PROGRESS REPORT

Studies of Multifrequency Phase-Resolved Fluorescence Spectroscopy for Spectral Fingerprinting

DOE Grant # DE-FG05-88ER13931

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Research Publications:

1. Millican, D. W. and McGown, L. B., "Fluorescence Lifetime Selectivity in Excitation-Emission Matrices for Qualitative Analysis of a Two-Component System", ANAL. CHEM., 61, 580-583 (1989).
2. Nithipatikorn, K. and McGown, L. B., "Fluorescence Studies of Energy Transfer in Sodium Taurocholate and Sodium Dodecyl Sulfate in Micellar Solutions", ANAL. CHEM., 61, 1405-1410 (1989).
3. Tu, X. M., Burdick, D. S., Millican D. W. and McGown, L. B., "Canonical Correlation Technique for Rank Estimation of Excitation-Emission Matrices", ANAL. CHEM., 61, 2219-2224 (1989).
4. Burdick, D. S., Tu, X.-M., McGown, L. B. and Millican, D. W., "Resolution of Multicomponent Fluorescent Mixtures by Analysis of the Excitation-Emission-Frequency Array", J. CHEMOMETRICS, 4, 15-28 (1990).
5. Meyerhoffer, S. M. and McGown, L. B., "The CMC Behavior of Sodium Taurocholate in Water", LANGMUIR, 6, 187-190 (1990).
6. Millican, D. M. and McGown, L. B., "Resolution of Spectra from Frequency-Domain Fluorescence Data Using Multiway Analysis", ANAL. CHEM., in press.
7. Hertz, P. M. R. and McGown, L. B., "Phase-Resolved Fluorescence Spectral Fingerprinting of Petrolatums", APPL. SPECTROSC., in press.
8. Meyerhoffer, S. M. and McGown, L. B., "Fluorescent Probe Studies of Metal Salt Effects on Bile Salt Aggregation", submitted to J. AMER. CHEM., 5/2/90.
9. Wu, K. and McGown, L. B., "A Modified Phase-Modulation Spectrofluorometer for Lifetime-Resolved Fluorescence-Detected Circular Dichroism", APPL. SPECTROSC., in press.

Doctoral Dissertation:

"Fluorescence Lifetime Selectivity in the Frequency Domain for Spectral Resolution",
David W. Millican, Duke University, 1990.

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Received
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INTRODUCTION

During the past two project periods (7/1/88 - 12/31/90), we have made significant advances towards our goal of characterizing complex samples in terms of their dynamic spectral characteristics through the use of phase-resolved fluorescence spectroscopy. Specific achievements are discussed in the following sections, each of which describes a particular area of focus in our studies.

Limits of resolution in PRFS: analysis of three-way data arrays.

In the past project period we have completed the first phase of our studies of resolution in simple, two-component systems. We have tested the limits of spectral resolution, in order to define those situations in which the addition of fluorescence lifetime selectivity becomes important as well as those cases for which lifetime selectivity fails. Novel data analysis strategies have been developed for the three-way, excitation-emission-frequency arrays (EEFA) that are generated in the PRFS experiment, including our most recent studies of evolving factor analysis for the frequency-domain data (manuscript in preparation).

PRFS fingerprinting of complex samples.

Petrolatums

During the past project period we have demonstrated the first application of multifrequency phase-resolved fluorescence spectroscopy (PRFS) to the characterization of complex samples. The specific samples used in the studies were a series of petroleum lubricants, or petrolatums. The incorporation of lifetime selectivity was found to significantly enhance discrimination between different petrolatum samples, relative to conventional, steady-state fluorescence techniques. Chemometric data analysis strategies were used to reduce noise contributions and to compare the dynamic spectral features of the petrolatums. In order to minimize the noise contribution to the data, each data matrix was decomposed into its eigenvectors and eigenvalues by singular value decomposition, followed by matrix reconstruction from the first rank n eigenvectors. The matrix rank n was estimated by canonical correlation technique with a technique recently described by Burdick and Tu, as part of a collaborative effort between our group and Professor Burdick's group in Statistics here at Duke.

Simple visual comparison of the steady-state EEMs and the phase-resolved EEMs (PREEMs) for the different petrolatum samples suggests that the PREEMs are better "fingerprints" than the steady-state EEMs. Quantitative comparisons between two samples were based on the normalized eigenvalues of the primary eigenvectors, which were obtained from singular value decomposition of the steady state EEMs and the PREEMs for replicate runs for the two samples. Several methods

of data analysis were used to compare the replicate runs associated with the two samples, including comparisons of single eigenvalues, pairs of eigenvalues and groups of three eigenvalues.

Several observations were made regarding the comparison of petrolatum samples. The PRFS data indicates that the shorter-lived emission components are responsible for the largest share of the variance (i.e., dominate the first eigenvector) in the spectra and provide the best discrimination between samples at the highest frequency. The longer-lived emission components appeared to dominate the second and third eigenvectors and were increasingly important for discrimination between samples as modulation frequency was decreased. Discrimination between samples based on two eigenvectors was significantly better than discrimination based on a single eigenvector. Relatively minor emission components in the intermediate lifetime range were found to be the best discriminators between the two samples, and the discrimination was greatly enhanced by the use of PRFS to amplify the relative contribution of the minor components.

These studies demonstrate the improved discrimination between samples afforded by selective enhancement of spectral features as a function of fluorescence lifetime, implemented in the frequency-domain through phase-resolution. Comparison of the best case of discrimination for the steady-state and PRFS data clearly indicated the advantage of fluorescence lifetime selectivity for the petrolatum samples.

Pooled Human Serum

We have continued our studies of the PREEM arrays of pooled human sera, including normal samples and samples that have been stripped of a particular component or enriched in a component. To date, we have collected three-way excitation-emission-frequency arrays (EEFAs) for a wide variety of such sera, including lipid-stripped and non-lipid-stripped preparations of normal sera, all male and all female sera, steroid-free sera, sera lacking particular steroids, hyperlipid sera, sera free of one or all of the thyroid hormones, and hyperlipid sera. We are currently in the process of analyzing the data, with emphasis on relating dynamic spectral characteristics to dynamic physicochemical interactions in the samples.

Studies of organized bile salt media.

We have continued our exploration of organized bile salt media as reagents in the characterization of complex samples. Our recent studies demonstrate that the bile salt aggregates are better at "individually wrapping" solubilized luminescent molecules in aqueous solution, compared to conventional detergents. For example, the fluorescence spectra of different crude oil are much more similar in aqueous bile salt solution than in cyclohexane, indicating that spectral features in the latter solvent are strongly influenced by dynamic interactions of fluorescent molecules with each other and with the sample matrix. Thus, by comparing the spectrum of a complex sample in an organic solvent to the spectrum in aqueous bile salt solution, we can achieve a better

understanding of the dynamic interactions that underlie the variations in spectral features between different samples. This, in turn, allows us to study the dynamic interactions and probe the distributions of fluorescent molecules between different microenvironments in complex samples.

Our studies in this area include fundamental studies of the cmc behavior of trihydroxy bile salts, comparison of anionic and nonionic salts, and the effects of metal cations on the aggregation properties of the bile salts as well as on the photophysical properties of luminescent molecules that are solubilized in the bile salt solutions. NMR techniques, including proton NMR and lanthanide shift, have been central to these studies.

Lifetime-resolved fluorescence-detected circular dichroism

Fluorescence lifetime and molecular chirality are both excellent probes of a molecule's microenvironment. Lifetime measurements provide a dynamic view of molecular rotation, diffusion, microviscosity, accessibility to quenchers, excited state complexation and energy transfer. Molecular chirality may be an intrinsic property of a molecule, or it may be induced or modified in a molecule that is tightly associated with a helical or otherwise asymmetric microenvironment, such as may occur in nucleic acids, proteins, membranes, micellar aggregates and cyclodextrins.

Because both the lifetime and chirality of a molecule are strongly influenced by its microenvironment, these characteristics are valuable in the fingerprinting and characterization of complex samples. We are already well advanced in our studies of fluorescence lifetime for these purposes, which is the core of this research project. As our next step, we recently initiated studies in which we combine frequency-domain fluorescence lifetime resolution with fluorescence-detected circular dichroism (FDCD) to explore the dynamic aspects of fluorescence emission that is excited by circularly polarized light. These are the first experiments to use fluorescence lifetime techniques in circular dichroism spectroscopy.

We recently completed the modification of a frequency-domain fluorescence lifetime spectrofluorometer for FDCD. The basic instrument is the same frequency-domain fluorescence lifetime apparatus used for our other studies on this project. In our FDCD-modified instrument, a xenon arc lamp is used to produce the excitation beam, which is passed through a monochromator for wavelength selection, a Pockels cell to create the intensity-modulated component of the excitation beam for the frequency-domain lifetime measurements, a linear polarizer to select horizontally or vertically polarized light, and a Babinet-Soleil compensator (BSC) which, in combination with the linear polarizer, produces right- or left-handed circularly polarized light. The intensity-modulated, circularly polarized excitation beam is then directed into the sample cell and the resulting emission is passed through an optional linear polarizer for magic angle correction of photoselection, an emission wavelength selector (monochromator or interference filter), a PMT

detector, system electronics, and an on-line IBM PC-AT microcomputer for data acquisition and analysis.

In order to establish that the steady-state FDCD signals obtained on our instrument are real and not the result of optical or other instrumental artifacts, several solutions of achiral fluorophores were measured. The FDCD signal should be zero for these optically-inactive solutions; thus, any significant, nonzero contributions indicate the presence of artifacts. Aqueous solutions of sodium fluorescein (NaF) and of pyrene yielded negligible FDCD signals, indicating the absence of significant artifacts in our measurements.

We demonstrated the absence of significant optical artifacts in the measurement of fluorescence lifetimes on the FDCD-modified frequency-domain instrument, using a solution of NaF in water. As expected for the optically-inactive solution, there is no significant difference between the lifetimes obtained with unpolarized excitation, right-handed circularly-polarized excitation and left-handed circularly-polarized excitation. The close agreement between the results for right-handed and left-handed excitation confirms the accuracy of the lifetime measurements with circularly polarized light.

We have also performed some *very preliminary* studies of pyrene in β -cyclodextrin. For free, achiral pyrene in solution, the lifetime results are the same for right-handed and left-handed excitation: in both cases, a long-lived pyrene signal dominates the emission and there is a minor contribution from a very short-lived component, probably pyrene excimer. *The agreement between right-handed and left-handed excitation for the achiral pyrene again indicates the accuracy of our lifetime-FDCD technique.*

In the presence of β -cyclodextrin, the lifetime of the long-lived pyrene component increases significantly for the left-handed excitation but remains the same as free pyrene for the right-handed excitation. This may reflect the selective formation of a left-handed inclusion complex between the achiral pyrene and the left-handed β -cyclodextrin helix. Also in the β -cyclodextrin solutions, the lifetime of the shorter-lived component increases by two orders of magnitude for all of the excitation orientations. This may indicate the formation of a longer-lived pyrene dimer in association with the cyclodextrins, as opposed to the very short-lived excimer formed between free pyrene molecules in the absence of cyclodextrin.

These early results indicate that lifetime-resolved FDCD will provide a wealth of information about complex samples, greatly increasing our ability to fingerprint and characterize that samples and study the dynamic processes that define the unique spectral properties of a given sample.

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