

Abstract: This application note demonstrates the use of the High Shear Couette Cell Accessory in performing linear dichroism experiments. Linear dichroism (LD) is an established technique for investigating the binding mode of ligands to DNA or proteins at lipid bilayers, for example. Applied Photophysics' high shear Couette Cell Accessory can be used to align biomacromolecules or deform vesicles, producing the anisotropy that is needed for an LD measurement. The LD signal is then used to deduce the orientation of absorbing species relative to the shear direction, and hence to the DNA axis or the membrane.

Authors:

BERNARD COSTELLO, PhD

Applied Photophysics Ltd.

MARTIN TEXTOR

Molecular Biophysics, University of
Kaiserslautern, Germany

SANDRO KELLER, PhD

Molecular Biophysics, University of
Kaiserslautern, Germany

CHIRASCAN SERIES APPLICATION NOTE

Linear Dichroism Applications with the High Shear Couette Cell Accessory (CCA)



INTRODUCTION

Many drug regimens include a compound that binds to and/or modifies DNA. In order to optimise the efficacy of drugs, as well as discover new drugs, it is important to decode the structural details of ligand-receptor complexes, to understand fully the binding interactions between the two entities.

More than 30% of the human genome codes for integrally bound membrane proteins, while at least as many again are likely to code for proteins that are peripherally bound. The mode of membrane binding of these proteins is therefore crucial to their function. Additionally, many drugs are proteins or peptides that adsorb to membranes or at receptor molecules that are attached to membranes, and the mode of action and potency of these drugs depends on how they interact with the membrane^[1]. Linear dichroism is able to distinguish between integral and peripheral protein membrane binding modes.

Linear dichroism (LD) is the difference in absorbance between light parallel to and perpendicular to an orientation axis^[2]. The anisotropy in a sample that gives rise to an LD response can be either intrinsic, as in liquid crystals for example, or induced, for example by an applied electric field or shear field.

KEYWORDS

▶ Linear Dichroism	▶ DNA-Ligand binding mode
▶ Couette Cell Accessory	▶ Retinoic Acid - Lipid Bilayer binding mode
▶ Shear	
▶ Alignment	

Linear dichroism is used to determine the direction of electronic transitions in oriented samples. This enables the binding mode of a ligand to DNA or of a protein at a lipid bilayer, for example, to be deduced. Here we give examples of both these applications.

LD has many other applications. Rodger and co-workers have used this technique to investigate the persistence length of DNA^[3]. This is an important property of polymers that depends on their flexibility and curvature; the persistence length of DNA is affected by its environment, the ionic strength and the ligands to which it is bound, for example. Nordén and co-workers used LD to help solve the filament structure of a human Rad51 protein^[4]. The Applied Photophysics high shear Couette Cell Accessory can be used to investigate the effect of temperature on each of these, and the effect of shear rate on the structure of biomacromolecules.

In this application note we show that linear dichroism spectra can be measured to wavelengths down to 180 nm, generating shear rates of up to 10,200 s⁻¹. We use the examples of ethidium bromide binding to calf thymus DNA and retinoic acid binding at a lipid bilayer to show how the binding modes can be deduced from LD measurements made using the Couette Cell Accessory.



Figure 1. Two-dimensional representation of a shearing motion.

A shearing motion is shown in two dimensions in Figure 1, where the rectangle, of height y , deforms to the parallelogram, with the top edge of the rectangle moving by a distance Δx in the direction of the arrow. The shear strain, γ , is then defined as $\Delta x/y$, and the shear rate, $\dot{\gamma}$, as $d\gamma/dt$, in the limit as y goes to zero. The strain is therefore dimensionless, and the shear rate has dimensions of reciprocal time (s⁻¹ in the S.I. system).

Shearing can cause large particles in the liquid to align or deform^[5]. For example rigid, rod-like, particles such as carbon nanotubes and glass fibres will align, whereas vesicles, micelles and flexible polymers will deform from their equilibrium conformations, extending in the direction of shear.

Linear dichroism is then used to determine the direction of net electron transfer in an absorbed chromophore relative to the shear direction, and thereby to the alignment direction of the absorbing species or membrane.

HIGH SHEAR COUETTE CELL ACCESSORY APPLICATIONS

Linear dichroism spectra and simultaneously measured absorbance spectra for calf thymus DNA at a concentration of about 0.12 mg/ml in water are shown in Figure 2.

The shear rates were from zero to 10,200 s⁻¹, the spectrum changed only very slightly at shear rates above 6000 s⁻¹ suggesting that full extension and alignment of the DNA molecules had been achieved. The wavelength range was from 360 to 180 nm, in increments of 1 nm, at a bandwidth of 1 nm and a temperature of 20°C. Each spectrum took about 2 minutes to acquire; the averages of three spectra at each shear rate are shown.

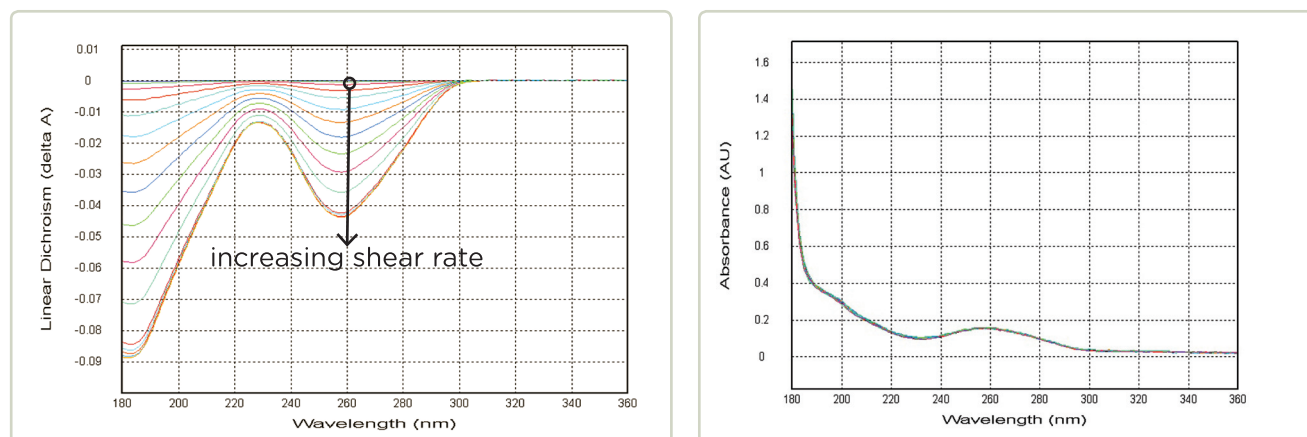


Figure 2. Left: LD spectra of calf thymus DNA in water at shear rates from 0 to 10. Right: the simultaneously measured absorbance spectra.

1. BINDING MODE OF ETHIDIUM BROMIDE LIGAND TO CALF THYMUS DNA

The original work on ethidium bromide (EtBr) binding to DNA using linear dichroism was performed by Tuite and Nordén^[6]. Linear dichroism was able to distinguish between the intercalation binding mode and the other general ways in which a ligand may bind to DNA, external binding to the phosphate backbone, binding in the minor groove, or binding in the major groove^[7].

Materials

Calf thymus DNA (ctDNA) sodium salt supplied by Sigma Aldrich Ltd was used throughout. DNA is a semi-flexible molecule with a persistence length obtained from light scattering of about 53 nm, i.e. around 150 base pairs^[8]. Since the number of base pairs is usually much greater than 150, DNA is easily extended by shearing.

Ethidium bromide, EtBr, was supplied by Sigma Aldrich Ltd. EtBr is a planar aromatic molecule (Figure 3).

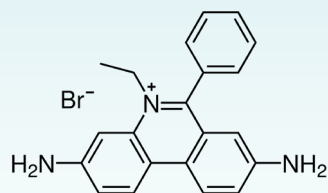


Figure 3. Chemical structure of ethidium bromide. Source: Wikimedia Commons.

Results

Results for EtBr binding to ctDNA are shown in Figure 4. DNA concentration was about 0.12 mg/ml in purified water, ethidium bromide concentrations from zero to about 70 μM . Shear rate was 240 s^{-1} , wavelength range 180 to 360 nm, extended to 600 nm for 50 and 70 μM EtBr, in increments of 1 nm, at a bandwidth of 1 nm and temperature of 20°C. Each spectrum took about 2 minutes to acquire; the averages of two spectra at each shear rate are shown.

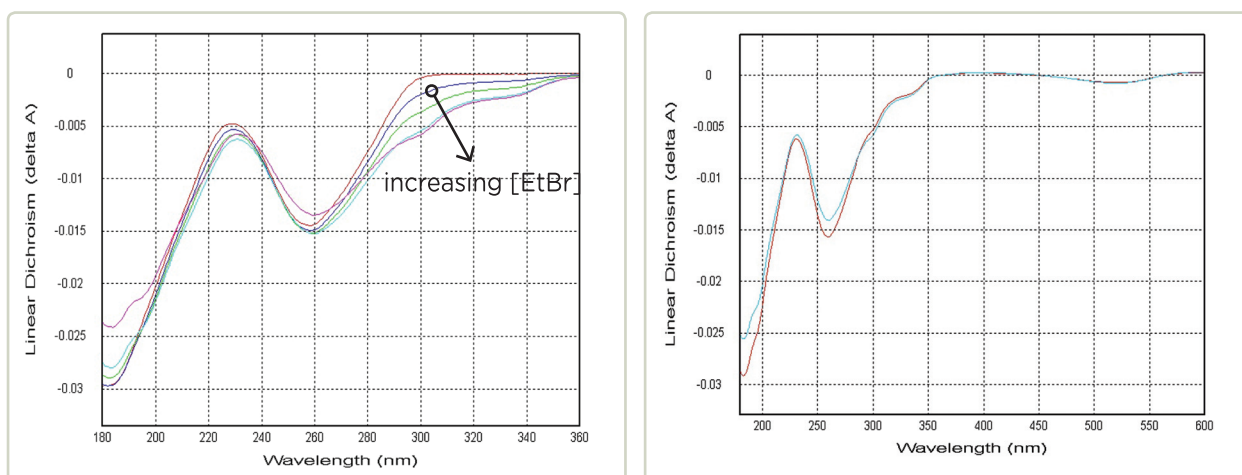


Figure 4. LD spectrum of ctDNA with intercalated ethidium bromide.

Data Interpretation

The negative peak at about 260 nm in the DNA spectrum without ligand indicates that the π - π^* transitions in the DNA bases at that wavelength are more perpendicular than parallel to the DNA helix axis. The transitions of EtBr enhance the negativity below 300 nm, and produce an additional negative peak at about 520 nm, indicating that its transitions are approximately parallel to the DNA bases, i.e. perpendicular to the orientation of the DNA long axis.

This suggests that the absorbance of EtBr on DNA is by intercalation between the base pairs. But note that the intercalation appears to saturate at a concentration of between 50 and 70 μM EtBr, resulting in a diminution of the negative 260 nm peak.

2. BINDING MODE OF RETINOIC ACID AT A LIPID BILAYER

Retinoic acid is a stiff, unsaturated molecule that may plausibly adsorb either at the bilayer surface, oriented in a direction parallel to the plane of the bilayer, or within a lipid leaflet, oriented in the direction perpendicular to the bilayer. If the bilayer forms the membrane of a spherical vesicle, then both orientations are globally randomised, and total linear dichroism response is zero. However, if the vesicle is deformed to an ellipsoid by shearing, then there is a globally preferential orientation of the retinoic acid, and the LD response becomes nonzero.

We used linear dichroism to confirm the binding mode of retinoic acid at a palmitoyl-oleoyl-phosphatidylcholine (POPC) bilayer, formed by extrusion as a large unilamellar vesicle (LUV) membrane.

Materials

The structures of retinoic acid and POPC are shown in Figure 5.

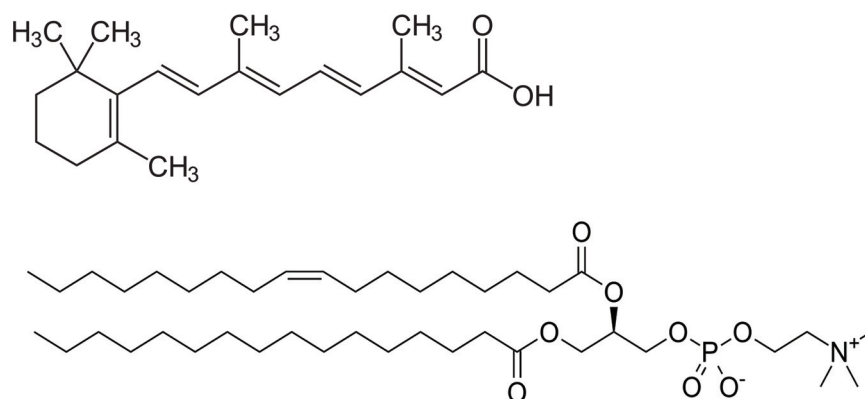


Figure 5. Chemical structures of retinoic acid (top) and POPC (bottom). Source: Wikimedia Commons.

Results

Results for retinoic acid binding to POPC vesicles are shown in Figure 6. Retinoic acid concentration was 50 μM , lipid concentration 5 mM, in 40% sucrose solution. Shear rate range was from 480 to 3360 s^{-1} , wavelength range was 250 to 450 nm.

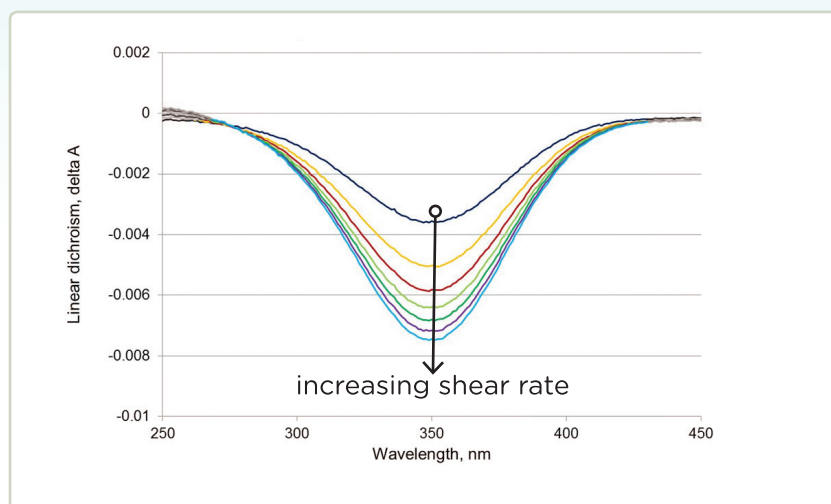


Figure 6. LD spectrum of retinoic acid in a POPC LUV membrane at various shear rates.

Data Interpretation

The negative peak at about 350 nm in the retinoic acid LD spectrum increases with shear rate, suggesting that the molecule is becoming preferentially orientated as the vesicle deformation increases. The negative sign of the peak indicates that the orientation is perpendicular to the shear direction, suggesting that the retinoic acid is absorbed perpendicularly to the plane of the bilayer, i.e. parallel to the orientation of the lipid acyl chains. In fact, it is known that retinoic acid absorbs in this way, rapidly flipping between the inner and outer leaflets^[10]. This allows retinoic acid to be used as a calibrant for the degree of vesicle deformation during shearing.

SUMMARY

The Applied Photophysics' high shear Couette Cell Accessory (CCA) can be used to align biomacromolecules or deform vesicles by shearing. Linear dichroism (LD) spectroscopy can then be used to deduce the orientation of absorbing species relative to the shear direction, and hence to the long axis of the aligned molecule or to the vesicle membrane.

The technique has been used to deduce the binding mode of ligands on DNA or proteins at vesicle membranes amongst other applications. Here we use the examples of an ethidium bromide ligand absorbed to DNA, and of retinoic acid adsorbed to a lipid bilayer membrane, to demonstrate the process. In both cases the negativity of the LD signal suggested that the direction of net electron transfer in the absorbing species was perpendicular to the shear direction. This confirmed that ethidium bromide is absorbed by intercalation between DNA base pairs, and that retinoic acid is absorbed perpendicularly to the plane of the membrane.

The Couette cell operates at shear rates of up to $10,200 \text{ s}^{-1}$, and to wavelengths down to 180 nm. The low gap to radius ratio ensures that the shear rate is almost independent of position within the cell, which is necessary for homogeneity throughout the sample and for comparison with other techniques. The cell temperature is controlled by thermostated circulating water closely coupled to the sample, to maintain a set temperature and avoid shear heating of the sample. The CCA offers the unique capability to simultaneously measure absorbance and linear dichroism and it allows a standard CD cell to be easily fitted in place of the LD cell to conduct CD measurements for comparative work.

APPENDIX 1:

HIGH SHEAR COUETTE CELL ACCESSORY DESIGN

The high shear Couette Cell Accessory (CCA) consists of two cylinders, a stationary inner cylinder (the stator) and a rotating outer cylinder (the rotor). The sample is situated in the annular gap between the two cylinders. As in all concentric cylinder systems, the shear rate varies across the gap, but in the Applied Photophysics design the shear rate variation is kept to a minimum by setting the gap to radius ratio as low as possible^[1]. This is essential to ensure that all particles in the sample are subjected to the same alignment or deformation forces. It is also necessary that the shear rate is known accurately, to allow for comparison with measurements obtained using other techniques.

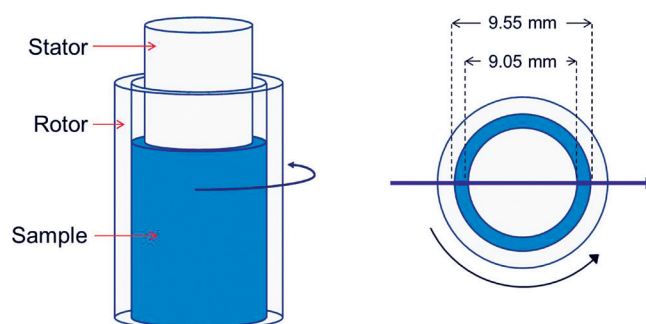


Figure 7. Schematic of the Applied Photophysics high shear Couette cell

A schematic of the APL Couette cell is shown in Figure 7, with the light path shown as the purple line: note that the light passes through the sample twice. R_o , the outer radius of the annular gap, is 4.775 mm, and R_i , the inner radius, is 4.525 mm; the gap width, ΔR , is therefore 0.25 mm, and the total pathlength is 0.5 mm.

Most low concentration solutions of proteins or DNA are close to being Newtonian liquids over a typical shear rate range, i.e. their viscosity is independent of the shear rate, and for such fluids the shear rate is proportional to the reciprocal of the radius squared, i.e. $\dot{\gamma} = 1/r^2$, where r is the distance of a point in the liquid from the rotation axis^[1]. This means that there is about an 11% difference in shear rate between the inner and outer surfaces of the sample, which is reasonable for practical purposes. The shear rate calculated by the Applied Photophysics software and quoted in this note uses the approximation:

$$\dot{\gamma} = \Omega R_o / \Delta R$$

where Ω is the angular velocity in radians per second. This gives a shear rate that is between those at the inner and outer surfaces.

The considerations for sample concentration for an LD experiment are the same as those for a circular dichroism (CD) experiment. The total absorbance, i.e. of the sample and buffer, should be below about 2 AU at all wavelengths, and the maximum signal-to-noise ratio occurs at an absorbance of about 0.9 AU^[12]. A typical LD signal is much stronger than a typical CD signal, often by several orders of magnitude, and it is often possible to obtain good LD data at lower concentrations than would be used for a CD experiment. The CCA offers the unique capability to simultaneously measure absorbance and linear dichroism and it allows a standard CD cell to be easily fitted in place of the LD cell to conduct CD measurements for comparative work.

There are various points to look out for. The flow should be laminar and circular, which means that each point in the sample follows a circular path. At high shear rates the flow may be laminar, but not circular. One such flow regime involves the formation of secondary flows known as Taylor vortices^[13, 14], in which each point in the fluid follows a closed helical path. Normally these vortices are only seen when the inner cylinder rotates, which is one reason why the CCA is designed with the outer cylinder as the rotor.

More importantly, at high shear rates the flow regime may become turbulent. The transition from laminar to turbulent flow is described by the Reynolds number, Re , which is the ratio of inertial to viscous forces:

$$Re = \dot{\gamma} \rho \Delta R^2 / \eta$$

where ρ is the density and η is the viscosity of the liquid.

The value for the critical Reynolds number for the Couette system is given in some textbooks as greater than 50,000^[15], but this is a value calculated for an ideal system of perfect components with no end effects and a completely homogenous sample. In reality, like the free energy, it represents a maximum value and for real systems, turbulence is likely to occur at a lower value of Re . However, on the CCA flow is stable for aqueous systems typically up to the maximum shear rate of 10,200 s⁻¹, giving a critical Reynolds number greater than about 1,200.

Another consideration is the effect of shear heating on the sample temperature. The shear energy, with units of J m⁻³ s⁻¹, is given by:

$$E_{\text{shear}} = \eta \dot{\gamma}^2 / 2$$

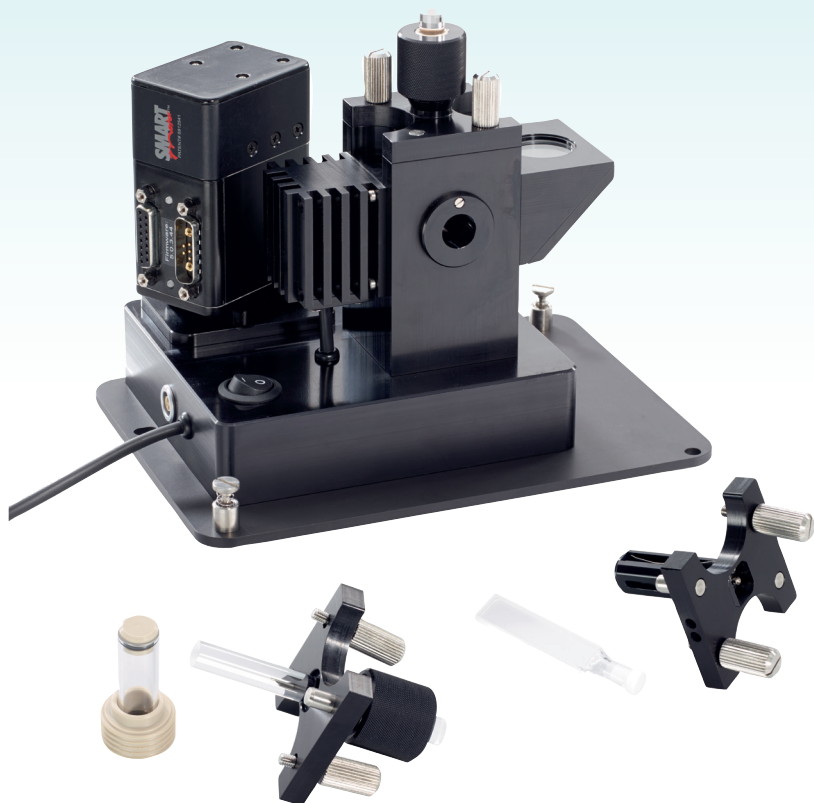
Under adiabatic conditions, for an aqueous sample with a viscosity of about $1 \times 10^{-3} \text{ Pa s}$, a heat capacity of about $4,200 \text{ J kg}^{-1} \text{ K}^{-1}$ and a density of about 1000 kg m^{-3} at 20°C , the temperature rise would be only about $10^{-2} \text{ }^\circ\text{C}$ per second at a shear rate of $10,000 \text{ s}^{-1}$. The conditions are of course not perfectly adiabatic, but since a spectrum usually takes several minutes to acquire, and multiple spectra at each shear rate are normally taken, without active cooling the temperature increase could be unacceptably high. The CCA also offers the facility for using thermostated circulating water to maintain a constant temperature.

Finally ease of use is a key aspect of the design of the CCA. The unit is very quickly and easily installed into the Chirascan or Chirascan-plus instrument. To enable even more rapid switching between absorbance and CD modes of measurement, a separate cuvette holder housing a standard CD cell can be fitted in place of the CCA within seconds. Additionally, since it is possible that bubbles can be trapped between the stator and rotor when installing the stator, an inspection light and mirror system allow the user to verify that the sample is homogenous without removing the accessory from the sample chamber.

REFERENCES

- [1] O.G. Mouritsen, "Life as a matter of fat - the emerging science of lipidomics", Springer-Verlag, 2005, Section 13.1.
- [2] A. Rodger, B. Nordén and T. Dafforn, "Linear Dichroism and Circular Dichroism", RSC, 2010, page 2.
- [3] M. Rittman, E. Gilroy, H. Koohya, A. Rodger and A. Richards, *Sci. Prog.*, 92, 2009, 163-204.
- [4] A. Reymer, K. Frykholm, K. Morimatsu, M. Takahashi and B. Nordén, *Proc. Natl. Acad. Sci. USA.*, 106, 2009, 13248-13253.
- [5] R.G. Larson, "The Structure and Rheology of Complex Fluids", OUP, 1998, page 281.
- [6] E. Tuite and B. Nordén, *Biorg Med Chem*, 3, 1995, 701-711.
- [7] Reference 2, pages 59-70
- [8] Reference 5, page 75.
- [9] S. Keller and M. Textor, unpublished data.
- [10] A. Hamilton, *J. Lipid Res.*, 31, 1998, 467-481.
- [11] R.W. Whorlow "Rheological Techniques", Ellis Horwood, 1992, pages 103-105.
- [12] L. Velluz, M Le Grand and M Grosjean, "Optical Circular Dichroism", Verlag Chemie, 1965, page 68.
- [13] G.I. Taylor, *Phil. Trans. Roy. Soc. Lond. Series A*, 223, 1923, 289-343.
- [14] C.D. Andereck, S.S. Liu and H.L. Swinney, *J. Fluid Mech.*, 164, 1986, 155-183.
- [15] e.g. J.R van Wazer, J.W Lyons, K.Y. Kim and R.E. Colwell, "Viscosity and Flow Measurement", Interscience, 1963, page 86.

ORDERING INFORMATION



Couette Cell Accessory with an LD cuvette (left) and a standard CD cuvette holder (right).

The new Couette Cell Accessory fits conveniently inside the Chirascan and Chirascan-plus sample chamber. The cell features concentric quartz cylinders whose rotation delivers a precise and homogeneous shear rate on a low volume liquid sample. The detection system employs the Chirascan photo-elastic modulator and standard CD detector operated at 100kHz to generate linearly polarised light.

Existing Chirascan and/or Chirascan-plus users can upgrade their system to gain LD capabilities. The upgrade, to be carried out by an Applied Photophysics engineer, involves fitting two key components; the new Couette Cell Accessory and a detection system for linearly polarised light.

Part Number	Description
CS/CCA	Couette Cell Accessory for Linear Dichroism studies. Includes high shear rate Couette Cell Accessory with 4 cells, Linear Dichroism calibration accessory, and triple CD/LD/ORD photomultiplier detector.
CS/LD	Linear Dichroism Option. Includes Linear Dichroism calibration accessory, and triple CD/LD/ORD PMT detector.

AppliedPhotophysics

Ultrasensitive Spectroscopy for the Life Sciences

4207Q151 v.1.00

Chirascan, Chirascan-plus and Chirascan-plus ACD are trademarks of Applied Photophysics Ltd

All third party trademarks are the property of their respective owners

© 2011 Applied Photophysics Ltd — All rights reserved

Applied Photophysics Ltd,

21, Mole Business Park, Leatherhead, Surrey, KT22 7BA, UK

Tel (UK): +44 1372 386 537

Tel (USA): 1-800 543 4130

Fax: +44 1372 386 477

Email: info@photophysics.com

Applied Photophysics was established in 1971 by
The Royal Institution of Great Britain
