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RESEARCH ARTICLE

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Couette flow fluorescence detected linear dichroism for analytes in lipid bilayers

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Abstract

Membranes are important sites of intermolecular interactions in biological systems. However, they present significant analytical challenges as they contain multiple analytes and are dynamic in nature. In this work, we show how a Jasco J-1500 circular dichroism spectropolarimeter can be used with a microvolume Couette flow cell and appropriate cut-off filters to measure excitation fluorescence detected linear dichroism (FDLD) of fluorophores embedded in liposomal membranes. The result is a spectrum that selectively probes the fluorophore(s) and eliminates the scattering that is apparent in the corresponding flow linear dichroism (LD) spectrum. The FDLD spectrum is opposite in sign from the LD spectrum with relative magnitudes modified by the quantum yields of the transitions. FDLD thus enables analyte orientations to be identified in a membrane. Data for a membrane peptide, gramicidin, and two aromatic analytes, anthracene and pyrene, are presented. Issues with the "leakage" of photons by the long pass filters used is also discussed.

KEYWORDS

anthracene, Couette flow, gramicidin, membranes, polarized light, pyrene

1 | INTRODUCTION

Membrane proteins comprise up to 30% of the protein complement of biological systems[1]; however, this is not reflected in the data available on membrane systems.[2] To bridge this gap, a range of complementary methods that probe membrane proteins in environments that at least mimic a biological system are required. Flow linear dichroism (LD)[3–7] has been successfully used to determine the orientation of protein secondary structures with respect to the membrane normal of a range of liposomes.[4,5,7–12] However, scattering from the liposomes is larger than the LD signals and correcting for the scattering component at lower wavelengths is challenging.[13] We have previously developed fluorescence detected LD (FDLD) to increase the selectivity of LD spectroscopy and successfully measure LD via measuring the fluorescence for molecules in and on polyethylene (PE) films,[14] DNA/drug systems,[15,16] and flow-oriented fibrous proteins.[15] This paper outlines the journey taken to collect the first reliable FDLD spectra for unilamellar liposomes with analytes embedded in the membrane (Figure 1).

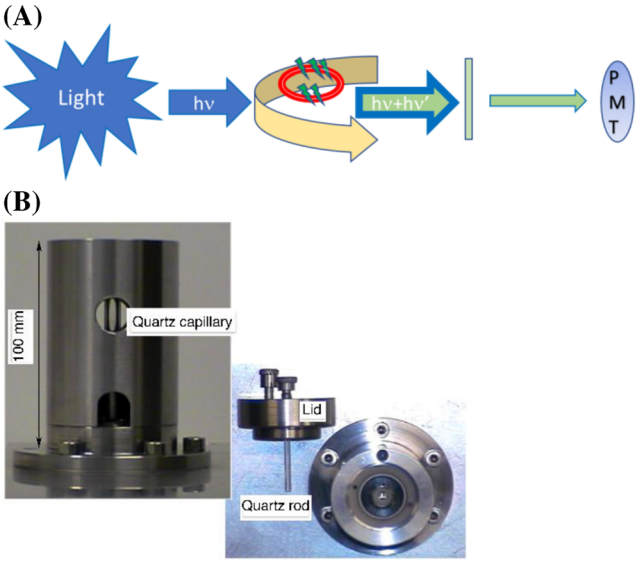

FIGURE 1 (A) Schematic illustration of the fluorescence detected linear dichroism (FDLD) experiment indicating a light source of higher energy than the emitted photons, flow orientation of liposomes (in red) leading to an elliptical shape for the liposomes and consequent orientation for embedded analytes (lightning bolts), and a long pass filter that allows the fluorescent photons (green) and some of the incident photons (blue) to pass through to the photomultiplier tube. (B) The microvolume Couette flow cell.

1.1 | Principles of FDLD

Most fluorescence in solution occurs by emission of light from the lowest electronic excited state with molecules finding non-radiative pathways to get from higher energy excited states to the lowest one. Thus, by scanning the excitation wavelength and inserting a long pass filter close to the exit window of the cell and as far as possible from the photomultiplier tube, we can measure fluorescence excitation spectra in a normal transmission spectrometer. The long pass filter is chosen to block the incident photons throughout the absorbance regime of the analyte(s) of interest yet to transmit any fluorescent photons. If the spectrometer is designed for circular dichroism (CD) or LD, we then measure fluorescence detected CD or LD. We have found that when we use a CD spectropolarimeter, the quality of FDLD spectrum is better when we collect the light that has transmitted through the sample rather than the conventional 90° detection, despite the challenge this makes for the long pass filter. It also simplified the equations required to interpret the data quantitatively.[14–16]

In this work, our focus is on microvolume Couette flow cell orientation[16,17] of liposome samples. The light path for the FDLD experiments is a simple linear one (Figure 1) where the incident light transmits through the sample with some photons being absorbed and consequently fluorescing, some being absorbed and returning to the ground state via a radiation-less pathway, and some simply passing through the solution. By analogy with LD, we define FDLD as

$$FDLD = F_Z - F_Y \quad (1)$$

where X is the direction of propagation of light and Y and Z are perpendicular to X with Z denoting horizontally polarized light. Y is therefore vertically polarized light.

As any photon reaching the detector in an FDLD experiment has previously been absorbed but any photon reaching the detector in an LD experiment has not been absorbed, we expect FDLD of a transition to be opposite in sign from its LD. In the fixed-high temperature (HT) mode adopted in this work (see Experimental)

$$LD^F(LD\,\text{trace}, \text{fixed}\,HT, \text{filter}) \approx -\phi I_0 J_2(\delta_0) LD \quad (2)$$

if an appropriate filter is chosen. We discuss below the complexities that result from the long pass filter not being 100% efficient.

2 | MATERIALS AND METHODS

2.1 | Materials

All chemicals, unless otherwise stated, including gramicidin D, anthracene, and pyrene were purchased from Sigma-Aldrich (North Ryde, NSW, Australia). Sucrose was purchased from CSR, Australia. Liposomes were prepared by codissolving the analyte with L-α-phosphatidyl choline (typeIV-S) from soybean (40%) in a glass flask, drying the sample to a thin layer with air or nitrogen, and resuspending the mixture by addition of water (18.2 MW.cm) followed by five cycles of 30 s sonication yielding cloudy white solutions.[5] We previously found using dynamic light scattering that this method produced an average particle size of 100 nm with quite a broad size distribution. Small molecules were prepared as approximately 100:1 molar ratios of lipid:analyte, and gramicidin was prepared at 50:1 ratio. We have previously found that some analytes adopt their final orientation over a few hours,[4,5] so all liposome samples were left to equilibrate for at least 1 day before data collection. It should be noted that we did not notice any change in spectra over this delay for the samples reported in this work. A Glad sandwich bag was used to provide a uniform polyethylene film that was stretched in a custom-made film stretcher.[18]

2.2 | LD and FDLD

Spectra were collected on a Jasco J-1500 (Hachioji, Tokyo, Japan) CD spectropolarimeter. Unless otherwise stated, parameters were set as HT = 600 V, 2 nm bandwidth, 2 s response time and 200 nm/min scan speed or 4 s response time and 100 nm/min scan speed.[19] The number of scans was chosen to give good signal:noise without wasting instrument time. More scans would have improved signal:noise levels. Nearly all data reported were collected in a custom-made microvolume capillary Couette cell (path length 0.5 mm, Crystal Precision Optics Ltd., Rugby, UK).[16,17] The Couette flow cell, illustrated in Figure 1B, induces Taylor–Couette flow. Unless otherwise stated, LD and FDLD spectra are the differences between 2.9 (~1000 rpm) and 0 V (0 rpm) spectra. Longpass filters were chosen to be beyond the absorbance wavelength range of the analyte of interest but as close to it as possible to maximize the photons collected. The filter was affixed to the exit window of the Couette cell with BluTack™. The orientation of the filter was adjusted to give as symmetric a beam image as possible. Ideally, the optical density of the filter should be at least 6, but, sometimes, 4 was the best we could find. In one case, we piggybacked two filters to increase the optical density while being aware that every additional optical element reduces the fluorescent photon count. The CD spectrum was collected in the Couette flow cell and in a 0.5 mm DMVBioCell.[19]

2.3 | Understanding the FDLD experiment

With the Jasco CD spectropolarimeters we have used to develop FDLD, we have the choice of working in fixed DC or fixed HT modes. Fixed DC is the normal operating mode for the Jasco instruments, tends to be less noisy, and gives greater dynamic range. With LD,

$$LD = \log_{10}\left(\frac{2+\mathrm{AC/DC}}{2-\mathrm{AC/DC}}\right) \approx \frac{\mathrm{AC}}{\mathrm{DC}}\log_{10}e$$
$$LD(\mathrm{DC}=1) \approx \mathrm{AC}\log_{10}e \tag{3}$$

where AC denotes alternating current and is the difference in signal measured for the two polarizations of light incident on the sample and DC denotes direct current, which is the sum. Equation (3) is what is usually plotted to give the LD trace. When we insert a filter to perform the FDLD experiment, the instrument still plots the same signals, but it is no longer measuring LD. The derivations of Wemyss et al.[15] lead to the following equations. If we use LD^F to denote what is plotted as LD when a filter is inserted for fixed DC and fixed HT modes, respectively,

$$LD^F(LD\,\mathrm{trace}, \mathrm{fixed\,DC}, \mathrm{filter}) \approx -2\log e J_2(\delta_0)\frac{LD}{\left(A_Z^{ex}+A_Y^{ex}\right)} \tag{4}$$

$$\begin{aligned} &LD^F(LD\,\mathrm{trace}, \mathrm{fixed}\,HT, \mathrm{filter}) \\ &\approx -\phi I_0 \ln(10) J_2(\delta_0) 10^{-\left(A_Z^{ex}+A_Y^{ex}\right)/2} LD \log(e) \\ &\approx -\phi I_0 J_2(\delta_0) LD \end{aligned} \tag{5}$$

where J_2 is the second order Bessel function of the first kind and δ_o is the maximum phase-shift the photoelastic modulator produces between the component of the incident light parallel to its stress axis compared with the component perpendicular to that axis. Thus, the fixed DC mode is a ratio of LD and absorbance (i.e., proportional to the reduced LD of the oriented sample), whereas the fixed HT retains the dependence on the quantum yield (ϕ) and I_o the incident intensity (which may be changed by the bandwidth and any masking of the cell) and follows the shape of the LD.

Since we can measure the absorbance by removing the filter and doing an LD experiment, at first sight, the fixed DC mode is the more attractive option. In our previous flow FDLD work (on DNA dyes and bacteriophage), we collected data in fixed DC mode.[13] However, it was only at high fluorophore concentrations that we actually measured the ratio signal of Equation (4). At lower concentrations, the fluorophore absorbance was dominated by stray light (including leakage through the filters and imperfections in the capillary and rod), so we measured a signal that was proportional to the inverse of the LD signal. In the case of liposomes, the orientation parameter is smaller than for DNA (~0.03[20] vs. ~0.1) and the light scattering larger. We therefore used the fixed HT mode in this work, unless otherwise stated, and so Equation (5) is appropriate.

3 | RESULTS AND DISCUSSION

3.1 | Gramicidin

Gramicidin D is a mixture of gramicidins A (80%), B (6%), and C (14%). These gramicidins are 15 residue linear peptides with alternating D and L amino acids including four tryptophans, which assemble in lipid bilayers into a helix.[5] The CD spectrum in Figure 2 has a sharp negative band at 230 nm indicating the gramicidin has adopted the intertwined double helix form.[22]

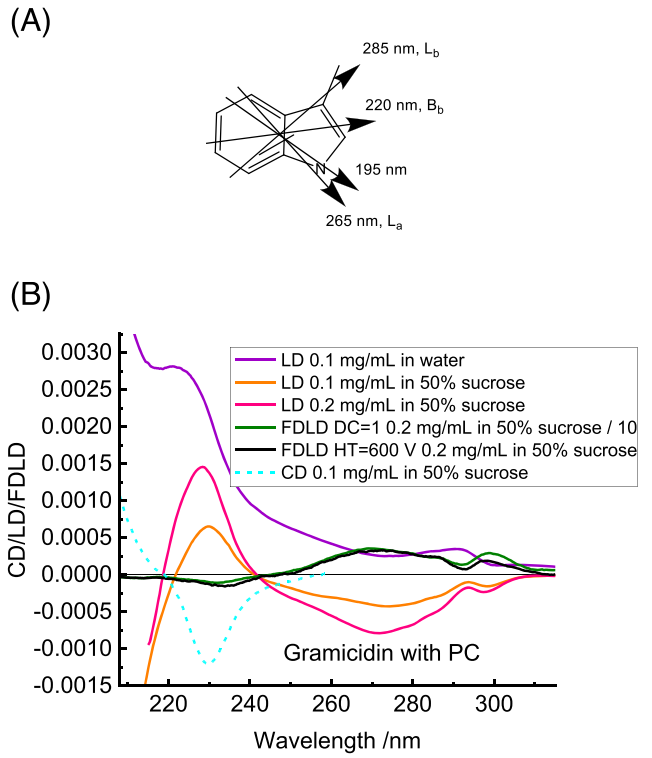

FIGURE 2 (A) Tryptophan transition polarizations as deduced from Norden et al.[21] and presented in Albinsson et al.[6] (B) Couette flow oriented fluorescence detected linear dichroism (FDLD) and linear dichroism (LD) of gramicidin with soy bean phosphatidyl choline (PC) at molar ratio ~1:50. LD and FDLD spectra are the differences between rotating (2.9 V, 1000 rpm) and non-rotating Couette flow. Circular dichroism (CD) and LD spectra were collected with direct current (DC) = 1 V for four scans with response time of 2 s and 2 nm band width. CD and LD are plotted in absorbance units. The FDLD spectra were collected with either high temperature (HT) = 600 V or DC = 1 for 22 or 18 scans with response time of 4 s, 2 nm band width, and a 325 nm Semrock EdgeBasic BLP01-325 long pass filter inserted on the exit side of the Couette cell.

Figure 2 shows the first liposome/peptide FDLD spectra (HT fixed at 600 V and 2 nm bandwidth and DC fixed at 1) ever to be published. All bands are at somewhat longer wavelengths than those of isolated tryptophans[6,21,23] (Figure 2A). The DC = 1 spectrum is an order of magnitude more intense than the fixed HT spectrum (whose DC ranges between 0.08 and 0.23). This spectrum divided by its DC trace overlays the fixed HT spectrum (data not shown). The FDLD of gramicidin in PC liposomes shows a clear broad positive signal centered at 274 nm for the tryptophan L_a band in accord with the average L_a band being parallel to the helix axis and the lipids. The components of the L_b band are apparent as less positive (i.e., negative) dips at about 293 nm and 283 nm tryptophan in accord with

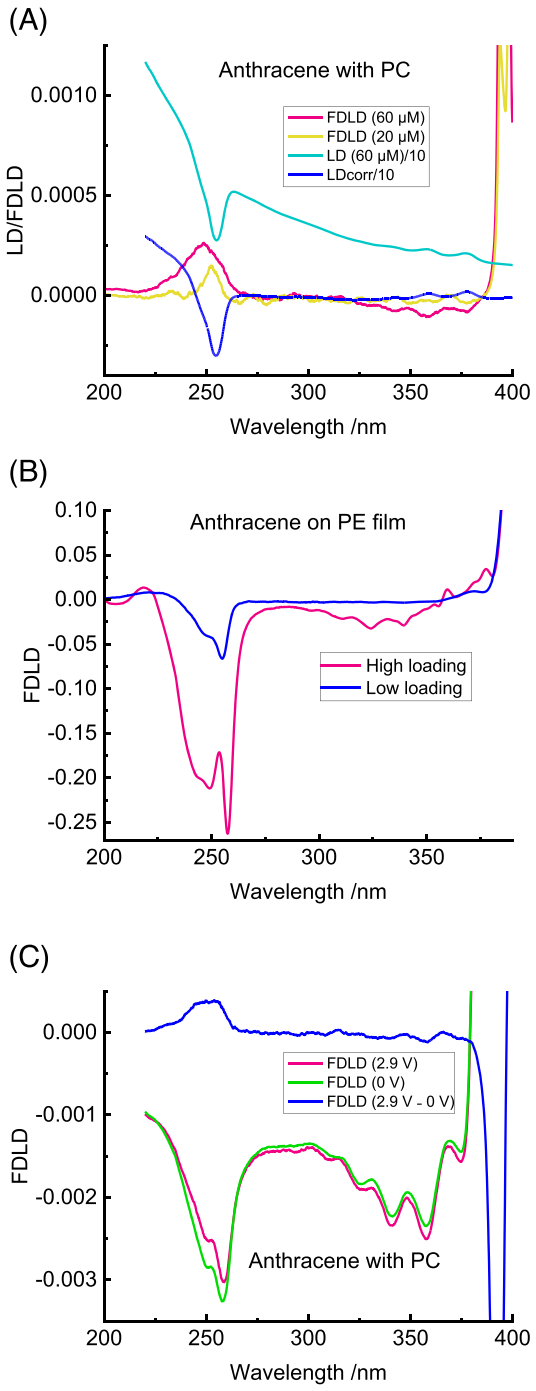

FIGURE 3 Legend on next page.

FIGURE 3 (A) Couette flow oriented fluorescence detected linear dichroism (FDLD) (high temperature [HT] = 600 V) and linear dichroism (LD) spectra of anthracene in phosphatidyl choline (PC) liposomes at molar ratio 1:100; 400 nm Thorlabs and 409 nm Semrock long pass filters were inserted on the exit side of the Couette cell for FDLD. LDcorr/10 is the LD spectrum minus 4.5xλ−2.8 (where λ is wavelength making an approximate correction for scattering) divided by 10; 2 nm bandwidth, 2 s response time, 30 scans. (B) FDLD of anthracene on stretched polyethylene (PE) film at different loadings; 400 nm Thorlabs and 409 nm Semrock long pass filters. HT = 600 V. (C) FDLD (2.9–0 V) of anthracene (80 μM) in PC liposomes (1:100) and the rotating and non-rotating raw data; 2 nm bandwidth, 2 s response time, eight scans.

these tryptophans transitions poking out from the helix. The 230 nm tryptophan B_b band is also negative. These signs mirror the LD spectra of Figure 2 and of Svensson et al.,[22] though the 230 nm FDLD is relatively smaller reflecting its lower quantum yield. Interestingly, the FDLD shows slightly better resolution (larger relative magnitude) of the L_b components under the L_a band than the LD, presumably reflecting different quantum yields of the two bands. In the FDLD, we only see tryptophan bands as it is the fluorophore, so the Figure 2 mirroring of the FDLD and LD spectra confirm the careful arguments of Svensson et al.,[22] which concluded that the 230 nm LD band belongs to tryptophan, not the protein backbone. CD spectra are in mdeg, and LD is reported as unitless being an absorbance and the FDLD is a photon count, which varies with the choice of parameters.

There are three gramicidin LD spectra available in the literature, one with PC lipids[5] and the other two with DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine) lipid vesicles.[4,22] The PC spectrum of Hicks et al.[5] shows a sloping baseline due to scattering as does the top trace in Figure 2. Following Svensson et al.,[22] we also measured the LD in 50% sucrose to match the liposome and solvent refractive indices, thus removing the scattering. The presence or absence of sucrose has no impact on the FDLD (data not shown)—scattering is not an issue for FDLD, which is one of its major advantages. However, as discussed further below, the fact that the filters are not perfect means that the FDLD plotted in Figure 2 is a small difference (<10%, see below) between the rotating and non-rotating spectra, which are themselves differences between horizontal and vertical polarized spectra. Repeat spectra all show broadly the same features but we are pushing the limits of the instrument. Careful positioning of the filter was essential for good data. Gramicidin (0.2 mg/mL) only increases the lower wavelength cut-off by ∼2 nm relative to 0.1 mg/mL.

3.2 | Anthracene FDLD

In order to understand flow-oriented liposome FDLD spectrum better, we collected data on anthracene embedded in PC liposomes. Anthracene has a distinctive spectral pattern, and despite its complexity,[14,18] its spectroscopy is quite well understood. The FDLD (HT fixed at 600 V) of anthracene in flow-oriented PC liposomes is overlaid with the LD of the same sample in Figure 3A. Although reduced LD values at two wavelengths are given in Ardhammar et al.[24] and film LD and FDLD data were given in Wemyss et al. and Razmkhah et al.,[14,18] this is the first published LD spectrum of anthracene flow oriented in liposomes as well as the first flow FDLD of anthracene. Assuming anthracene inserts parallel to the lipids, we expect the 254 nm long axis LD to be negative and the 380 nm short axis LD to be positive and conversely for the FDLD. This is precisely what is observed in Figure 3A (see below for further discussion of the data).[18]

To show the value of the absence of any scattering effects in FDLD, an LD spectrum of the same sample as the FDLD that has been corrected by approximate removal of the turbidity contribution to the LD[25] to help separate the absorbance LD from the scattering is also shown in Figure 3A.

There are some interesting features about the anthracene FDLD experiment that should be noted. First, although we saw the sign patterns we expected based on transition polarization, this is not what is observed with stretch polyethylene film orientation. On the film, the 254 nm band couples with the short axis band (300–400 nm) sufficiently strongly to cause the short-axis LD to be positive[14] and the FDLD to be negative below 359 nm (Figure 3B). In the lipidic environment, however, the coupling with the higher energy transition is suppressed to the extent that the LD of all the components of the short axis band are the same sign. Second, there is no evidence of the π–π stacking interactions between anthracenes that on the film gives a sharp dip in the spectrum at 254 nm (Figure 3B) at high loading.[18] Finally, we should note that the higher concentration samples (Figure 3A,C) show a distorted 254 nm peak, indicating the importance of variable concentration experiments.

Figure 3C shows the raw data for a rotating and non-rotating experiment overlaid on their difference, which is the FDLD spectrum. The raw data are dominated by the photons that leak through the filters with the leakage being negative indicating that more *Z*-polarized (horizontal) photons do not reach the detector than *Y*-polarized (due to the imperfections of the Couette cell, predominantly the rod). Working in

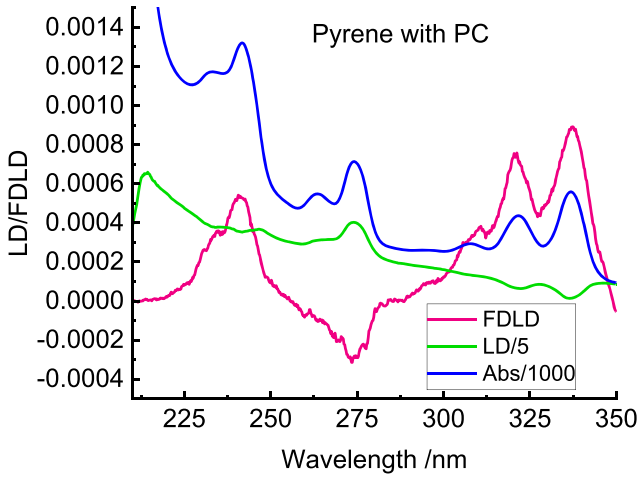

FIGURE 4 Couette flow fluorescence detected linear dichroism (FDLD) (high temperature [HT] = 600 V), linear dichroism (LD), and absorbance of pyrene (140 mM) in phosphatidyl choline (PC) liposomes (1:100 ratio). Spectra were collected for six scans with response time of 2 s and 2 nm band width. A 355 nm Semrock long pass filter was used to collect the FDLD spectrum.

fixed-HT mode means one does not need to consider the stray light contributions to the denominator in Equation (4).

3.3 | Pyrene FDLD

As a final example, we measured the flow LD and FDLD of pyrene in PC liposomes (Figure 4). A pyrene flow LD spectrum has been published,[24] and our LD spectrum is consistent with that, though shows much less scattering (due to the design of the microvolume Couette cell) and much better signal to noise resulting from instrumentation developments in the last 20 years. As a consequence of these instrument developments, we have collected data below 250 nm, resulting in an extra band being observed. The FDLD mirrors the LD as expected, without the complication of a sloping baseline. The 264 nm peak in the absorbance spectrum (blue) is much more of a shoulder in the LD and FDLD presumably reflecting vibronic coupling with the opposite signed higher energy (240 nm) band as observed by Thulstrup et al. in their 1970 publication.[26]

4 | CONCLUSION

In this work, we have shown how FDLD can be measured for analytes embedded in liposomes in order to detect and understand the orientations of fluorophores bound to the membranes. The FDLD spectra selectively probe any fluorophore(s) in the membrane and eliminate the scattering that is apparent in the corresponding flow LD spectra. The FDLD spectra are opposite in sign from the corresponding LD spectra, though the relative magnitudes of bands are modified by the quantum yields of the transitions. In this proof of concept work, we presented data for the linear peptides in gramicidin D and two aromatic analytes, anthracene and pyrene. The leakage of photons through the long pass filters used and the differential scattering of the linearly polarized photons by the Couette cell mean that the FDLD is the small difference between two larger signals measured for the rotating and non-rotating Couette cell. This meant that a fixed high tension voltage mode rather than the more commonly used fixed DC mode has been used with these samples. This has the advantage of giving a spectrum that is proportional to the LD spectrum but the disadvantage of a reduced dynamic range. The use of a CD spectropolarimeter that has been adapted to also measure LD spectra for this work means it is only possible to measure excitation spectra.

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DATA AVAILABILITY STATEMENT

Data may be obtained from the corresponding author.

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