Breaking the 200 nm Limit for Routine Flow Linear Dichroism Measurements Using UV Synchrotron Radiation

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ABSTRACT   The first synchrotron radiation flow linear dichroism spectra are reported. High-quality spectral data can be collected from 450 nm down to 180 nm in contrast to the practical cutoff of ~200 nm on benchtop instruments. State-of-the-art microvolume capillary Couette flow linear dichroism was successfully ported to a synchrotron radiation source. The sample volume required is < 50 μL. A characterization of the synchrotron radiation linear dichroism with known DNA and DNA-ligand systems is presented and the viability of the setup confirmed. Typically, wavelengths down to 180 nm are now routinely accessible with a high signal/noise ratio with little limitation from the sample concentration. The 180 nm cutoff is due to the quartz of the Couette cell rather than the beamline itself. We show the application of the simultaneous determination of the sample absorption spectrum to calculate the reduced linear dichroism signal. Spectra for calf thymus DNA, DNA/ethidium bromide, and DNA/4′,6-diamidino-2-phenylindole systems illustrate the quality of data that can be obtained.

INTRODUCTION

All biological processes are fundamentally interactions between molecules, mainly between macromolecules or between macromolecules and small molecules. Despite significant advances in our ability to characterize such interactions and the single-molecule revolution that is taking place, we still struggle to measure key properties of molecular interactions. The work reported in this study directly addresses this need. We have developed a novel implementation of flow linear dichroism (LD) that uses small sample volumes (< 50 μL) in a synchrotron beamline to quickly and easily give LD spectra of high quality down to 180 nm. Our current limitation is the quartz of the cell rather than the synchrotron, and so we anticipate being able to go much lower in wavelength in the not too distant future.

Recent applications of microvolume Couette flow LD have confirmed that the technique can be used to investigate a wide range of systems providing relative orientations of chromophores (1–5). Most importantly, it can be applied to significant classes of biological molecules that have been difficult to study by established structural techniques such as crystallography and NMR. These classes include long polymeric structures such as DNA (6,7), DNA-ligand complexes (8,9), fibers including fibrous proteins and peptides (10–12), and membrane proteins (13,14). The desire to probe the time dependence of interactions including enzyme kinetics, fiber assembly, or protein insertion into membranes introduces an added dimension of complexity. Ultraviolet (UV) LD of solutions has gained a wider audience with the advent of dedicated Couette cell units. Major hurdles have now been overcome mainly with respect to the sample size and volume,

hence allowing routine measurements to be performed on < 50 μl volumes (Supplementary Material, Data S1) of often valuable and hard to obtain proteins and DNAs (1–4).

LD is the difference in absorbance of light polarized in planes parallel ($A_{//}$) and perpendicular ($A_\perp$) to the direction of orientation, as follows (15):

$$LD = A_{//} - A_\perp. \tag{1}$$

The key information that LD provides is the orientation of subunits of a system with respect to the orientation axis—which, in the case of flow orientation, is the long axis of the system (see Fig. S3 in Data S1). LD can be used to deduce kinetic and structural information about a wide range of systems; the only requirements are that the systems can absorb radiation in the accessible wavelength range and can be oriented. Flow LD requires samples to be long enough to be oriented by shear forces in solution and is ideally suited to polymeric DNAs and fibrous proteins; lipid-bilayer vesicles (liposomes) can also be aligned in shear flow because the liposomes elongate in the flow. LD, as with other spectroscopic techniques, may have samples in near-native environments. With the use of UV-visible flow LD on an adapted Jasco J-715 circular dichroism spectropolarimeter (Jasco, Tokyo, Japan) or a Bio-Logic Modular Optical System 450 (MOS-450; Bio-Logic, Grenoble, France), we can probe the interaction over a wide wavelength range (ideally 800−185 nm, although in practice the lower limit is often above 200 nm). Thus, polarized spectroscopy can be used to provide more information about the nature of the biomolecules and their interactions (1).

It has recently become apparent to us, especially with samples in which the solvent/buffer absorbs or when the samples scatter light (both of which are frequent problems for liposome/membrane protein systems and fibers of any kind), that we cannot collect sufficiently high-quality data at the low

wavelength end of the spectrum to probe intermolecular interactions on our existing instrumentation. The obvious solution was to have a much-increased light throughput such as is provided by a synchrotron light source. The ASTRID synchrotron source (ISA, Denmark) has been established as a light source for circular dichroism spectroscopy that is simple to use, and its data for this purpose have been validated (17). Thus, adapting the ASTRID circular dichroism beamline for LD seemed the best way forward. In this study, we report how the beamline and sample holders were modified and show the success of this method using DNA and DNA-ligand systems (see Fig. S1 in Data S1).

RESULTS AND DICUSSION

The first phase of the work was to determine whether the Astrid synchrotron radiation (SR) LD system was consistent with available benchtop instruments. Fig. 1 shows an overlay of DNA LD collected using the SR high-energy grating with the DNA LD spectrum collected from the Jasco J-715 in-
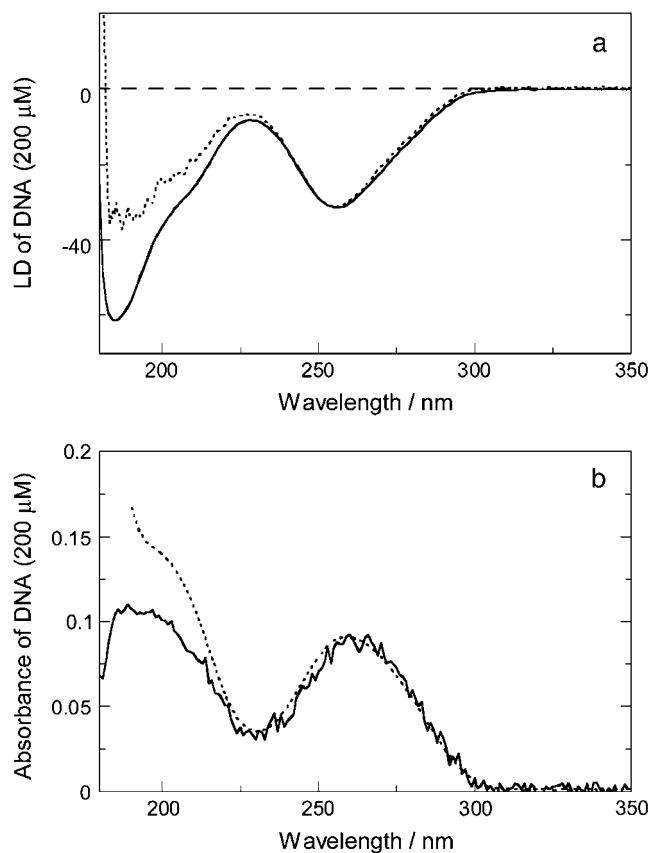

FIGURE 1 (*a*) LD of calf thymus DNA (200 μM base) from SRLD collected with the high-energy grating setup (*solid line*) in the same microvolume Couette flow cell (500 μm pathlength; Crystal Precision Optics, Rugby, England; also available from Kromatek, Essex, UK) as LD of ct-DNA from a Jasco J-715 spectropolarimeter (Jasco) (*dotted line*). (*b*) Corresponding absorbance calculated from SRLD (Data S1, *solid line*) and measured on a Jasco V-550 (Jasco) (1 mm pathlength, *dotted line*).

strument (Jasco) located at the University of Warwick. The main differences between the SR and conventional instrument data are at low wavelengths (Fig. 1). The apparent cutoff for the Jasco J-715 (Jasco) spectrum is $\sim$190 nm (as determined by the high-tension voltage being < 600 V, but see comment below) and that of the SRLD is 180 nm. In the case of the Jasco circular dichroism spectropolarimeter (Jasco), the spectrometer is the limiting factor; in the case of the SR system, the cutoff was due to the quality of the quartz cell in the cell, not the beamline radiation or optics. The SRLD signal/noise ratio was extremely good right down to the cutoff point. It is, however, apparent that the two spectra do not overlay below $\sim$210 nm, which means at least one of them is not providing a true spectrum. In our attempts to collect DNA LD data below $\sim$210 nm on benchtop instruments (Jasco J-715 and Bio-Logic MOS-450), we have found that the magnitude of the LD signal is variable and becomes larger (that is, more negative) the better we focus all the light onto the photomultiplier tube and remove stray light. Because light throughput was not an issue for the SR system, we believe the SR spectrum is the true one. In support of this conclusion, we note that Dougherty, Causley, and Johnson (18) measured the flow LD of three bacterial DNAs to just < 180 nm and, in each case, the $\sim$185 nm band was approximately twice the magnitude of the 260 nm band as is the case for our SRLD data but not the bench top data. The shoulder observable in the SRLD spectrum at 205 nm illustrates the high sensitivity of the SRLD signal because, essentially, no noise masks the spectral structure. The signal/noise ratio of the SRLD means that the dynamic range of LD is orders of magnitude larger than on a benchtop machine over the entire range of the grating being used. In other words, it is now possible to simultaneously monitor transitions associated to chromophores in varied concentrations such as transitions in aromatic amino acids (a few percent of residues) and in the amide chromophores of the backbone (100% of residues) without having to change the cell pathlength or sample concentration.

To probe the full wavelength range that we anticipated would be usable from the SRLD system as currently configured, we measured the spectrum of two DNA-ligand systems. Ethidium bromide is an intercalator whose transitions, when bound to DNA, are oriented perpendicular to the DNA helix axis and so giving negative LD signals. Fig. 2 *a* shows an overlay of DNA plus ethidium bromide collected with the high-energy grating and with a Jasco J-715 spectropolarimeter. The longest wavelength band of ethidium bromide when bound to DNA occurs at 516 nm (2). This wavelength is not accessible to the SRLD system, and so the focus of our analysis is the ethidium bromide bands that appear as long wavelength shoulders on the 260 nm DNA band. The significant result is that the benchtop and SR data agree from 350 nm to 220 nm, which is the overlap region of the high-energy grating used on the SRLD and the reliable J-715 (Jasco) wavelength (see above). The data collected for 4′,6-dia-midino-2-phenylindole (DAPI, Fig. 2 *b*), a DNA groove
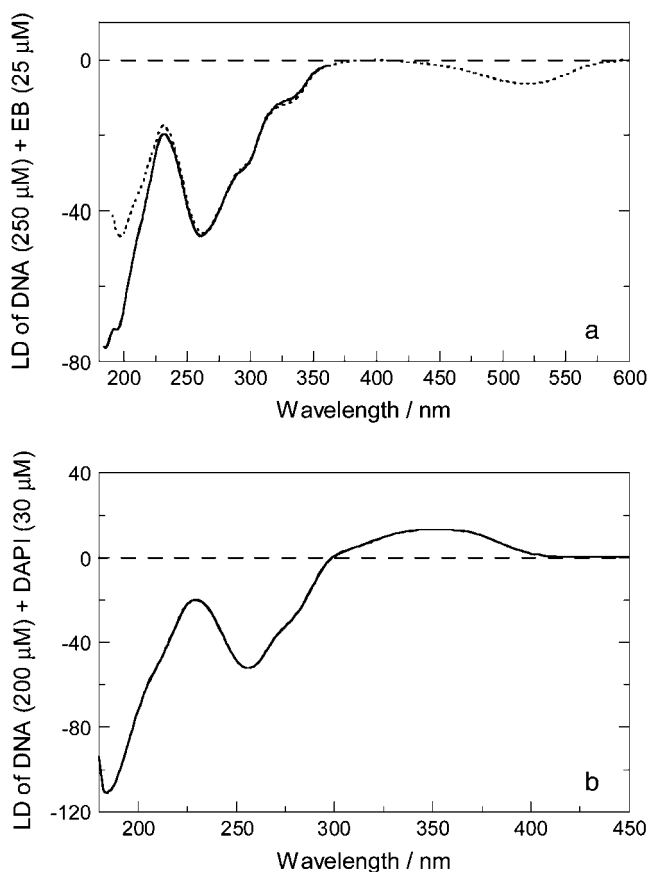
FIGURE 2   LD of calf thymus DNA with a ligand in a microvolume Couette flow cell measured on the SRLD setup (*solid line*) and on a Jasco J-715 spectropolarimeter (*dotted line*). (*a*) ct-DNA (250 μM) + ethidium bromide (EB, 25 μM) using the high-energy grating setup. (*b*) ct-DNA (200 μM base) + 4′,6-diamidino-2-phenylindole (DAPI, 30 μM)) using the low-energy grating setup.

binder, were obtained with the low-energy grating to enable a wider wavelength range from 450 nm to 180 nm without any apparent reduction in data quality. The long axis of DAPI lies along the DNA minor groove making an angle of ~45° to the helix axis (19). Thus, its long axis polarized transition at 350 nm has a positive LD signal as illustrated in Fig. 2 *b*.

CONCLUSION

In conclusion, we have shown that flow LD using a microvolume cell can be successfully adapted to a synchrotron radiation source. SRLD spectroscopy allows reliable data collection to much lower wavelengths than benchtop instruments with good signal/noise performance until the quartz cutoff point. The lower wavelength limit is currently determined by the quality of the quartz components of the cell to be 180 nm, and the upper wavelength limit is the 450 nm of the available low-energy grating. The significantly extended wavelength and dynamic range expands the possibilities for DNA-drug systems and their structural characterization. Perhaps

more significantly, the proof of principle using DNA opens up the field to applying SRLD to other samples that can be oriented but in which the low wavelength cutoff is particularly limiting. We anticipate that studies on membrane peptide and protein systems will benefit most from this principle because their key transitions lie below 210 nm, whereas the practical LD cutoff for model membrane systems on a benchtop instrument is typically well above this wavelength (20). Access to the n-π* bands to gain information on the environment effects (21), as well as to the stronger π-π* transitions characteristic of the structural content (4), will provide a more complete picture of the behavior of biomolecules in solution. Work is in progress to introduce quartz components into the microvolume LD cells that will extend the wavelength range significantly with anticipated further increases in information available. The demonstrated added value of synchrotron radiation flow LD for a wide range of samples will open the doors in a way similar to the revolution started with synchrotron radiation circular dichroism. The significance of this approach is illustrated by the increasing number of synchrotron circular dichroism facilities worldwide, all of which can potentially be adapted for LD.

SUPPLEMENTARY MATERIAL

To view all of the supplemental files associated with this article, visit www.biophysj.org.

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