Ultraviolet (UV) synchrotron radiation circular dichroism (SRCD) spectroscopy has made an important contribution to the determination and understanding of the structure of biomolecules. In this paper, we demonstrate an innovative time-resolved SRCD (tr-SRCD) technique, overcoming limitations of current broadband UV SRCD setups, to access ultrafast (down to nanoseconds) time-scales, previously measurable only by other techniques, such as infrared (IR), nuclear magnetic resonance (NMR), fluorescence and absorbance spectroscopies and small angle X-ray scattering (SAXS). The tr-SRCD setup takes advantage of the natural polarisation of the synchrotron radiation emitted by a bending magnet to record broadband UV CD faster than any current SRCD setup, improving the acquisition speed from 10 mHz to 130 Hz and the accessible temporal resolution by 11 orders of magnitude. We illustrate the new approach by following the photoisomerization of an azopeptide. This breakthrough in SRCD spectroscopy opens up a wide range of potential applications to the detailed characterisation of biological processes, such as protein folding, protein-ligand binding and DNA nano-structure formation.
Time resolved transient circular dichroism spectroscopy using synchrotron natural polarisation

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Abstract

Ultraviolet (UV) synchrotron radiation circular dichroism (SRCD) spectroscopy has made an important contribution to the determination and understanding of the structure of biomolecules. In this paper, we demonstrate an innovative time-resolved SRCD (tr-SRCD) technique, overcoming limitations of current broadband UV SRCD setups, to access ultrafast (down to nanoseconds) time-scales, previously measurable only by other techniques, such as infrared (IR), nuclear magnetic resonance (NMR), fluorescence and absorbance spectroscopies and small angle X-ray scattering (SAXS). The tr-SRCD setup takes advantage of the natural polarisation of the synchrotron radiation emitted by a bending magnet to record broadband UV CD faster than any current SRCD setup, improving the acquisition speed from 10 mHz to 130 Hz and the accessible temporal resolution by 11 orders of magnitude. We illustrate the new approach by following the photoisomerization of an azopeptide. This breakthrough in SRCD spectroscopy opens up a wide range of potential applications to the detailed characterisation of biological processes, such as protein folding, protein-ligand binding and DNA nano-structure formation.

Keywords: time-resolved spectroscopy, circular dichroism, synchrotron, biomolecular dynamics

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Introduction

Circular dichroism (CD) is an optical property of molecules having chiral structure(s) and/or spatially oriented arrays of chromophores. It manifests as a difference in absorption for left- and right-circularly polarised light. In the ultraviolet (UV) range, this feature has been exploited for many decades for characterisation of organic molecules, materials with supramolecular chirality, and in protein conformation determination, where there are distinctive spectral signatures for each secondary structure type, i.e., α-helices and β-sheets. Thus, CD spectroscopy is an important biophysical tool for characterising native and modified proteins. In the biomedical context, protein misfolding can have dramatic consequences on cell physiology, causing serious neurodegenerative diseases, as found in Alzheimer’s and Parkinson’s. Structural and kinetics studies of protein folding, using time-resolved approaches, are providing crucial insights at the molecular level into the aetiology of these diseases.

There are two main ways to measure CD spectra: the ellipsometric method and the direct absorption difference detection method. The former is based on quantifying the variations of the ellipticity and azimuth orientation of a highly elliptically polarised beam passing through a dichroic sample. In 2012, Eom et al. innovatively adapted the ellipsometric method to a heterodyne-detection technique, providing both the CD spectrum and the optical rotation dispersion spectrum, by analysis of the phase and the amplitude of the transmitted orthogonal electric field of the incident light polarisation. More recently, Hiramatsu et al. coupled the heterodyne detection technique to a singular value decomposition analysis. This improvement removes linear dichroism and linear birefringence artifacts, allowing accurate time-resolved CD (tr-CD) measurements in the visible range with sub-picosecond temporal resolution. The second way to acquire a CD spectrum is based on absorption difference
measurement. The light is alternately circularly right and left polarised using an optical or acousto-optic modulator. Then the detection system records intensity variations and allows CD determination. This method has been extensively refined, and can provide sub-picosecond resolution for monochromatic measurements. Hache and colleagues used this technique to probe ultra-fast kinetics in bio-molecules and achieved 800 fs temporal resolution. Trifonov et al. have developed a setup for picosecond transient broadband CD measurements in the visible range. They used a combination of a Pockels cell and a light continuum generator to obtain a pulsed white light source alternately circularly right and left polarised. Both ellipsometric and absorption difference broadband techniques have advantages and disadvantages, but they also share the same spectral restriction to the visible range, due to the lack of stable continuum light sources in the UV range. This limits the application of these broadband tr-CD spectroscopies and makes them unsuitable for UV CD measurements.

The synchrotron radiation circular dichroism (SRCD) technique was developed in 1980 and. Since its first use for protein structure determination, it has been used in a wide range of applications. Indeed, the brilliance of the synchrotron radiation (SR) in the vacuum UV (VUV) range and its stability enables one to measure CD spectra of samples dissolved in buffer down to 160 nm with an acceptable signal to noise ratio for the heterodyne detection.

In this paper, we describe the development of a different approach for SRCD measurements, utilising the natural polarisation of the SR emitted by a bending magnet. The DISCO beamline provides a SR beam composed of two parts (Supplementary Information Fig. 1), which are above and below the accelerated electron’s orbital plane in the synchrotron storage ring. These two continuum beams are similarly elliptically polarised but with opposite direction (Supplementary Information 2). Schiller and Hormes have previously demonstrated that the natural elliptical polarisation of the SR can be used for CD spectroscopy. However, they measured the CD signal sequentially, wavelength by wavelength; so the scanning process
of the monochromator limited the temporal resolution of their setup significantly. We have developed a spectrograph that measures simultaneously the intensity of both (oppositely polarised) parts of the SR beam, allowing one to determine a whole UV CD spectrum in just a single measurement. While current SRCD setups only take advantage of the wide spectral range and the brilliance of SR, our setup also uses its natural polarisation and its temporal distribution. This broadband single measurement approach gives access to a combination of a temporal and a spectral range previously inaccessible with the current broadband CD setups, enabling new insights into biomolecular dynamics.

Method

The tr-SRCD setup is annexed to the existing SRCD endstation at the DISCO beamline 19. The optical layout is shown Figure 1. The beamline excitation monochromator is used first order for the wavelength calibration of the detection spectrograph (Supplementary Information Fig. 3). With the zero order, we get on the sample a pulsed white beam containing all the wavelengths between 120 nm to 600 nm; this pulsed broadband source is used for the tr-SRCD measurement, limited by the CMOS spectral response. The beam is spatially defined by a spatial filter (labelled S1 in the figure), then centred by the two plane mirrors (M1, M2) before being refocused on the sample. In order to avoid damage from beam irradiation, this optical system is designed to allow variation of the illuminated area on the sample. The spherical mirror M3 and the cylindrical mirror M4 refocused the beam 2975 mm after the monochromator slits; the calculated beam diameter at the focal plane is about 300 µm (FWHM). By moving the sample cell by 200 mm along the optical axis, we get a controllable probe beam diameter from 300 µm up to 3 mm. Adjustment of the beam diameter to the exposure time helps to minimise the sample irradiation dose. Beyond the sample, the cylindrical mirror M5 and the spherical mirror M6 horizontally focused the beam on a
secondary slit (S2). This secondary slit at 3900 mm is used to define the final spectral resolution of the setup. The flat M7 mirror reflected the beam on a spherical flat field grating with 580 grooves per mm (Horiba Jobin-Yvon) that diffract and focus the incident UV/vis light horizontally onto the 2D detector.

Figure 1: Optical layout of the tr-SRCD setup.

The tr-SRCD setup acquisition frequency is defined by the camera frame rate, which can be set from 0.033 Hz to 100 Hz in full resolution mode and can be increased further by reducing the pixel array size. This range of sampling frequency allows one to study reaction kinetics from tens of milliseconds to minutes through real-time recording and sequential measurements. The image intensifier can be synchronized with the camera output clock and with an external function generator providing trigger signals with a higher frequency. When the intensifier is synchronized with the output clock, the temporal resolution of the measurement
corresponds to the duration of the amplification gate or the light pulse length if the gate is sufficiently short (Supplementary Information 4). However, if an external device triggers the image intensifier at a higher frequency than the camera frame rate, the exposure time of the camera defines the temporal resolution of the setup.

The sample was held in a CaF$_2$ flow cell with a path-length of 24 µm (Aquaspec, Microbiolytics GmbH, Freiburg, D). The sensitivity ranges of 2D detectors, such as CDD or CMOS, are usually limited to 200 nm. We may also use an image intensifier (HS–IRO, LAVISION) to reach lower wavelength range and to increase time resolution. To recover the main part of the visible light provided by the HS-IRO phosphor, a combination of two 50 mm focal length infinity corrected objectives was used. A CMOS detector (Prime 95-B, PHOTOMETRICS) records the phosphor screen image. For the applications that do not need the picosecond temporal resolution, the Prime 95-B was used without intensifier.

Equation 1 defines CD at a given wavelength. The variables $A_L$ and $A_R$ correspond to the absorption for left and right-handed circularly polarised light, respectively. All the spectra associated with the tr-SRCD setup in this study were determined by the following equation.

$$CD = (A_L - A_R)$$  \hspace{1cm} \textit{Equation 1}

In this case, the absorption difference between left-handed and right-handed circularly polarised light can be associated with the absorption difference between the upper and lower part of the SR (Supplementary Information Note 5).

**Results**

We chose camphorsulfonic acid (CSA), purchased from Sigma-Aldrich, as our calibration sample, as it has two strong peaks in the VUV/deep UV range: the first at 290 nm and the second at 192 nm. It is commonly used as a calibration standard for CD spectrometers
To validate that the recorded signal is CD, the two enantiomers must exhibit opposite signed spectra.

The resulting spectra for D-CSA and L-CSA, measured between 190 nm and 315 nm for 30 mg/mL solution concentration, are shown in Figure 2A. The CMOS detector has been used without the image intensifier. It integrated the light for 500 µs and the images were acquired at 20 Hz. As expected, we observe two oppositely signed spectra with identical amplitudes. The ratio between the amplitude of the two peaks should be about 2\(^2\); we obtained 2.11 +/- 0.11 in the tr-SRCD spectra (curve (d) of Figure 2A). The signal-to-noise ratios at 190 nm for the D-CSA spectra (a), (b), (c) and (d) in Figure 2A are 6.6, 12.7, 39.8 and 44.40, respectively.

**Figure 2:** a. CD spectra of D-CSA (black) and L-CSA (red) acquired with the tr-SRCD setup at 20 Hz with 500 µs temporal resolution. 1, 10, 50 and 100 measurements were integrated to obtain the data shown in curves (a), (b), (c) and (d) respectively. b. CD spectra of D-CSA (black) and L-CSA (red) from single pulse measurements acquired with the tr-SRCD setup at 500 Hz with 82 ps temporal resolution; 5000 measurements were integrated.

In order to establish that the highest theoretical temporal resolution of the setup is attainable experimentally, we added an image intensifier, thereby obviating the temporal
resolution limitation of the CMOS detector. We performed this steady state experiment on the CSA with the maximal temporal resolution as a proof-of-principle. The intensifier was triggered with an external trigger and its amplification was reduced to its shortest value, 100 ns. The measurements were made using the 8 bunch mode of the synchrotron SOLEIL which provides 82 ps pulses at 7.14 MHz. The intensifier amplified only one pulse per gate. So its temporal resolution corresponds to the pulse length, which, in this case, is 82 ps. In Figure 2B, each spectrum comes from ten images; 500 separated pulses were integrated for each image. The spectral range was reduced to between 255 nm and 300 nm, in order to optimize the setup for the detection of the peak at 290 nm. The signal-to-noise ratio at 290 nm for the d-CSA spectrum is 10.33.

![Figure 3: Schematic representation of the FK-11-X molecular system for both cis and trans conformation. Its amino acid sequence is Ac-Glu-Ala-Cys^{AZO}-Ala-Arg-Glu-Ala-Ala-Arg-Glu-Ala-Ala-Cys^{AZO}-Arg-Gln-NH_{2}.](image-url)

We tested the capacity of the setup to follow reactions in real time by studying a photocontrolled and reversible system, the azobenzene crosslinked peptide FK-11-X\textsuperscript{22,23}. It is
composed of a 16 amino acid peptide cross-linked to a photoswitchable molecule, the azobenzene (Figure 3). This ligand can be isomerised from the trans to cis and cis to trans conformation using a 370 nm and a visible light source (460 nm), respectively. Several groups have studied the dynamics of this photo-induced reaction and the isomerisation of the azobenzene appears to occur within one picosecond \(^{24-27}\). This conformational change constrains the peptide structure and triggers its folding and unfolding processes. Theoretical \(^{22,28}\) and experimental \(^{29,30}\) studies agree that, following the azobenzene isomerisation, peptide conformational changes occur on the microsecond scale. The azobenzene cross-linked peptide was synthesized as described previously \(^{22}\) (Supplementary Information Note 6). The aim of our study was to follow the change in concentration of the unfolded (cis azobenzene) and folded (trans azobenzene, ground-state) FK-11-X peptide. We triggered the isomerisation with two continuous diodes, at 370 nm (5W) and at 460 nm (5W). The concentration of FK-11-X solution was 10 mg/mL in phosphate buffer (70 mM, pH 7). The sample was alternately irradiated with the two diodes, for 2.5 seconds for each. The transient CD and absorption spectra of the cis to trans isomerisation were followed, measuring spectra at 130 Hz with a 500 \(\mu\)s temporal resolution; the measurement has been integrated over 20 cycles. The variations in the CD and absorption are shown in Figure 4.
Figure 4: a. 2D representation of the evolution of the absorbance over time; 650 spectra are shown. b. Concatenation of the 650 absorption spectra into 8 averaged spectra. c. Evolution of the CD over time with a 425 ms time-step.

We observe a decrease in the concentration of the cis-conformation species from the first milliseconds, as shown by the CD and the absorption changes (Figure 4). The absorption decreases progressively and reaches a plateau after the 2.5 seconds of irradiation with the 460 nm diode. The CD spectrum corresponds to the signal of the α-helical structure and the increasing amplitude reflects the change of the species concentration, from cis to trans-conformation, i.e., from unfolded to folded structures. The total CD changes from the initial state and final state measured with the tr-SRCD setup and from steady state measurement (cis and trans CD spectra) in the literature\(^\text{29}\) are similar. This comparison shows that being able to
calibrate the SR polarisation, we can measure transient CD spectra similar to those measured with steady state setups. The only difference arising from the elliptical polarisation is the amplitude of the CD, which is linearly correlated to the circularity of the polarisation.

On going experiments involve the kinetic study of the refolding of model proteins after thermal denaturation.

Our study demonstrates that the new tr-SRCD setup developed at the DISCO beamline in the SOLEIL synchrotron offers new opportunities for the investigation of biomolecular structure and dynamics. The setup is based on the use of the natural properties of the synchrotron radiation provided by the DISCO beamline. It uses both spectral and polarisation characteristics to reduce the time required for the measurement of a transient CD UV spectrum. Our tr-SRCD measurements of the CSA show that an integration time of 500 µs is enough to obtain an acceptable signal-to-noise ratio, i.e., greater than 6. We performed a proof-of-principle high temporal resolution steady state experiment on CSA performed using an image intensifier allowing resolution down to a few tens of picoseconds. The FK11 experiment demonstrates the capacity of the tr-SRCD setup to follow dynamics in real time, with a temporal resolution of 500 µs and acquired at 130 Hz. These values do not correspond to the hardware cap and can be easily improved, by opening the spectrograph slits and integrating more photons in shorter time period and/or coupling the system with the image intensifier. This breakthrough in the SRCD spectroscopy will enable the study of many biological and chemical reactions crucial for our understanding of biomolecular phenomena.

The development of new CaF2 microlenses windows for sCMOS camera would grant access to direct detection and measurement of spectra on the full DUV range down to 160 nm.

In the near future, the setup could easily be coupled to other reaction triggers, such as a T-jump, lasers and microfluidic stop-flow equipment. Thus, this new approach will provide
exciting insights into the dynamics of biomolecules, as well as for molecular and materials systems more broadly. This method will enable one to follow the behaviour of molecules through high quality SRCD spectra on a temporal range from the picoseconds to minutes. Indeed, it covers time scales consistent with the fluctuation and domain motions of proteins suspected to play a fundamental role in their activity.

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Author Contributions M.R., F.W., F.J. and A.G. came with the original idea. M.R., A.G. and J.D.H. conceived the research. F.P., B.L., D.D., and F.A. conceived the optical scheme. F.A., A.G., F.W. and F.J. performed the experiments. B.N. and S.Z. conceived and prepared the peptides samples. C.M. conceived the detection scheme. F.A., J.D.H., A.G. and M.R. wrote the manuscript. All authors commented on the drafts of the manuscript.

Competing Interest None.

Data availability statement Raw data were generated at the synchrotron SOLEIL large scale facility and are available from the corresponding author upon reasonable request.

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SUPPLEMENTARY INFORMATION

Time resolved transient circular dichroism spectroscopy using synchrotron natural polarisation

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Contents

1. SR beam intensity image
2. Polarisation characterisation
3. Wavelength calibration
4. Scheme of the detection using an intensifier
5. Data processing algorithm
6. Purification of FK-11-X

1. SR beam intensity image

![Image of the projected synchrotron radiation white beam recorded with the CMOS. The grayscale (12 bits) corresponds to the intensity of the light.](image)
2. Polarisation characterisation.

In order to select two areas on the detectors having similar but opposite elliptical polarisation, we measured the fourth Stokes parameter for each pixel of the array. We used a combination of a broadband quarter-wave plate (QWP) (BERNHARD HALLE) and a Wollaston prism (BERNHARD HALLE) to measure both the left-handed and right-handed components. Two intensity measurements were made, the first with the QWP and the analyser oriented respectively at 0° and 45° and a second at 0° and 135°. The normalised $S_3$ is determined with the following equation, $\theta$ corresponds to the orientation of the analyser and $\alpha$ to the orientation of the QWP:

$$S_3 = \frac{I_{\theta=0^\circ, \alpha=45^\circ} - I_{\theta=0^\circ, \alpha=135^\circ}}{I_{\theta=0^\circ, \alpha=45^\circ} + I_{\theta=0^\circ, \alpha=135^\circ}}$$

Figure S2 shows the measured $S_3$ value of the synchrotron radiation at 265 nm in function of the vertical beam divergence.

![Figure S2](image)

**Figure S2:** $S_3$ value in function of the vertical beam divergence at 265 nm.

3. Wavelength calibration

We use the beamline monochromator to record images of the monochromatic beams in order to determine the corresponding wavelength for each pixel of the detector array.
**Figure S3**: 6 monochromatic rays selected with the beamline monochromator.

4. **Scheme of the detection using an intensifier**

The intensifier gate only amplifies the light coming from one SR pulse. The intensifier allows one overcoming the limitation brought by the minimal exposure time of the CMOS (50 µs) and so measure the intensity of one pulse at once (Figure S3). We use this protocol for the results presented in Figure 1B. We integrated 500 pulses for each recorded image for this measurement.

**Figure S4**: Detection sequence of the single pulse measurement presented in the Figure 1B.
This protocol might allow one to follow triggerable dynamics down to nanoseconds time scale (Figure S4). Indeed, shifting progressively the delay $\delta$ between the measurement and the trigger, one could build sequentially a dynamic having the minimal delay increment as sampling limitation and the synchrotron radiation pulse width as temporal resolution limitation.

![Diagram](image)

**Figure S5:** Detection sequence for single pulse measurement combined to a triggered system.

5. **Data processing algorithm (MATLAB R2016b)**

The processing steps of the data to obtain the transient SRCD spectra are:

- Wavelength calibration of the measurement.
- Creation of the mask for both upper and lower beam having a similar average $S_3$ absolute value.
- Determination of the reference intensity from the frame measured before the reaction trigger.
- Determination of the transient intensity from the frames measured during the reaction.
- Determination of the transient absorption spectra for both upper and lower beam.
- Normalization of the absorption spectra.
- Determination of the transient CD, subtracting the absorption spectra of the upper and lower beam.

The processing steps of the data to obtain the CSA SRCD spectra are:

- Wavelength calibration of the measurement.
- Creation of the mask for both upper and lower beam having a similar average $S_3$ absolute value.
- Determination of the reference from the intensity measurement with the sample cell loaded with water.
- Intensity measurement with the sample cell loaded with CSA enantiomers and a racemic mix.
- Determination of the absorption spectra for both upper and lower beam for the two enantiomers and the racemic mix.
- Calculation of the CD, subtracting the absorption spectra of the upper and lower beam.
- Correction of the measurement subtracting the CD spectrum of the buffer to the CD spectra of the enantiomers.

6. Purification of FK-11-X

FK-11-X was purified by reversed-phase HPLC on a Ultimate 3000 chromatographic system (Thermo Scientific) using a Luna C18(2) column ($250 \times 4.6$ mm, 5 $\mu$m, 100 Å, Phenomenex). The separation was performed at 40°C using the following gradient of water + 0.1% formic acid (mobile phase A) and acetonitrile + 0.08% formic acid (mobile phase B) at a flow rate of 1 mL.min$^{-1}$: linear increase from 15% B to 32% B within 12 min followed by linear increase to 100% B in 3 min. The peptide purity was checked by LC-MS on an Ultimate 3000 Micro-
HPLC system (Thermo Scientific) connected to an ESI-Q-TOF mass spectrometer (Q-STAR Pulsar, AB Sciex) equipped with an IonSpray source. The separation was achieved on a C18 column (Uptispher® WTF, 5 µm, 150 × 1.0 mm, Interchim) using the following gradient of mobile phases A:B at 40 °C and 40 µL.min⁻¹: linear increase from 10% B to 60% B within 30 min followed by linear increase to 100% B in 5 min. The purified FX-11-X was dried under vacuum and conserved at -20°C until use. FX-11-X was dissolved at in phosphate buffer, 70 mM, pH 7. The peptide concentration was determined from absorbance measurements at 365 nm, using the extinction coefficient provided at 367 nm for the dark-adapted azo group, ε = 28 000 M⁻¹.cm⁻¹ [1].

[1] Flint D. G.; Kumita J. R.; Smart O. S; Woolley G. A. Using an Azobenzene Cross-Linker to Either Increase or Decrease Peptide Helix Content upon Trans-to-Cis Photoisomerization. *Chem Biol* 2002, 9, 391–397.
