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Wallace, Bonnie A. (2019) The role of Circular Dichroism Spectroscopy in the era of integrative structural biology. Current Opinion in Structural Biology 58 , pp. 191-196. ISSN 0959-440X.

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***Short Title*:** Circular Dichroism Spectroscopy and Structural Biology
***Keywords*:** Circular Dichroism Spectroscopy, Bioinformatics, Tools and Resources, 3-Dimensional Structures, Protein Secondary Structures

Abstract:

Circular dichroism (CD) spectroscopy has been used widely in structural biology for literally a half century, primarily to examine the secondary structure, folding and interactions of proteins in solution. With recent developments in instrumentation, it is now possible to apply CD to many additional types of sample environments, including oriented membranes, films, and dehydrated samples. In addition, developments in bioinformatics have made validated CD spectra and metadata available for novel analysis methods on additional types of samples such as membrane proteins, intrinsically disordered proteins, multiple fold types, and multicomponent, macromolecular complexes. New software has also enabled increased inter-operability of CD with other structural biology methodologies, contributing to their use in joint studies of protein structures at various levels of organization.

Highlights:

- Instrumentation developments are enabling an enhanced range of measurements
- The PCDDB provides spectra and metadata for traceability and methods development
- New software developments are enabling novel types of analyses
- CD spectroscopy can now examine more types of proteins with important biological roles
- CD spectroscopy is being used in many interdisciplinary and structural biology applications

Introduction:

This review focuses on new developments in electronic circular dichroism (CD) spectroscopy of proteins. CD is a well-established technique that has recently gained popularity due to developments in data archiving, validation, analyses, and types of samples that can be studied due to new instrumentation (such as synchrotron radiation circular dichroism (SRCD)). CD is now finding use as one of a cadre of methods that can be used to examine the structure, dynamics and similarities of different individual proteins, macromolecular complexes, and protein families. Indeed, a noticeable trend is not only the growth in the number of studies using CD, but in how many of them use CD in conjunction with other methods as part of integrative programs in structural biology. In the past 5 years, more than one-third of the published papers incorporating CD studies of proteins also used another structural biology technique (such as X-ray crystallography, electron cryomicroscopy (cryoEM), nuclear magnetic resonance (NMR) spectroscopy, small angle X-ray and neutron scattering, or molecular dynamics and computational modeling). The interrelationships between these methods have been enhanced during this time period due to new developments in CD data accessibility and bioinformatics that are described in this review.

New Instrumentation Enabling Spectroscopic Measurements:

In recent years instrumentation developments have enabled new and improved CD spectroscopic measurements. The most notable of these developments has been SRCD, in which ultraviolet (UV) and vacuum UV wavelength radiation produced by a synchrotron is used

as the light source instead of the Xenon arc lamps usually found in conventional CD instruments [1]. Such a very bright synchrotron light source has been critical for many new applications – including both new protein sample types and new sample physical characteristics. SRCD beamlines are currently in operation at many sites in Europe [Denmark (ISA) [2], France (Soleil) [2], Germany (ANKA [3] and Bessy), the UK (Diamond [4]), and in Asia ([China (BSRF) [2], Japan (HiSor [2], and Taiwan (NSRRC) [2]).

SRCD beamlines have a number of advantages over conventional lab-based CD instruments. Firstly, their high flux permits penetration of light through samples that have high absorbances, thus enabling measurements of optically-dense materials (including samples which include high salt concentrations, additives, or molecules such as lipids that do not produce chiral signals but absorb in the wavelength range of the measurements). This high penetration enables spectral measurements on samples such as membranes, films, and proteins under crowded conditions, more similar to those found *in vivo*, or, for example, more comparable to those conditions used for crystallization of proteins. Secondly, and seemingly paradoxically, the high light flux also means that measurements can be made from samples with lower absorbances, as the SRCD signal-to-noise levels are much higher than in conventional CD instruments, thereby enabling measurements from very small amounts of sample (an advantage for precious materials), or more rapid measurements (requiring shorter dwell times to record measurements at comparable signal-to-noise levels).

In addition, the much higher light flux profile of the beam allows measurements at much lower wavelengths (effectively ~170 nm in aqueous solutions and 130 nm for dehydrated samples [5]) [Figure 1] than those generally attainable by conventional CD spectrometers. This is particularly advantageous for samples such as those containing polyproline-II-like structures [6] or intrinsically disordered proteins [5,7], in which the main characteristic peaks occur at wavelengths below 188 nm, a wavelength that is usually close to the low wavelength cut-off for measurements in a conventional CD instrument. Because the lower wavelengths also include additional information on peptide bond transitions, obtaining them can enable analyses that separately identify more distinct types of secondary structures, and in some cases, provide information on protein fold motifs [8]. The enhanced sensitivity associated with SRCD also enables examination of subtle structural consequences associated with formation of protein-protein or protein-ligand complexes [9]. Amongst the most novel uses yet for SRCD include the definition of the complete structure of the C-terminal domain of a sodium channel using only SRCD [10] and the demonstration (in conjunction with crystallography and small angle scattering (SAXS)) of the nature of a coiled-coil protein domain that acts as a molecular ruler [11].

Other significant advances in instrumentation include “front end” features such as sample chambers, which enable measurements of oriented samples (oCD) [12], particularly useful in identifying the disposition of protein secondary structure features relative to membranes (i.e., transmembrane helices vs. helices tilted at various angles or those aligned with the membrane surface). These as well as the new “periscope” at ISA (with a perpendicular beam direction, which enables measurements on horizontal Langmuir-trough-like films or dense samples that normally separate out and settle in vertical cells) have been established at SRCD beamlines. A further advantage for SRCD beamlines is that they enable placement of samples adjacent to the light detectors, thus effectively eliminating many of the scattering artefacts apparent when long sample to detector distances are used [13]. In addition, new sample handling systems, such as stopped- and continuous-flow devices enable kinetic measurements.

Such developments are not exclusive to SRCD instruments (although in a number of cases their development may have been pioneered at SRCD beamlines). For instance, conventional, benchtop CD instruments can be modified by realignment of detectors close to sample holders to enable the examination of scattering samples, the use of hydrated cells allows measurements of films [14], and oCD and stopped-flow measurements are now available options. A recent type of development for both conventional [15] and SRCD [16] instruments has been high throughput sample handing, which enables more automated (less personnel-requiring) measurements of samples. This may find particular use in industrial settings for examining proteins under different conditions or for drug screening.

Altogether, developments in instrumentation have had a significant effect on the utilization

and utility of a technique for which the first-lab based instruments became commercially available in the 1960's. These and the computational methods described in the next section, are now enabling measurements of new physical types of samples, and are extending the classes of proteins that can be examined (such as membrane proteins, fibrous proteins, disordered proteins).

The Protein Circular Dichroism Data Bank, a New Tool for CD Bioinformatics:

Although traditional CD spectroscopy was used primarily to estimate the secondary structural contents of proteins, advances in bioinformatics software and analysis methods, connect and complement CD results with other types of structural biology characterizations. This has, significantly, been enabled by a very important new development in CD spectroscopy, the Protein Circular Dichroism Data Bank (PCDDB) [17,18]. The PCDDB was developed based on the data sharing concept of the well-established and highly used Protein Data Bank (PDB) [19-21] for crystal, NMR and cryoEM structures. The PCDDB is a freely accessible databank of user-deposited CD spectra plus metadata, not only including the spectral measurements but also sample sources and conditions. In recent years, the PCDDB has contributed not only to traceability and availability of results, but has also enabled the development of new tools and methods of analyses. Very importantly it includes validation tools, found in both the linked and stand-alone versions of the VALIDICHRO package [22], which ensure that the user (and depositor) are fully aware of the data quality and completeness. More than one million data sets have already been downloaded from the PCDDB by >10,000 different users.

New Software for Structural Analyses of CD Data:

These bioinformatics resources have meant that CD data are now widely available for mining and development of new software for novel types of analyses. Such methods include, for example, secondary structure analyses: DichroWeb [23], based, in part, on the original CDPro package [24], augmented by new bioinformatics-defined reference data sets (SP175 for soluble proteins [25], and SMP180 for membrane proteins [26], and a variable scaling factor for concentration corrections [27]), plus BeStSel [28] (which includes novel analyses for beta-sheet proteins), and Capito [29] (which is optimized for analyses of multiple data sets). All of these empirical methods use reference data sets from proteins of known structures. In addition, the DichroCalc webserver [30] produces CD spectra from *ab initio* dynamics and matrix calculations. There are also new tools for comparative analyses, enabling identification of spectral nearest neighbours (DichroMatch@PCDDB) [31], and PDB2CD [32,33], which permits the calculation of spectra directly from PDB coordinates. DichroMatch can identify related structures (even those without any significant levels of sequence identity) that adopt similar types of secondary and tertiary structures, and so can also be used for fold identification and for identifying proteins with potential functional relationships [31]. PDB2CD can be used to predict spectra of proteins with known crystal and NMR structures [32], which can then be used for comparative studies with related proteins and results from protein dynamics [33].

Examination of Additional Types of Protein Samples:

Whilst new instrumentation methods are enabling examination of different physical types of samples, and bioinformatics advances have led to enhanced analyses, only recently CD studies have expanded to cover additional classes of proteins with important biological roles [Figure 1].

Intrinsically disordered proteins and globular proteins with intrinsically disordered regions are obvious examples, as they have been identified as existing widely across genomes, and tend to form hub complexes for biological processes. Because they are dynamic structures and do not adopt persistent or typical secondary and tertiary structures, and tend not to crystallize, they have been challenging to characterize structurally. These types of proteins do, however, have signature CD spectra [34], so that combinations of CD and, for example, NMR spectroscopy, can be used to examine conformational changes associated with binding partner interactions [35].

Membrane proteins are significant components in all cell types and organisms, and whilst they constitute around 30% of the gene products in many organisms and more than 50% of all current drug targets, there has been a proportional dearth in the structures of membrane proteins

that have been determined and deposited in the PDB relative to the number of soluble proteins, and so they may benefit from other methods of characterizations such as CD spectroscopy. In the past, membrane proteins have also been difficult targets for CD spectroscopic studies, but with the physical and bioinformatics developments described above, they have become viable candidates for CD analysis [13].

Likewise, fibrous proteins have proven problematic for CD studies in the past due to their potential for producing light scattering artifacts and due to their secondary structures which have extended chains and secondary structures (phi, psi angles) atypical for globular proteins. But these are now being added to the cadre of proteins amenable to analysis by CD spectroscopy [7,36].

Combining CD Spectroscopy with Other Structural Biology Methods:

The new and wider ranges of target proteins/preparations suitable for CD spectroscopic analysis, enabled by the instrumentation and computational methods described above, have now enhanced the coordinated use of CD spectroscopy with other computational and structural biology methods, thereby yielding more thorough descriptions of the structure, dynamics and function of proteins. Recent examples include joint studies with crystallography [37], solid-state and solution NMR [38,35], molecular dynamics simulations [39], molecular design and modelling [40,41], *ab initio* folding studies [42], small angle x-ray and small angle neutron scattering [43], cryoEM [44], and atomic force microscopy [45].

An example of the interoperability of CD with other methods is the use of thermal unfolding studies to examine drug/ligand binding. Although there are many physical techniques (such as thermophoresis/fluorophore assays) which can be used for such studies and which are both more sample- and time-efficient than CD, often the physical conditions of interest (i.e., in membranes or suspensions) are not suitable for those methods, but can be examined using CD spectroscopy [46]. A recent example is that thermal melt CD studies of membrane proteins have been useful in identifying amphipathic environments (detergents, amphipols) that are suitably stabilizing for use in crystallization or for producing cryoEM samples [47].

Furthermore, circular dichroism has had recent impacts in widely diverse fields such as origins of life/space [48] and pharmaceutical sciences [49,50]. For the latter, new possibilities for drug formulation studies have been explored by CD studies, which have demonstrated that dehydrated proteins [5] and proteins associated with gelatine (as in capsules) [51] can retain their structural integrity. Additionally, CD spectroscopy, which has in the past been identified as one of the techniques recognized by the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use, is now playing an increasingly important role in quality control characterizations for biological medicines such as antibodies. Spectra in both the near and far UV wavelength regions are acceptable means of characterizing protein higher order structures (referred to as HOS features) indicative of correct folding. Hence CD spectroscopy is now widely used as a type of characterization included in patent filings for biological therapeutics. This should, in the future, provide new opportunities for development of computational methods comparing batch and conditional quality control/reproducibility screening assays, which will require new metrics and software to be developed for such CD analyses.

Conclusions:

Bioinformatics and instrumentation developments, and the availability of an accessible repository of spectral and metadata are enhancing the use of CD spectroscopy in studies of proteins of different types, in wider ranges of environments, and in conjunction with other structural biology techniques. Recent CD studies provide new understandings of structure, dynamics, and interactions between macromolecules in solution, in amphipathic complexes including membrane bilayers, and in oriented and dehydrated films. Comparisons of their structures in these and other conditions such as those used in crystallography, cryoEM, NMR and small angle scattering, are adding to a more holistic understanding of the inter-relationship between structures and their environments.

Acknowledgements:

I express my thanks to Drs. R.W. Janes, A.J. Miles, and L. Whitmore, and the many Wallace lab members (past and present) for their help in developing, testing and showing the utility of the methods and software we have produced over the years, and for their stimulating discussions on all things CD; to my many colleagues (too numerous to list) with whom we have collaborated on projects of biological interest; and to the SRCD beamline scientists around the world, with whom I have had the pleasure to meet and work with on developments to this technique, especially Drs. S.V. Hoffman and N. Jones (ISA, Aarhus, Denmark), Dr. J. Sutherland and John Trunk (NSLS, Brookhaven, USA), Dr. D. Clarke and A. Brown (SRS Daresbury, UK), Dr. Frank Wien (Soleil, France), Dr. Jochen Buerk and Sigmar Roth (ANKA, Karlsruhe, Germany), and Dr. Ye Tao (BSRF, Beijing, China), and to the late Jack Aviv (Aviv Biomedical) for modifying our lab-based CD instruments and software to enable some of the measurements/developments described in this review.

This work has been supported by grants over the past many years from the U.K. Biotechnology and Biological Sciences Research Council (BBSRC) (currently grant numbers P024092 and J019135) to my lab and as linked projects with the RWJ lab.

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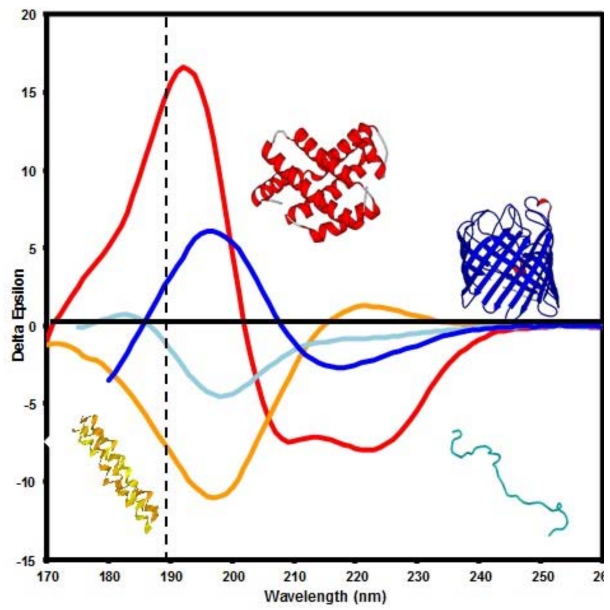
Supplementary Material: Websites for CD Bioinformatics Resources:

Protein Circular Dichroism Data Bank: http://pcddb.cryst.bbk.ac.uk
Validichro (spectral validation website):
http://valispec.cryst.bbk.ac.uk/circulardichroism/ValiDichro/upload.html
CDToolX *(data processing and thermal analyses)* [52]
http:// http://www.cdtools.cryst.bbk.ac.uk/
Dichroweb *(secondary structure analyses):* http://dichroweb.cryst.bbk.ac.uk/html
BeStSel *(secondary structure analyses):* http://bestsel.elte.hu/index.php
Capito *(secondary structure analyses*): http://capito.nmr.fli-leibniz.de.
CDPro *(secondary structure analyses*): http://sites.bmb.colostate.edu/sreeram/CDPro/
Dichrocalc *(secondary structure ab initio calculations):*
http://comp.chem.nottingham.ac.uk/dichrocalc
PDB2CD *(CD spectra from PDB coordinates):* http://pdb2cd.cryst.bbk.ac.uk
Dichromatch@PCDDB *(Identification of protein spectral nearest neighbours):*
http://pcddb.cryst.bbk.ac.uk/dichromatch.php
You Tube videos *(on measurements, instrument calibration, analysis methods, and databank access and deposition of CD and metadata):* https://www.youtube.com/user/ThePcddb

Figure Legend:

Figure 1. *Spectral Characteristics of Different Classes of Proteins:* Synchrotron Radiation Circular Dichroism (SRCD) spectra of a mostly helical globular protein (myoglobin) [spectrum in red, corresponding crystal structure in red in upper centre] [25], of a beta barrel membrane protein (Fhu) [spectrum in blue [26], crystal structure in blue at upper right]**,** an intrinsically disordered proten (Tarp) [spectrum in light cyan [35], structure depicted in cyan at lower right], and a polyproline-II rich protein (bovine collagen) [spectrum in yellow [7], structure in yellow at lower left]. The corresponding CD spectra (as opposed to the SRCD spectra) would, in general, terminate to the right of the dashed line (at around 188 nm). This figure indicates the additional information present in an SRCD spectrum, and illustrates that the spectra for proteins with different types of secondary structures tend to change direction or sign at around the CD wavelength endpoint. The additional information in the low wavelength data available in SRCD spectra can thus make their deconvolution analyses more accurate [8].



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