

Advantages of synchrotron radiation circular dichroism spectroscopy to study intrinsically disordered proteins

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Abstract The unordered secondary structural content of an intrinsically disordered protein (IDP) is susceptible to conformational changes induced by many different external factors, such as the presence of organic solvents, removal of water, changes in temperature, binding to partner molecules, and interaction with lipids and/or other ligands. In order to characterize the high-flexibility nature of an IDP, circular dichroism (CD) spectroscopy is a particularly useful method due to its capability of monitoring both subtle and remarkable changes in different environments, relative ease in obtaining measurements, the small amount of sample required, and the capability for sample recovery (sample not damaged) and others. Using synchrotron radiation as the light source for CD spectroscopy represents the state-of-the-art version of this technique with feasibility of accessing the lower wavelength UV region, and therefore presenting a series of advantages over conventional circular dichroism (cCD) to monitor a protein conformational behavior, check protein stability, detect ligand binding, and many others. In this paper, we have performed a comparative study using cCD and SRCD methods for investigating

the secondary structure and the conformational behavior of natively unfolded proteins: MEG-14 and soybean trypsin inhibitor. We show that the SRCD technique greatly improves the analysis and accuracy of the studies on the conformations of IDPs.

Keywords Secondary structure · Intrinsically disordered protein · Protein conformation · Synchrotron radiation circular dichroism spectroscopy

Introduction

The intrinsically disordered proteins (IDPs) (Dunker et al. 2001; Uversky 2014) are a ubiquitous and highly abundant group of functionally active proteins in nature that fail to form a rigid three-dimensional structure (lacking a stable structural content) under physiological conditions. The outstanding interest in the IDPs in recent decades (Tompa 2012) might be associated with the discovery of their involvement in many human diseases (Korsak and Kozyreva 2015; Uversky et al. 2008), and also with the multifunctional roles these proteins can fulfill within the organisms (Kragelund et al. 2016), participating specially in signaling (Bürgi et al. 2016), recognition (Wright and Dyson 2015), and regulation reactions (Galea et al. 2008), but as well interacting with multiple partners (Vavouri et al. 2009), even simultaneously (Money et al. 2009), promoting the binding and/or accelerating interactions between bound partners (Uversky 2013).

Also called intrinsically unfolded proteins, the IDPs are found as dynamic ensembles or protein clouds which are described by a sum of many interconverting conformations, which could be attributed to the remarkable structural flexibility of these proteins (Dunker et al.

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2008). Upon binding to their targets, however, many IDPs undergo transitions to more ordered conformations (Moritsugu et al. 2012). Similarly, the unordered content of these proteins can be affected by chemical or physical factors, such as the presence of organic solvents, removal of water, temperature, or pH changes.

In order to characterize this chameleon (Uversky 2003) nature of an IDP, several spectroscopic techniques can be employed. Circular dichroism (CD) spectroscopy (Ranjbar and Gill 2009; Wallace and Janes 2009) is a powerful technique that stands out from the other methods to analyze the secondary structure content of a polypeptide chain, or to monitor its conformational behavior, check protein stability, and detect ligand binding. Moreover, the use of the synchrotron radiation as the light source for the CD measurements (Sutherland et al. 1992), known as synchrotron radiation circular dichroism (SRCD) spectroscopy, has presented a series of advantages over conventional CD (cCD) (Wallace 2000; Miles and Wallace 2006) due to its feasibility of taking faster measurements, the small amount of sample required, capability of sample recovery (sample not damaged), and specially accessing lower wavelengths in the vacuum-UV region that were not possible with the conventional method (Wallace and Janes 2001). Among the advantages of SRCD over the cCD method are the extended interval for collecting CD data, the increased signal-to-noise ratio due to the higher flux of photons in the SRCD, the possibility of taking measurements in high absorbing buffers or even non-physiologic conditions, and the possibility of better characterizing significant or even subtle conformational changes, for instance as the one caused by a single amino acid residue change (Powl et al. 2009).

In this study, we develop a series of comparative experiments probing several of these advantages when working with intrinsically disordered proteins in solution, using conventional and synchrotron radiation circular dichroism methods on two intrinsically disordered proteins: the soluble domain of MEG-14 (a micro-exon gene protein, isolated from the genome of *Schistosoma mansoni*) (Lopes et al. 2013) and STI (soybean trypsin inhibitor, a partially folded protein containing high content of disordered regions on its secondary structure). The proteins used in this comparative study, STI and MEG-14, are examples of a partially and a fully unfolded protein, containing nearly 50% and almost 100% of residues in a disordered conformation, respectively (secondary structure predictions are in Suppl. Figure 1). The disordered conformation in a polypeptide chain do not present a regular repeating pattern of backbone residues, it lacks the defined dihedral angles found in the canonical secondary structure (α -helix, β -strands, etc.). All the comparisons of SRCD and cCD spectra were done under the

same conditions to demonstrate the advantages achieved by using the SRCD method.

Materials and methods

Intrinsically disordered proteins

The soluble domain of MEG-14 (UniProt M1GUG5) was obtained as described in Lopes et al. (2013), and soybean trypsin inhibitor (STI) (PDBID 1AVU, UniProt P01071) was purchased from Sigma-Aldrich. Phospholipids were purchased from Avanti Polar lipids. All reagents and solvents were analytical grade.

cCD and SRCD measurements

Conventional CD spectra measurements of MEG-14 (10 μ M) and STI (10 μ M) were recorded on a J-815 spectropolarimeter (Jasco Instruments, Tokyo, Japan) over the range from 270 to 184 nm, in 1-nm steps, as an average of three scans, at 25 °C, in 10 mM sodium phosphate, 10 mM Tris-HCl, and 10 mM HEPES buffer (all pH 7.4) using a 0.1-cm quartz Suprasil cuvette (Hellma Analytics). Additionally, the cCD spectra of both IDPs (50 μ M) were also obtained on a J-720 spectropolarimeter, using a short path length (0.0097 cm) quartz Suprasil cuvette (Hellma Analytics, optical path calibrated by interferometry), and the same conditions as described above. SRCD spectra of STI and MEG-14 (50 μ M) were obtained under the same conditions described for the cCD measurements (number of scans, temperature, short path length cuvette, etc.), except for the wavelength from 270 to 170 nm.

cCD/SRCD spectra of the IDPs in 10 mM sodium phosphate buffer (pH 7.4) were also obtained in the presence of 25, 50, and 75% trifluoroethanol (TFE), or in the presence of 100 mM NaCl, 10% glycerol, 5 mM of β -mercaptoethanol (reducing agent), or in the presence of unilamellar vesicles of 1-palmitoyl 2-oleoyl phosphocholine (POPC), used at a 1:100 and 1:200 protein-to-lipid molar ratio, at 25 °C. Moreover, cCD/SRCD spectra of the IDPs were measured over the temperature range of 20 to 80 °C, in 10 °C steps, with 5-min equilibration and collecting three scans at each temperature. CDTool (Lees et al. 2004) was used for all CD/SRCD data processing, which was performed with the average of the three individual scans, the subtraction of the spectrum of the corresponding baseline of each sample (containing buffer and all the others additives, except the proteins), smoothing with a Savitsky-Golay filter and zeroing in the 263–270 nm region. Final processed spectra were expressed in delta epsilon units ($\Delta\epsilon$) using mean residue weights of 99 and 111.6 for MEG-14 and STI, respectively. The average of the three

consecutive scans of each sample was used for the calculation of errors bars (one standard deviation). The high tension (HT) signal (or dynode voltage) was collected simultaneously with each cCD/SRCD spectrum over the same wavelength range.

Results and discussion

IDPs in aqueous solutions

The conventional circular dichroism (cCD) spectra of STI (Fig. 1a) in three different aqueous solutions assayed present a small positive peak at 225–230 nm, which is found in many disulfide-containing proteins (Hider et al. 1988; Tetenbaum and Miller 2001) or due to contributions of aromatic side chains of Trp (Woody 1994), and a huge negative peak around ~198 nm, which can be attributed to the significant contribution of the unordered content on the native

state of this protein (Chemes et al. 2012). STI presents a CD spectrum of a protein lacking any content in helix, but presenting contributions of β -strands together with a high amount of disordered elements, known as β_{II} class proteins (Sreerama and Woody 2003). For the protein MEG-14, as a result of its quite flexible and almost fully disordered state (Lopes et al. 2013), no spectral bands were observed at ~225-nm region (lacking any ordered content), but a negative peak of ~198 nm with similar shape was observed (Suppl. Figure 2a). Therefore, the negative 198-nm peak appears to be the most representative hallmark of the disordered conformation in a protein (a fully or a partially disordered protein) by circular dichroism spectroscopy. As the major CD peak for analyzing the disordered structure on an IDP occurs at wavelengths <200 nm, it is reasonably important to be able to reach accurately the low-UV wavelength region. The usual light sources in conventional CD instruments (Xenon lamps) provide low light flux below 190 nm, hampering analysis at low-UV wavelengths. The

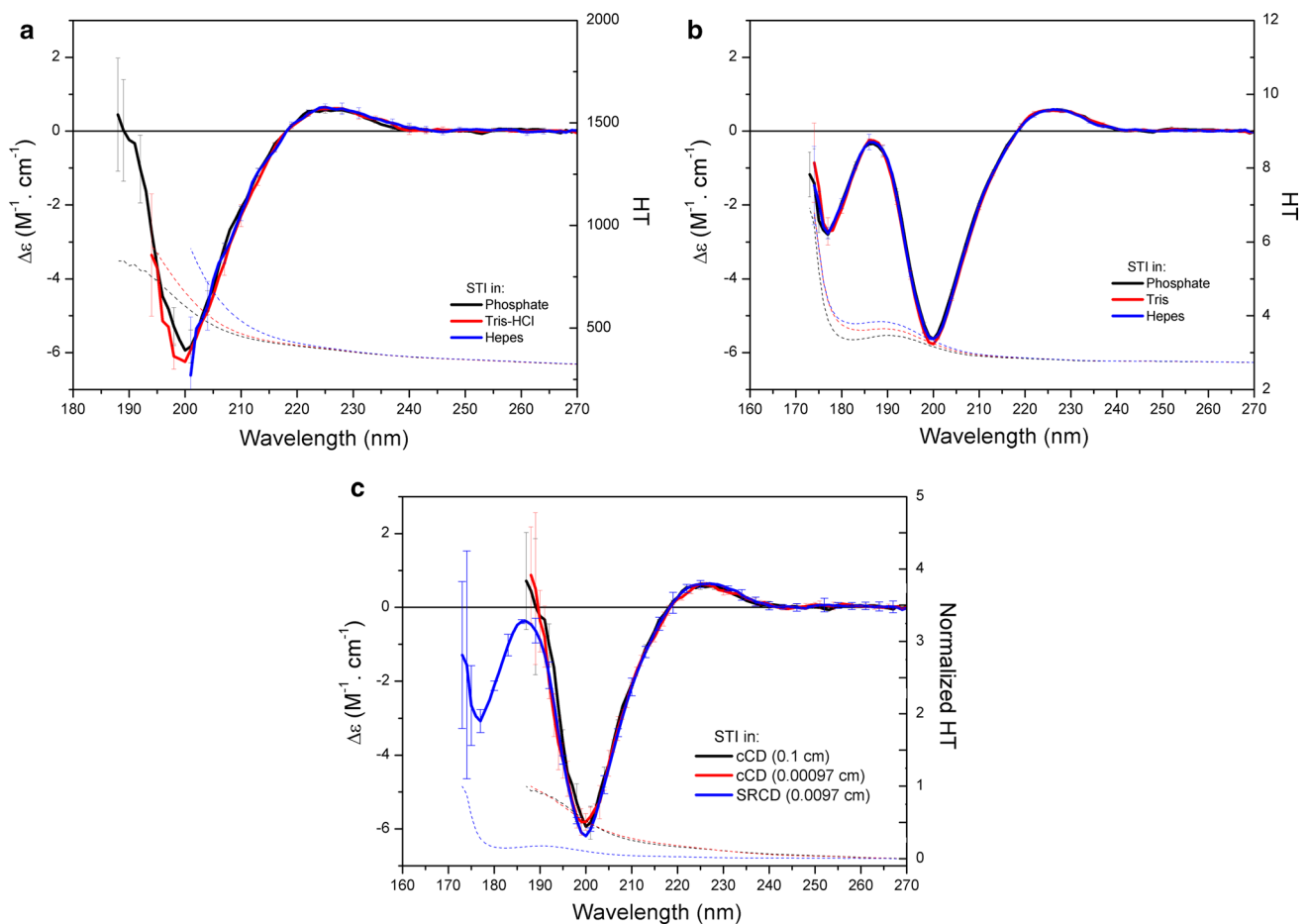


Fig. 1 cCD/SRCD spectra of IDPs in aqueous solutions. **a** cCD and **b** SRCD spectra of STI in 10 mM sodium phosphate (black), 10 mM Tris-HCl (blue), 10 mM HEPES (red), pH 7.4, at 25 °C. **c** Comparative cCD (black) and SRCD (blue) spectra of STI obtained at the

same conditions, and cCD spectra obtained with a 0.1-cm path length cuvette (red). Error bars and HT curves (dash) are shown for each measurement

higher flux of photons achieved in synchrotron facilities accurate data collection down to wavelengths of ~ 170 nm in aqueous solutions (Wallace and Janes 2010).

It is possible to note that although the cCD spectra in all the three different buffers were quite similar, the cut-off limit for accessing the low-wavelength UV region and, consequently, the region where spectral uncertainty increases (large error bars) were not equivalent in both proteins. Phosphate buffer gave the lowest cut-off among the three solutions (~ 186 nm); however, dilute Tris-HCl buffer was also efficient for monitoring the negative peak of the IDPs (cut-off ~ 192 nm). A limitation to access the negative peak appeared when using HEPES buffer (even at low molar concentration, as used here), which gave a low cut-off ~ 202 nm. Some buffers have already been listed as “bad choices” for measuring CD of globular/ordered proteins (such as piperazine sulphonic acid derivative or carbonyl buffers) because they generally absorb strongly at or below 200 nm (Kelly et al. 2005; Greenfield 2006; Wallace and Janes 2009). Therefore, they are less favorable buffers when working with disordered proteins as they interfere with the analysis of the low-wavelength peak at ~ 198 nm, which is the major (and sometime unique) spectral band in the cCD spectrum of an IDP.

In the SRCD spectra of STI (Fig. 1b), the lowest wavelength (cut-off limit) reached was ~ 173 nm in phosphate buffer. This wavelength extension clearly improved the observation of the full peak at ~ 198 nm and the accuracy of measurement < 200 nm (reduced errors bars) in all buffers. Interesting to note is that the SRCD method enabled accessing the peak at 198 nm when STI was in HEPES buffer, showing the method overcomes some of the limitation of highly absorbing buffers. Moreover, a small band at ~ 184 nm could be observed in the SRCD spectra of IDPs,

appearing as a shoulder for STI and a small positive peak for MEG-14 (Suppl. Figure 2c). This spectral band can also be attributed to the unordered content of the protein (Wallace and Janes 2009; Lopes et al. 2013). Therefore, the extended wavelength range obtained with the SRCD spectrum of an IDP gives access to additional electronic transitions, provides higher information content in the data, and provides more accurate data in the low-wavelength region, which can improve the structural analysis of this group of proteins.

As an alternative, cCD measurements for STI and MEG-14 were also obtained using a short path length cuvette in the conventional spectropolarimeter. A small increment (~ 3 nm) in the lowest UV wavelength accessed was achieved with the short-path length cell, however it is not equivalent to the improvement observed by the SRCD method (Fig. 1c), which was ~ 20 nm in the low-UV region due to the high flux photons of the SRCD method.

Tracking conformational changes in an IDP

The addition of the solvent TFE in an aqueous solution of a polypeptide is known to promote conformational changes on protein secondary structure, inducing the protein to acquire a more ordered content (Jasanoff and Fersht 1997; Cammers-Goodwin et al. 1996). Both cCD and SRCD methods were able to monitor the appearance of two minima at 208 and 222 nm and a positive maximum at 192 nm (Fig. 2), which is indicative of a significant induction of an α -helix structure in the presence of the organic solvent. The disordered CD spectrum observed for STI in aqueous buffer (Fig. 2a) was replaced by spectral bands assigned for the α -helix at 25% of TFE, and the spectral bands were gradually increased in

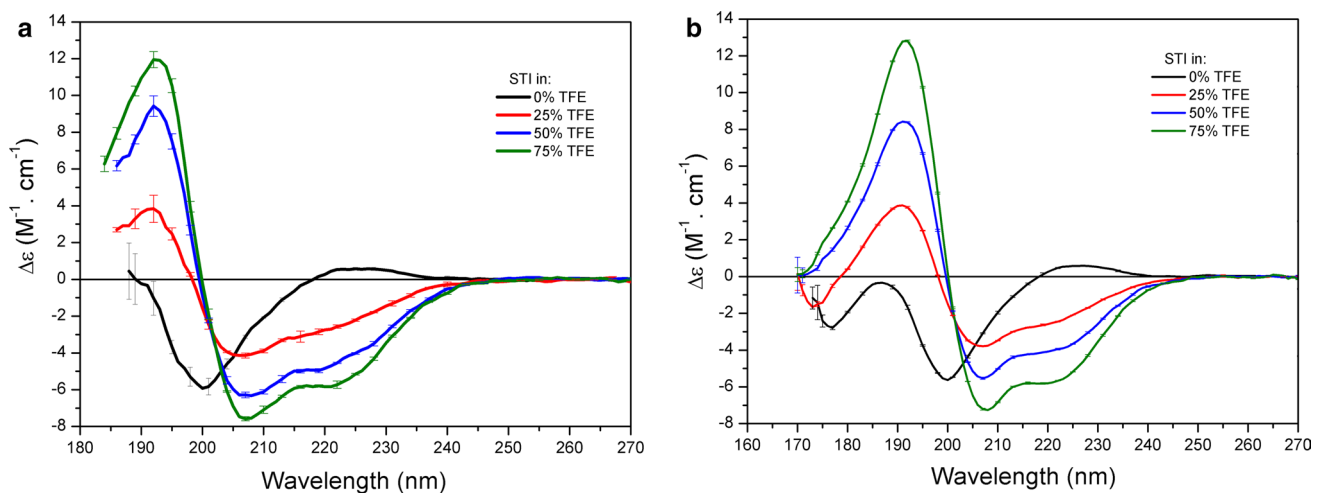


Fig. 2 Induction of ordered content in IDPs. **a** cCD and **b** SRCD spectra of STI in 10 mM sodium phosphate buffer (black), and in the presence 25% (red), 50% (blue), and 75% (green) of TFE. Error bars are shown for each measurement

the presence of 50 and 75% of the solvent. The changes on the 208/222-nm peaks were clearly monitored by the conventional method; however, the large error bars of <200 nm affected the analysis of the 192-nm peak. As the SRCD method extended the accuracy at low-UV wavelengths, it was possible to perform more accurate analysis of the changes on the entire peak at 192 nm, and on the additional transitions at the 184-nm region, both assigned for the disordered content of the proteins (Fig. 2b). The signal-to-noise ratio in the cCD spectra in Fig. 2 were clearly improved in the SRCD spectra (also in agreement with error bars of both methods). The high signal-to-noise achieved in the SRCD technique makes possible the acquisition of good-quality data with a number of repeated scans smaller than that required in the cCD method, consequently reducing the time for data collection.

Intrinsically disordered proteins underwent disorder-to-order transitions as an effect of temperature increase (Uversky 2009) in response to increased strength of hydrophobic interactions at high temperatures. Subtle changes are expected to be observed on a fully IDP (Uversky 2009), such as the reduction of the negative 198-nm peak and the increase of the 222-nm band observed on the melting assay of MEG-14 (Fig. 3a, b). Both cCD and SRCD spectra were able to detect these changes, but only the SRCD method enabled the visualization of the changes at the small positive peak at 184 nm, which was increased in intensity and red shifted to 202 nm during heating. Analysis of the thermal melt series of STI by cCD and SRCD methods showed equivalent changes, in which analysis at regions <200 nm could

be better assigned when the SRCD method was used (Suppl. Figure 4).

Working with IDPs in absorbing/scattering systems

The addition of 10 mM of NaCl, 5 mM of β -mercaptoethanol, and 10% glycerol in the aqueous solution of both STI (Fig. 4a) and MEG-14 (Suppl. Figure 5) reduced the wavelength range over which the CD spectrum could be measured, limiting the lower wavelength data. When NaCl or glycerol were added, the cCD spectra of STI showed merely part of the negative peak at 198 nm, due to the truncated form of the spectra. Adding the reducing agent β -mercaptoethanol in solution has limited even more the CD spectral range to \sim 203 nm, hampering the observation of the negative peak. Once again, the use of the SRCD method could overcome these limitations for accessing the negative peak region for both IDPs (Fig. 4b), representing an alternative way for accessing low-wavelength information on an IDP in solutions containing absorbing additives. Therefore, SRCD represents a better approach to evaluate the unstructured content of an IDP in solution, especially when any of these absorbing additives is present.

Another example is the study of protein–lipid interactions by tracking the conformational behavior of a protein in the presence of membrane models (micelles, small or large phospholipid vesicles). The presence of membrane models in a cuvette can interfere with the quality of the CD spectra at low-UV wavelengths due to scattering effects, especially when lipids are used at high concentrations (Wallace and Janes 2009; Wallace and Miles 2016). In Fig. 5, it is possible to observe that scattering effects

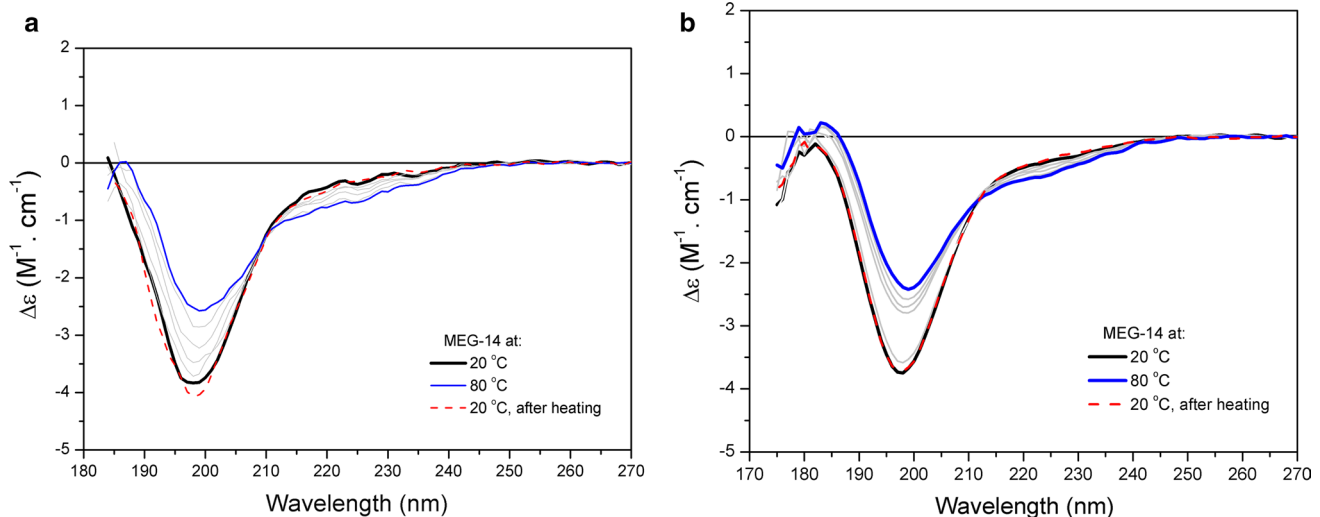


Fig. 3 Conformational changes caused by the increase of temperature. Thermal melt series for MEG-14 in sodium phosphate buffer (pH 7.4) from 20 °C (black) to 80 °C (blue), in 10 °C steps (interme-

diate temperatures in grey), obtained by **a** cCD and **b** SRCD methods. The spectrum obtained at 20 °C after heating is in red dashed line

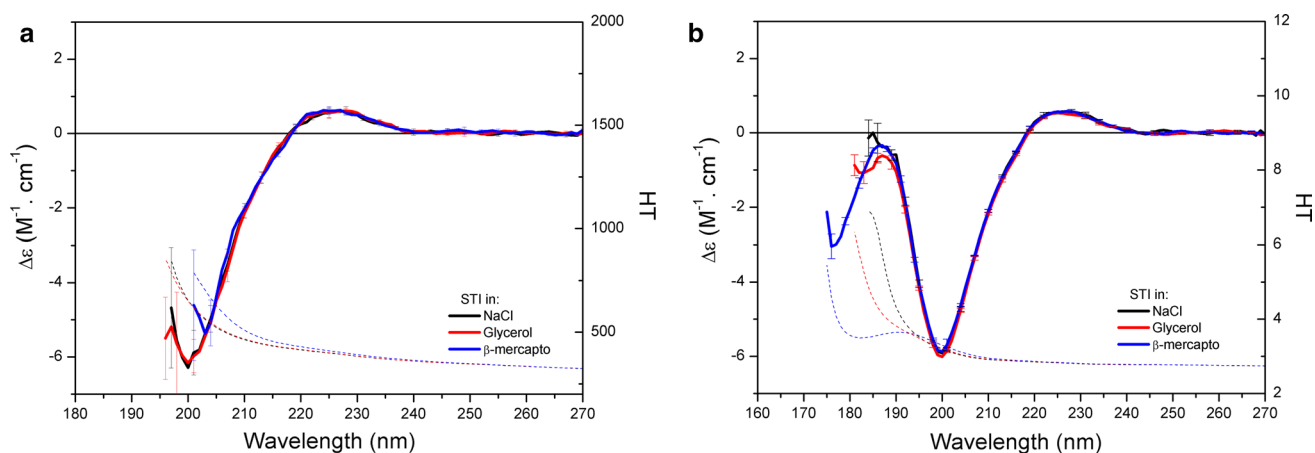


Fig. 4 cCD/SRCD spectra of IDPs in buffers with absorbing additives. **a** cCD and **b** SRCD spectra of STI in 10 mM sodium phosphate buffer (pH 7.4) with 100 mM NaCl (black), or 10% glycerol (red), or reducing agent 5 mM β -mercaptoethanol (blue)

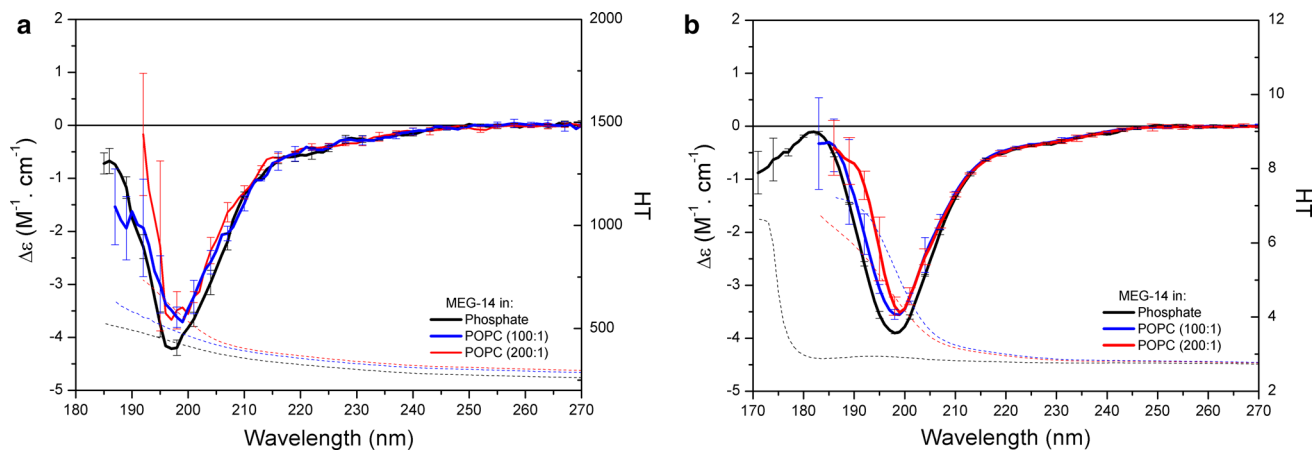


Fig. 5 Working with IDPs in scattering systems. **a** cCD and **b** SRCD spectra of MEG-14 in 10 mM sodium phosphate buffer (black) and in the presence of phospholipid vesicles of POPC at a 100:1 (blue)

and 200:1 (red) lipid to protein molar ratio. Error bars and HT curves (dash) are shown for each measurement

disturbed the monitoring of the negative band at 198 nm in the cCD of MEG-14. Similar scattering effects were noted also for STI (Suppl. Figure 6) in the presence of the vesicles. Nonetheless, the high flux of the SRCD method improves the analysis of IDPs in scattering systems, like high concentrations of membrane models, and enabled the accessing of the 198 nm peak for the disordered proteins at the molar ratios used (1/100 and 1/200, protein to lipid).

Conclusions

Circular dichroism spectroscopy stands out from other techniques to characterize the intrinsic disordered state of an IDP and to track the disorder-to-order transitions of its secondary structure as a response to changes in many

external factors. As IDPs are multifunctional proteins, they may interact with many different partners. Evaluating the interaction of an IDP with other biomolecules by CD spectroscopy is an assay widely performed. Adjusting for the best experimental conditions for the binding may require the use of buffers, absorbing additives, and/or scattering systems that can hamper the acquisition of a good-quality spectrum in the low-wavelength region when using conventional CD spectroscopy. In the present study, we observed that SRCD spectroscopy can clearly overcome most of these limitations in several experimental conditions tested.

Intrinsically disordered proteins present CD spectra with bands positioned in the vacuum-UV wavelength region, a major negative peak at 198 nm, and a small positive band at ~184 nm. The higher light flux and increased signal-to-noise ratio associated with synchrotron radiation at the

low-UV wavelengths are improvements especially good for the studying of an IDP by circular dichroism spectroscopy, since the major peak on the CD spectra of a full IDP is expected to be positioned at this low-wavelength region (~198 nm). In the same way, the extended wavelength in the vacuum-UV region makes possible the monitoring of additional spectral bands for this group of proteins below the 190-nm region. All improvements are important to increase accuracy in detecting changes in a SRCD spectrum of an IDP, improve better estimation of its disordered content, and to discriminate between the partially folded states of a natively unfolded protein.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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