein-ligand complex, BSA-2,6-AQDS. To our knowledge, this is the first example of a clear and quantitative observation of a low field MARY in a protein system. BSA is a typical drug delivery protein, and 2,6-AQDS binds in the deep pocket of the protein. It is interesting to discuss the relationship between the molecular dynamics of RPs in the pocket of the protein and the low field magnetoreception mechanism. The details of the relationship between the spin dynamics and the molecular motion are probed by magnetic field effects (MARY spectroscopy), pulse magnetic field effects (SEMF-techniques), and molecular dynamics simulations.

4. L. M. Antill* and E. Vatai*, RadicalPy: radical pair spin dynamics for the masses, *near submission*

The interaction of a pair of radicals, known as a radical pair (RP), was first observed as anomalous line intensities in EPR spectroscopy by Fessenden and Schuler in 1963 and in 1967, Bargon, Fischer, and Johnsen and independently Ward and Lawler observed similar anomalies in NMR spectroscopy. These phenomena are known as chemically induced dynamic electron polarisation (CIDEP) and chemically induced dynamic nuclear polarisation (CIDNP), respectively. In 1969, a new theory was postulated to explain these anomalous intensities by Closs and independently by Kaptein and Oosterhoff, which is known as the radical pair mechanism (RPM). Since the 1970's, there have been considerable advances in the theoretical framework and many methods and experiments have been developed, creating the field of Spin

Chemistry. This field extends from chemical systems encompassing the solid and liquid state, to both biological systems and material science, with the application of magnetic field strengths ranging from very weak fields of tens of micro-Tesla up to tens of Tesla.

In recent times, the RPM has gained popularity with non-specialists through its proposed involvement in biological magnetoreception. Traditionally, successful spin dynamics simulations have only been accessible by select research groups and the complexities of the quantum nature of the RPM often perturbs non-specialists, which can oftentimes lead to incorrect or over-simplified theoretical modelling of their experimental observations. With this in mind, we aimed to democratise spin dynamics simulations by developing an intuitive open-source framework in the Python programming language, to which we name RadicalPy.

RadicalPy includes both quantum and classical simulation methods. The toolbox offers an atom and molecule database (including spin multiplicities, gyromagnetic ratios, and hyperfine coupling constants) allowing the solution of the Liouville von Neumann equation to be solved, in both Hilbert and Liouville space, with relative ease. General kinetic and relaxation superoperators, such as, Haberkorn and Jones-Hore, and singlet-triplet dephasing (STD) and random fields relaxation (RFR), respectively, are easily incorporated.

The software also contains classical simulations, such as random walk Monte Carlo methodologies for modelling the molecular diffusion of free or encapsulated RPs (e.g., micelles or vesicles). The results of which, and molecular dynamics (MD) trajectory import capability, can be used to calculate the time evolution of the exchange and dipolar couplings, and estimate spin dephasing rates. Transient absorption experiments can be simulated with radical pair kinetic equations providing both ΔA and ΔΔA time traces. Other experimental techniques, including, but not limited to, isotropic and anisotropic magnetic field effects (MFEs), with $B_{1/2}$ calculation, can be simulated via various quantum mechanical approaches.

We hope this versatile and intuitive open-source framework provides the means for non-specialists to perform correct and complex radical pair dynamics simulations with relative ease.

(2)特許出願:該当なし

(3)その他の成果(主要な学会発表、受賞、著作物、プレスリリース等)

1) Invited article: Magnetosensitive fluorescence fluctuation spectroscopy, Lewis M Antill, 電子スピンサイエンス (SEST) vol. 20, 通号 39 号, 2022/10.

2) Best Presentation Award, The 3rd Annual Meeting of the Quantum Life Science Society Conference (第3回量子生命科学会), Title: Exploring magnetic field effects in biomolecules with high-sensitivity fluorescence spectroscopy, 2021/09.

3) “Magnetosensitive radical pair dynamics in protein binding pockets”, RIKEN CEMS Excited-State Chemistry of Functional Materials, 2021/01/28.

4) “Understanding the photoinduced structural dynamics of cryptochrome proteins”, The 58th Annual Meeting of the Biophysical Society of Japan, 2020/09.