



# Chapter 6

## Enzyme–Ligand Interaction Monitored by Synchrotron Radiation Circular Dichroism

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### Abstract

CD spectroscopy is the essential tool to quickly ascertain in the far-UV region the global conformational changes, the secondary structure content, and protein folding and in the near-UV region the local tertiary structure changes probed by the local environment of the aromatic side chains, prosthetic groups (hemes, flavones, carotenoids), the dihedral angle of disulfide bonds, and the ligand chromophore moieties, the latter occurring as a result of protein–ligand binding interaction. Qualitative and quantitative investigations into ligand-binding interactions in both the far- and near-UV regions using CD spectroscopy provide unique and direct information whether induced conformational changes upon ligand binding occur and of what nature that are unattainable with other techniques such as fluorescence, ITC, SPR, and AUC.

This chapter provides an overview of how to perform circular dichroism (CD) experiments, detailing methods, hints and tips for successful CD measurements. Descriptions of different experimental designs are discussed using CD to investigate ligand-binding interactions. This includes standard qualitative CD measurements conducted in both single-measurement mode and high-throughput 96-well plate mode, CD titrations, and UV protein denaturation assays with and without ligand.

The highly collimated micro-beam available at B23 beamline for synchrotron radiation circular dichroism (SRCD) at Diamond Light Source (DLS) offers many advantages to benchtop instruments. The synchrotron light source is ten times brighter than a standard xenon arc light source of benchtop instruments. The small diameter of the synchrotron beam can be up to 160 times smaller than that of benchtop light beams; this has enabled the use of small aperture cuvette cells and flat capillary tubes reducing substantially the amount of volume sample to be investigated. Methods, hints and tips, and golden rules to measure good quality, artifact-free SRCD and CD data will be described in this chapter in particular for the study of protein–ligand interactions and protein photostability.

**Key words** Circular dichroism, Ligand binding, Titration, Binding constant, UV denaturation, Protein stability, Data processing

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## 1 Introduction

Circular dichroism (CD) is a powerful technique which enables the monitoring of local and global changes in the structure and

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conformation of proteins. CD spectroscopy enables the selective monitoring of specific chromophores of the protein including peptide backbone in the far-UV region (180–250 nm), aromatic side chains of amino acid residues and dihedral angles of disulfide bonds in the near-UV region (250–350 nm), and prosthetic groups (hemes, flavones, carotenoids) in the visible region (400–800 nm). Bespoke benchtop CD instruments commonly use a xenon light source. Synchrotron radiation circular dichroism (SRCD) beamlines utilize the light produced at synchrotrons as the light source that is brighter than standard xenon light sources [1], and with much higher photon flux in the vacuum-UV region (130–200 nm). The highly collimated microbeam generated with the SRCD beamline B23 at Diamond Light Source (DLS) had enabled to reduce the amount of sample required for standard CD measurements by using lower volume cuvette cells with 1–2 mm diameter aperture [1, 2] and the high-throughput CD (HTCD) using 96 plates [3]. The higher photon flux of B23, however, can affect the photostability of the investigated protein by inducing structural denaturation [1]. Although this effect can be minimized or eliminated by reducing the slit width of the double-grating monochromator of B23 or rotating the cuvette cells around the axis parallel to the incident beamlight [4, 5], the UV protein denaturation has been used as an assay to determine the photostability and also qualitatively to determine ligand-binding interactions for UV-transparent ligands like fatty acids and carbohydrates [4, 5]. The rate of UV denaturation is significantly affected, usually decreased, if the ligand is interacting with the protein, otherwise, the rate with and without ligand is unaffected [4, 5]. In addition to qualitative investigations into ligand-binding interactions, recent advances saw the development of small-volume titration techniques that are most important when investigating the ligand-binding interactions of limited materials and has been well demonstrated with membrane proteins [6–15].

A large number of SRCD spectra can be collected during a single experiment, particularly when using the HTCD with 96 plates or UV denaturation assays at B23, and for Users who are allocated a specific number of 8-h shifts, it is important to be able to analyze on-the-fly to ensure that the experimental conditions are suitable for data collection. For this reason, software has been developed at B23 which allows for the analysis of CD data in .ols, .csv, and .txt format [16], making it compatible with data collected on other CD instruments off-site.

CD instruments measure the difference in absorbance of left- and right-circularly polarized light of chiral molecules as a function of wavelength expressed in nanometers (nm) otherwise known as CD spectroscopy [17]. Absorbance is therefore an important experimental parameter which must be considered when planning an experiment, and such considerations are made by application of the Beer-Lambert law  $A = \epsilon \cdot c \cdot l$ , where  $A$  is absorbance,  $\epsilon$  is specific molar extinction coefficient which is

specific for the chromophore studied,  $c$  is concentration, and  $l$  is pathlength. In most cases an optimum  $A$  (also known as optical density OD) of 0.8 is recommended, with a maximum of 1.5. For proteins, the amide bond is the main chromophore that absorbs in the far-UV region (180–250 nm) [18], while the aromatic side chains of tryptophan, tyrosine, and phenylalanine amino acid residues and disulfide bonds are the chromophores that absorb in the near-UV region. Prosthetic groups such as hemes, NAD and FAD cofactors, and carotenoid pigments are the chromophores that extend the absorption of light in the visible region (400–800 nm). The folding of proteins is promptly determined by SRCD/CD spectroscopy in the far-UV region for which the content of secondary structure can be estimated using a variety of algorithms [18–21] that are also available in several suites of programs [16, 22–24]. The CD/SRCD in the near-UV region is sensitive to the local environment of the aromatic side chains of Trp, Tyr, and Phe, and dihedral angle of disulfide bonds of cystine residues and has been used successfully to probe qualitatively and quantitatively ligand-binding interactions [25].

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## 2 Materials

### 2.1 Fused Silica Cuvettes

Common cuvettes employed for measurements of SRCD/CD spectra in the far-UV region are cylindrical or rectangular cells with a 0.02 cm pathlength made of fused silica. Low-volume titrations are conducted with a cell with a small-diameter channel which leads down to a small-volume reservoir. The reservoir window is made of black fused silica to mask accordingly the area of the incident light passing through the sample.

For measurements conducted in the near-UV region using larger pathlengths, e.g., 1 cm, these cells have black walls and small aperture (2 mm × 2 mm) windows. This restricted surface for light entry permits the use of small sample volumes (for a 1 cm pathlength, 70  $\mu$ L of solution is required for these specialized cuvettes compared to a standard cuvette requiring a sample volume >1 mL).

### 2.2 Buffer Systems Specificity

#### 2.2.1 Buffering System of Choice

For measurements in the far-UV region, sulfonate- (HEPES, MES, MOPS, PIPES) [17] and carboxylate-rich (glycine, acetate, citrate) [26] buffering systems are not recommended as these buffers tend to absorb at 200 nm due to the  $n \rightarrow \pi^*$  electronic transitions of the S=O and C=O bond, respectively, and alternative buffers should be used instead. Regularly, phosphate buffer (concentrations of <25 mM) is suggested to be the most appropriate buffer system for CD measurements, and low concentrations of Tris or Tris-acetate-EDTA (TAE) (<25 mM) are also acceptable, provided that pH adjustment is not done with HCl as this would introduce or increase the existing content of chloride anions ( $\text{Cl}^-$ ), which absorb in the far-UV region. pH adjustment of buffers should be

achieved using more UV-transparent phosphoric acid, nitric acid, acetic acid, citric acid, and borate [17].

### 2.2.2 Salts

As discussed previously, the presence of high concentrations of  $\text{Cl}^-$  is undesirable for studies in the far-UV region below 200 nm. If monovalent salts are required for maintenance of the ionic stabilization in the system to be studied, UV-transparent fluoride anions ( $\text{F}^-$ ) should be substituted for  $\text{Cl}^-$  [17, 25]. However, it is important to ascertain that the fluoride anions is not affecting the protein folding compared to that with chloride anions, which can be conducted by comparing the CD spectra in the overlapping transparent region that usually can be achieve down to 210–205 nm. Sulfate salts can also be used as an alternative [17, 25]. For a 0.02 cm and longer pathlengths, less salt concentration is better for CD measurements; otherwise the 200 nm or higher wavelength cutoff will prevent accurate secondary structure estimations. For pathlengths of 0.02 cm and longer, no more than 50 mM NaCl should be used, which could be increased to 100–150 mM NaCl with 0.01 cm. Higher concentrations of NaCl that need to be used (e.g., >500 mM) will require even narrower cell pathlengths that can be achieved only with demountable cells of 0.0050 or 0.0020 cm as it will be otherwise impossible to clean standard cuvette cells of these pathlengths. Of course, it will require higher protein concentration following the Beer's law. For example, as a rule of thumb, an average protein concentration of 0.4 mg/mL (for proteins highly helical the concentration could be as high as 0.5 mg/mL, while for disordered or highly beta-sheet proteins, the concentration would be 0.3 mg/mL as the UV absorption is higher) will enable a good signal-to-noise quality CD spectrum using a cuvette cell of 0.02 cm pathlength. Therefore, halving the pathlength to 0.01 cm will require double the protein concentration to obtain the same CD spectrum. With B23, the smallest pathlength available for standard measurements is 5  $\mu\text{m}$  which requires a protein sample volume of 2  $\mu\text{L}$  at a concentration of 16 mg/mL.

### 2.2.3 Buffer Additives

Addition of buffer additives should also be carefully assessed based on their UV absorption properties. For protein samples which have been purified using high concentrations of imidazole, such as from affinity purification columns, buffer exchange of the protein to remove all imidazole is required as it absorbs too strongly in the ~210 nm region due to its large extinction coefficient [17]. The addition of histidine in the protein formulation should be therefore avoided for the same reason. Similarly, urea and guanidine denaturant agents should be avoided unless the protein denaturation is the aim of the experiment. High concentrations of glycerol (<10% (v/v)) should also be avoided to enable the CD to be measured below 200 nm [22]. The four carboxylate groups of ethylenediaminetetraacetic acid (EDTA) absorb strongly below 200 nm and therefore should not be used in concentrations above 1 mM for measurements in the far-UV region

using a 0.02 cm pathlength [17]. For higher EDTA concentration, a smaller pathlength should be used but a compromise has to be reached as the protein concentration should be increased as well that will decrease the EDTA-protein molar ratio.

If a reducing agent is required, beta-mercaptoethanol (BME) is preferred to dithiothreitol (DTT). DTT strongly absorbs across the UV region— $\text{DTT}_{\text{red}}$  (the active for which maintains the reduced state of proteins) below 260 nm across the far-UV region and  $\text{DTT}_{\text{ox}}$  (the result of the eventual oxidization of DTT over time from the environment) below 340 nm and across the near-UV region [27, 28]. Tris (2-carboxymethyl) phosphine-HCl (TCEP-HCl) is also compatible with CD but is highly acidic; therefore, the suitability of a potentially acidic environment for the protein should be considered (no more than 1 mM) [29, 30]. DMSO should also be avoided for measurements in the far-UV region as it absorbs strongly in the UV region ( $\lambda_{\text{max}}$  220 nm, cutoff is <260 nm) and therefore is unsuitable for far-UV measurements [31, 32]. If an organic solvent is required, most other types, e.g., hexane and trifluoroethanol, are transparent down to 185 nm; however, it is important to note that these solvents may induce structural changes to the protein being studied [22].

### 2.3 Protein Samples

Protein samples should be soluble, homogenous, and pure; therefore, purification techniques including size-exclusion chromatography (SEC) should be used in addition to standard immobilized metal affinity chromatography (IMAC) to ensure highly pure samples which can be checked using gel electrophoresis and mass spectroscopy [22]. Solid particles can lead to light scattering and distortion of measured CD spectra [17]; therefore, effort should be made to remove them from samples before data collection. Centrifugation of the sample and only using the supernatant for measurements or filtering samples through a 0.2  $\mu\text{m}$  filter before loading into the cuvettes for data collection can be adopted to ensure samples are solid free [17]. Protein concentration determined spectroscopically absorbance should follow the Beer-Lambert law measuring either the absorbance at 280 nm for proteins containing aromatic amino acids or at 205 nm for nonaromatic amino acid containing peptides/proteins [18]. This should be performed with the final protein product (after purification and removal of solid particles) prior to any CD measurement.

### 2.4 CDApps Software

CDApps software has been developed to analyze SRCD and CD data (.ols, .csv, .txt format). Developed at Diamond B23 beamline, the software is available to use on-site for analysis, and can be copied on a USB stick along with video tutorial files for analysis and training off-site. Information regarding beamline software including manuals can be found on the beamlines software page <http://www.diamond.ac.uk/Instruments/Soft-Condensed-Matter/B23/manual/Beamline-software.html>.

The software has progressed from its original conception as detailed in [16] which focused on the planning and data processing of standard, thermal melt, UV denaturation and titration experiments to have a more intuitive interface as well as the processing capability for high-throughput CD (HTCD) data sets from a 96-well plate [3] and CD imaging applications [33].

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## 3 Methods

Circular dichroism experiments conducted at B23, Diamond Light Source, can all be planned and processed using CDApps [16].

For all experiments, background measurements (background buffer equivalents) should be collected which can be subtracted from all experimental data files. The most recent upgrades for the data collection software include the option to collect UV absorption data which can be used as an internal control for each sample measured. Optimum absorbance readings are  $\sim 0.8$ , for titrations the range recommended is from 0.4 to a maximum of 1.5–1.6 [34]. Within these limits, the chromophore concentration will be sufficient for detection and without being too high to saturate the detector distorting the spectral profile. A recommendation before starting a series of measurements is to know the absorbance in terms of intensity magnitude and wavelength maxima for all components of the solutions such as solvent (buffer), solute (protein/peptide), additives, ligands, and any other chemical agent present therein (*see* Subheading 2.2.1 and **Notes 1–4**). CDApps can help to calculate the absorption of mixtures from the information input into the software (pathlength to be used, extinction coefficient at specific wavelengths, and molecular weights of materials) for the individual components during planning of the experiments [16].

Before commencing measurements, an air baseline should be recorded for the instrument once the experimental parameters (wavelength range, wavelength increment, integration time) have been set up. This will allow for the data collection software to calculate the absorbance simultaneously during CD signal collection.

### 3.1 Standard CD Measurements

Standard CD measurements can be performed at the B23 beamline at DLS. Compared to standard benchtop CD instruments, the synchrotron light source is comparatively tenfold brighter due to the higher photon flux in a smaller diameter beam [1]. These characteristics open up new opportunities for CD measurements which are unattainable using conventional CD instruments, some of which will be discussed throughout this chapter.

Standard CD measurements can be used to qualitatively assess the conformational property of proteins in terms of secondary structure and local tertiary structural changes occurring upon ligand-binding interactions.

Below will be described the basic experimental setup which can be changed for the sample available, using the principles of the Beer-Lambert law for guidance [35]. For protein solutions of a concentration higher than the standard described previously (about 0.4 mg/mL in 0.02 cm cell pathlength) smaller pathlengths are required, whereas for more dilute solutions larger pathlengths are used [35] (*see Notes 5 and 6*).

In the far-UV region, protein secondary structure conformational changes as a function of environment (solvent composition and polarity [8, 22], pH [8, 22], ionic strength [8, 22], surfactants [36], pressure (up to 200 MPa) [37], temperature (−170 °C to +350 °C) [38], and ligand interaction [8]) can be promptly monitored. Standard parameters are as follows:

1. A protein solution at concentration of 0.4 mg/mL, measured using a cuvette of 0.02 cm pathlength. These cuvettes (both cylindrical and rectangular designs) require to be filled at least with 40  $\mu$ L of solution.
2. Default parameters for CD measurements in the far-UV region (180–260 nm) using B23 beamline:
  - (a) 1 nm increments.
  - (b) 1 s integration.
  - (c) 1.2 nm bandwidth (bw) corresponding to the monochromator slit width of 0.5 mm (for benchtop CD instruments the recommended bw is 1 nm).
3. The number of scans can be tailored to the sample/experimental design (*see Note 6*).

In the near-UV region, protein conformation changes with respect to the local environment of aromatic side chains of Trp, Tyr, and Phe residues and disulfide bond of Cys residues as a function of solvent composition and polarity [8], pH [8], ionic strength [8], surfactants [39, 40], pressure (up to 200 MPa) [41], temperature (−170 °C to +350 °C) [42], and ligand [8] interaction can be promptly monitored. Standard parameters are as follows:

1. Protein solution with an absorbance at 280 nm of ~0.8 is desirable (for ligand titration, the recommended absorption range is from 0.4 to 1.6).
2. Data collection occurs in the near-UV region (260–350 nm) using the following parameters:
  - (a) 1 nm increments.
  - (b) 1 s integration.
  - (c) 1.8 nm bandwidth (corresponding to slit width of 1 mm). For benchtop instruments, the recommended bandwidth is 2 nm.

3. The number of scans can be tailored to the sample/experimental design (*see Note 6*).

For all types of measurements concerning proteins, clean cuvettes by (*see Note 7*):

1. Flushing the cell with deionized water followed by 96% (v/v) ethanol.
2. Place the ethanol wet cell in conc. nitric acid in a fume hood, and leave for 10 min.
3. Remove the cell and flush with deionized water to remove the acid.
4. Wash with 96% (v/v) ethanol and dry under nitrogen or using a vacuum pump.

### **3.2 UV Denaturation of Proteins Monitored by Circular Dichroism in the Far-UV Region (180–260 nm)**

In the far-UV and vacuum-UV regions, the intense photon flux of the highly collimated microbeam of B23 beamline has the potential to cause protein denaturation [5, 43–45]. UV photo denaturation experiments have been used successfully as a novel assay for the assessment of protein photostability that can be used to determine ligand-binding interactions, in particular for ligands with weak or devoid of any UV chromophores in the far-UV region [5].

For these measurements, samples are loaded into cuvettes following the guidelines mentioned in Subheading 2.1. Repeated continuous scans, usually 20, 30, or in case of stable materials up to 100 are collected from the sample (*see Note 8*).

The general protocol is as follows:

1. For a cuvette (either cylindrical or rectangular cell) of 0.02 cm pathlength, 40  $\mu$ L of a protein solution at about 0.4 mg/mL is loaded into the cuvette.
2. Standard data collection parameters in the far-UV region (180–260 nm) include 1 nm increments, 1 s integration time, and 1.2 nm bandwidth. At this point a standard CD measurement can be collected.
3. For the UV denaturation method, repeated continuous measurements are scanned. The number of scans to be collected is dependent on the system under study as the protein sensitive to UV light (photostability) varies from protein to protein. A suggested range is 20–30 scans that correspond to a total time of 60–90 min in order to obtain a good denaturation rate trend within a reasonable time-scale which is often a key consideration especially for Users of the B23 beamline at Diamond Light Source where allocated experimental time is limited.
4. For data analyses *see* Subheading 3.4.



### 3.3 Low-Volume Titrations for Ligand Binding Monitored by Circular Dichroism

CD titration experiments enable the determination of the quantitative binding interactions of host-ligand systems. It has been successfully employed for the determination of the dissociation constant  $K_d$  for ligand binding to membrane proteins and soluble proteins [6–15]. This was done by monitoring the CD spectral changes of the protein upon the incremental addition of small but accurate aliquots of ligand corresponding to an increased molar ratio until no more changes were observed.

CD titration experiments conducted in the near-UV region (250–350 nm) have the potential to use a large volume of sample due to the larger pathlength which are often employed to monitor low-concentration solutions. A fused quartz cuvette (Starna) with a window aperture of 2 mm × 2 mm for a 1 cm pathlength requiring only 70  $\mu$ L to be filled is routinely used at B23 due to the smaller footprint of the synchrotron light source beam that can be conveniently varied from 0.05 to 1 mm in diameter. The larger beamlight of benchtop CD instruments, from about 3 to 8 mm in diameter, makes the use of such a small aperture 1 cm cell very impractical. On the contrary, this cell is routinely used for CD titration with B23 in particular for membrane proteins of which yields are notoriously smaller than most soluble proteins under the same expression and purification conditions. Another advantage of the small aperture 1 cm cell is the fact that the addition of the ligand aliquots and their mixing with the protein can be conducted rather gently reducing the equilibration time when detergents are used to solubilize the membrane proteins. CDApps can also be used to calculate the absorption of the solution at each titration step point using the molar extinction coefficient for both the protein and the ligand to ensure the total absorption in the cuvette is within the acceptable 0.4–1.5 absorption limits [16] (*see Notes 9–11*).

For these experiments, the following protocol is used:

1. Measure the concentration of protein solution at  $A_{280}$ . Use this value to calculate the dilution factor for the stock solution for the working solution. The concentration of the protein to be used during the experiment is in part determined with respect to the UV absorbance contribution of the ligand at each titration point which should not be more than 1.5–1.6 (*see Notes 9–11*).
2. Aliquots of ligand are added to the host mixture in the cuvette, pipetting up and down to thoroughly combine the components (*see Notes 12 and 13*).
3. Mixture incubated for a user-defined period of time (e.g., 20 min) to allow for equilibration to occur (*see Note 14*) and ensure the appearance of the solution is homogenous before measuring (*see Notes 13, 15 and 16*). This is particularly important in the presence of detergents as the addition of

aliquots affects the vesicle morphology and requires equilibration to obtain reproducible CD baselines. The way to determine the incubation time is to scan repeated consecutive CD spectra until the spectral shape is stable. The incubation time can be calculated as the product of the number of scans multiplied by the time to scan a single spectrum.

4. Measurements are collected for each titration point, after the determined incubation time.
5. CDApps is encouraged for the analysis, using the experimental plan as the template for the analysis as it contains information regarding volumes, concentration, and dilution factors of the mixture components in addition to pathlength(s) used to monitor the mixtures (*see* **Notes 10–12**).
6. CD spectrum of the Buffer under the same incubation time is measured to be used as baseline to be subtracted from each titration point spectrum, followed by the CD spectrum of the relevant proportion of the maximum-titrate ligand concentration. This is essential if the ligand is chiral. For achiral ligands, this is not necessary as the ligand will be devoid of any CD and the CD spectrum of the baseline will be sufficient for the data processing.
7. Difference in the CD signal at each point is plotted against the respective ligand concentration, fitting with a Hill function for determination of the binding dissociation constant  $K_d$  and the stoichiometry of the interaction using a nonlinear regression analysis [46].

### **3.4 Processing of Circular Dichroism Data Collected at B23**

Data is processed using CDApps available at the B23 beamline for use on-site and as a USB downloadable copy for use off-site (found at Computer > Software (W:) > cd\_programs > CD Apps > USB). For processing, the following steps are recommended.

#### *3.4.1 Loading CDApps for Processing of Circular Dichroism Data Collected at B23*

Below are the initial steps required to load CDApps software:

1. Open the CDApps software by:
  - (a) On-site—Computer > Software (W:) > cd\_programs > CD Apps > CD Apps.exe. Double click the CD Apps icon.
  - (b) Off-site—double-clicking the CD Apps icon.

The following options page enables the User to select the type of analysis appropriate for the data set.

- (a) CD Measurement (including Automation)—analysis options available include 1 ton spectra, titration, UV denaturation, thermal melt using both the Quantum Peltier (+5 °C to +95 °C) and Linkam MDS 600 (−150 °C to +350 °C) controllers and 96-well format.

(b) CD Titration.

The analysis using both of these methods shall be discussed.

For all types of analyses, the most important bits of information for subsequent analyses including conversion of units and secondary structure estimations (SSE) are concentration, pathlength, concentration and molecular weight and  $\Delta\epsilon$  (the average amino acid molecular weight).

3.4.2 Analysis Using  
the “CD Measurement  
(Including Automation)”  
Option

- (a) Select the “CD Measurement (including Automation)” (highlighted in Fig. 1).
- (b) Click on the “Sample Description” section of the sample line to be completed and enter details, e.g., name of the sample (Fig. 2).
- (c) To add experimental data to the spreadsheet:  
Select the “Experiment Data” tab, selecting the appropriate button for the type of file to be uploaded (.ols or .csv/.txt).

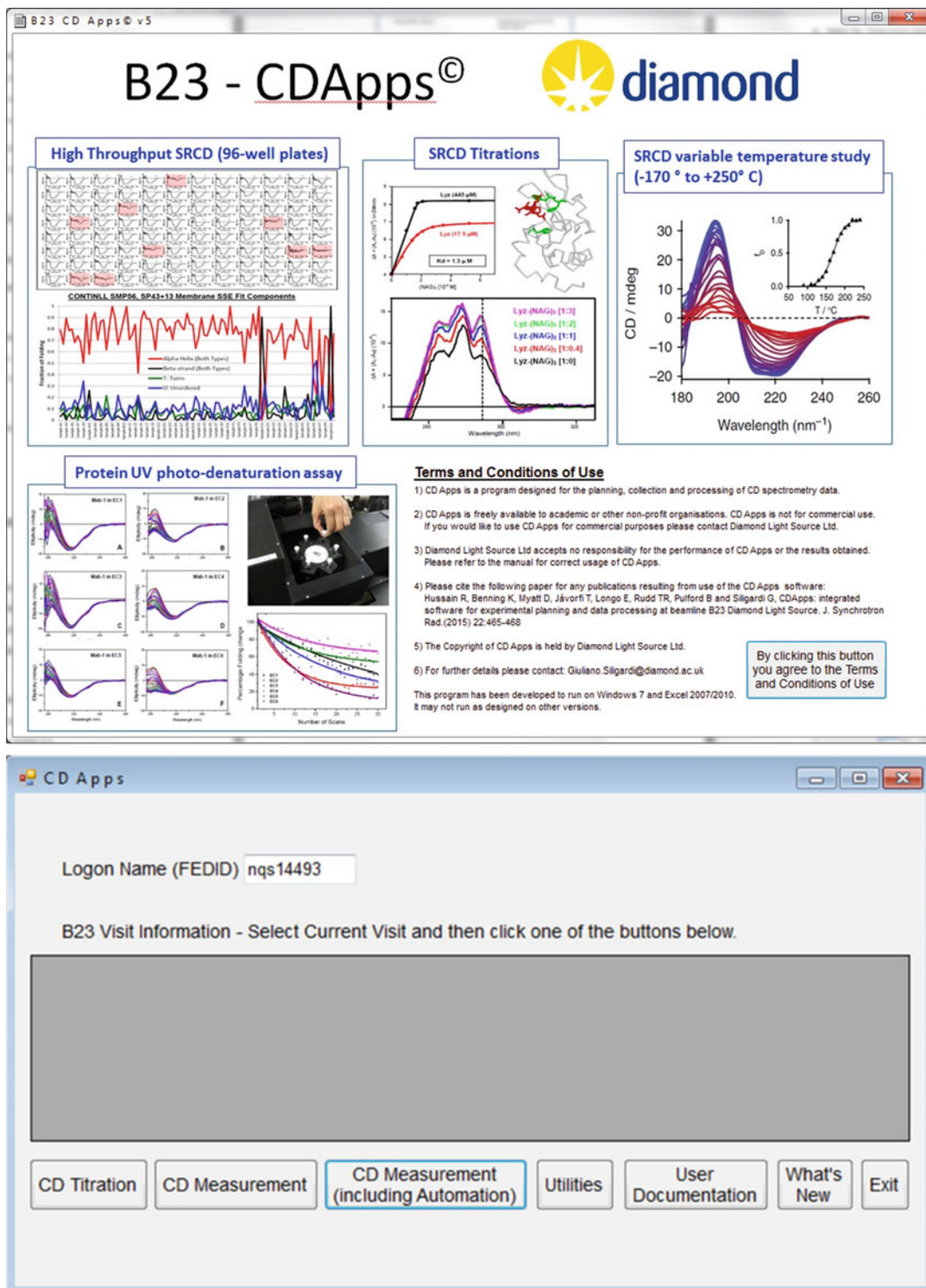
Generally, files collected at the beamline are saved as .ols files. Files collected using both the benchtop Chirascan instrument and the beamline modules can be converted into .csv format.

Attach the appropriate data file to the sample line by clicking on the file to highlight (line becomes blue), then drag and drop the file across to the sample line.

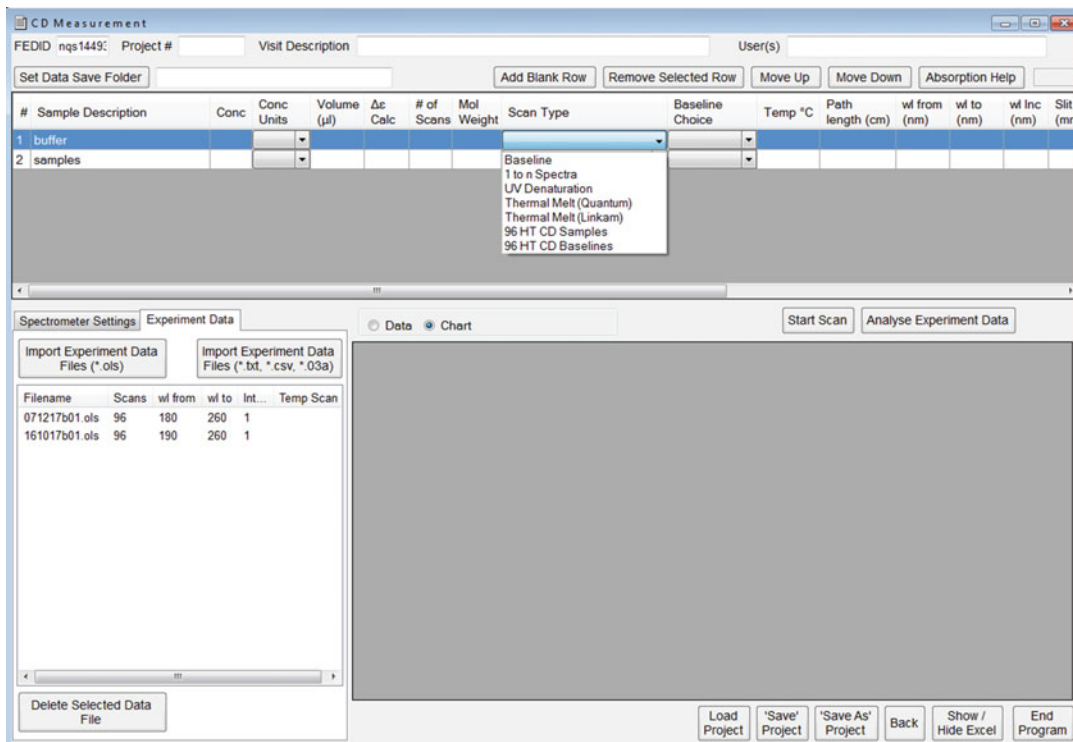
Experiment details (number of scans, wavelength ranges, wavelength increment) are automatically completed for the attached file.

- (d) Complete the remaining empty experiment details (concentration, concentration units (mg/mL or  $\mu\text{M}$ ), volume ( $\mu\text{L}$ ),  $\Delta\epsilon$  calculated, molecular weight (Da), temperature ( $^{\circ}\text{C}$ ), pathlength (cm), slit (mm), and integration time(s)).
- (e) New rows can be created by selecting “Add blank row” and repeating **steps 3–5**. If all of the details of a row are to be replicated for additional rows, this can easily be done by a single right-click and selecting the “replicate” option. Appropriate data can then be attached using **step (d)**.

Note: For any type of analysis, the correct scan type needs to be selected and appropriate baseline allocated. Baseline is assigned to background or buffer measurements, which will be subtracted from all other measurements selected in the group during the analysis. Depending on the analysis selected, the software will subtract the baseline differently. For 1 to  $n$  Spectra multiple entries should be used during the analysis, one per sample measured which can each contain multiple scans per loaded file. The 1 to  $n$  Spectra option creates an



**Fig. 1** (Top) Terms and conditions page which appears upon opening. (Bottom) Initial options page which opens upon loading of the program



**Fig. 2** Opening screen for CDApps. From here all of the experimental details including data type and experimental parameters are added ready for the analysis. From here the initial information is input before progressing through the analysis steps by pressing “Analyse Experiment Data”

average scan for each loaded file and subtracts the baseline from each sample. UV Denaturation analyses subtract the average spectra of the baseline from every individual scan in the file.

- (f) From here, a range of analyses are available for different types of data sets. The type of analysis is selected using the “Scan type” drop-down menu for each sample line (*see Note 17*). Each analysis option will be discussed in Subheading 3.4.

#### 1 to $n$ Analysis

Multiple samples can be analyzed by completing the required number of sample lines as detailed in Subheading 3.3, **step 2**, and highlighting the desired number of samples before selecting the “Analyse Experimental Data.”

- (a) Complete the workbook with the appropriate number of sample lines and data.

Ensure that the data files to be analyzed are within the same wavelength range and increments. For analyses in the far-UV region (180–260 nm) where secondary structure estimations (SSE) are desired as part of the analysis, ensure that loaded files are collected in 1 nm increments.

Select “1 to n Spectra” option from drop-down menu for “Scan type.”

- (b) Highlight (blue) samples to be analyzed by clicking on the desired sample line and click “Analyse Experimental Data.”
- (c) Select the zeroing region for the data which will be used to off-set all data sets. This is often in a region where no CD signal is observed. Click “Graph Data.”
- (d) Graphs will appear in the Excel Workbook, detailing the different units for CD presentation ( $\Delta\epsilon$ ,  $\Delta A$ ).

Note: Only after the zeroing has been performed can SSE be calculated. A new tab will appear under the zeroing section (Fig. 3b, c).

- (e) For SSE, select the appropriate algorithm (CONTINLL [19–21], CDSSTR, or SECLON3 [20]) and database (SP29, SP23, SP1, SP37, SP2, SP42, SMP50, SP43, SDP48, SMP56, CLSTR).
- (f) Data will be output into an Excel Workbook containing graphical and numerical outputs. Graphs can be used as they are by copying, or numerical outputs can be used for further analysis and redesigning of graphs for data presentation.

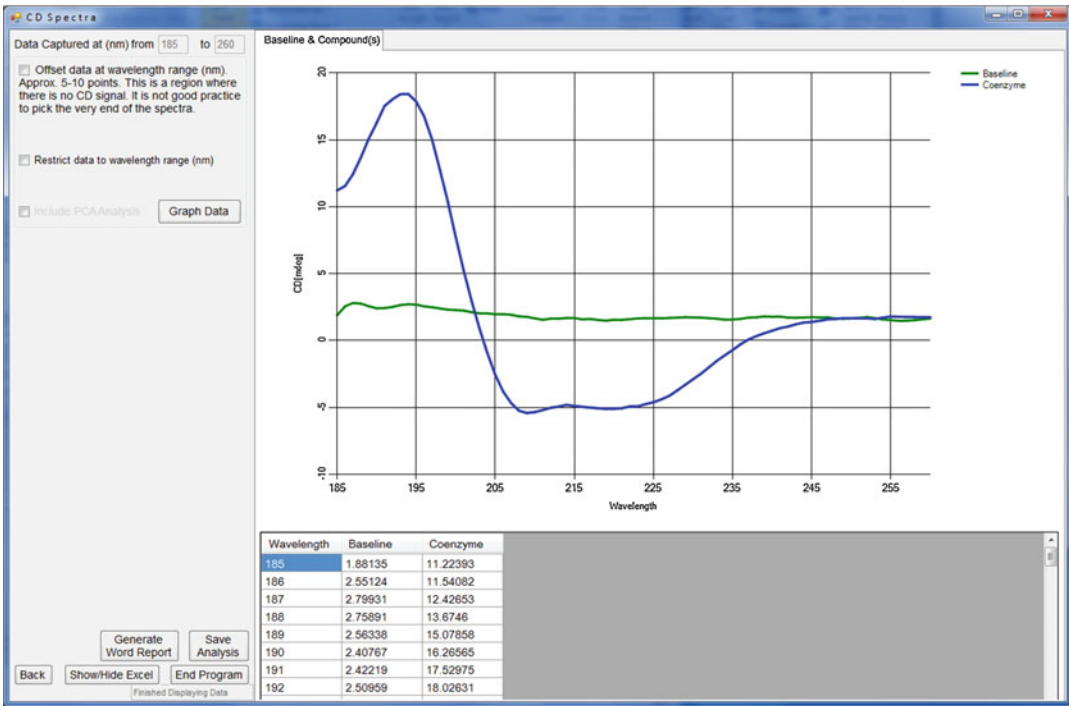
#### UV Denaturation Analysis

UV denaturation analyses investigate the unfolding process of a protein as a result of exposure to UV light from the synchrotron light source during a CD experiment.

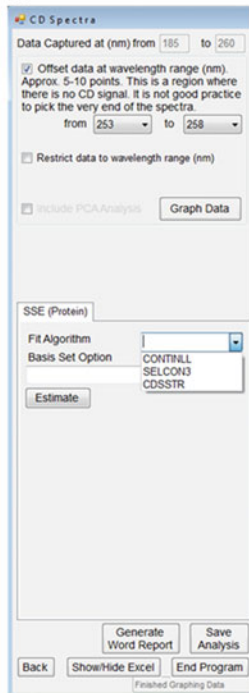
1. Select UV denaturation option from drop-down menu for “Scan type” (Fig. 2).
2. Highlight the Sample and Baseline sample lines. Highlighted lines will appear blue.
 

Note: Unlike the 1 to  $n$  analysis option, only one sample can be analyzed at a time.
3. Click “Analyse Experiment Data.”
4. Set the off-set region for the data (where no CD signal is observed).
5. Select the wavelength to monitor for UV denaturation by either clicking on the region of the observed signal in CDApps or selecting the wavelength from the drop-down menu (Fig. 4).
6. Click “Graph Wavelength to Monitor” button.
7. Under the UV Denaturation Fitting tab in CDApps, select the most appropriate fit for the data points (Fig. 4).
8. Alternatively, copy the data from the “Folding Change” tab of the Excel workbook for analysis using alternative software.
9. Under the SSE (Protein) tab, select appropriate algorithm for protein secondary structure estimation (Fig. 5).

A.



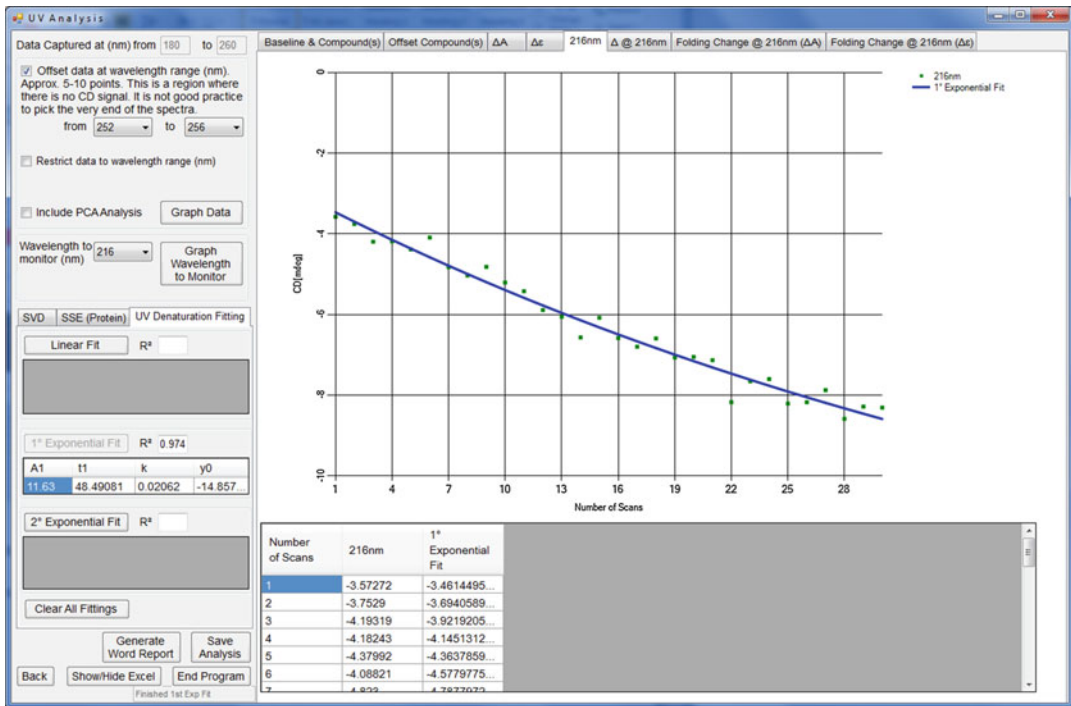
B.



C.

Basis Set	Ref Set	Proteins	Secondary Structures	Wavelength
1	SP29	29-Soluble	H1, H2, S1, S2, T, U	178-260
2	SP23	23-Soluble	H, 3-10, S, T, P2, U	178-260
3	SP1	29-Soluble	H, S, P2, T, U	178-260
4	SP37	37-Soluble	H1, H2, S1, S2, T, U	185-240
5	SP2	37-Soluble	H, S, P2, T, U	185-240
6	SP42	SP37+5 Denatrd	H1, H2, S1, S2, T, U	185-240
7	SMP50	SP37+13 Membrane	H1, H2, S1, S2, T, U	185-240
8	SP43	43-Soluble	H1, H2, S1, S2, T, U	190-240
9	SDP48	SP43+5 Denatrd	H1, H2, S1, S2, T, U	190-240
10	SMP56	SP43+13 Membrane	H1, H2, S1, S2, T, U	190-240
11	CLSTR	Soluble/Denatrd	H1, H2, S1, S2, T, U	190-240

**Fig. 3 (a)** Analysis page of CDApps after pressing “Analyze Experimental Data” button for “1 to  $n$ ” function. **(b)** After setting the data offset a SSE tab appears from which the appropriate algorithm and **(c)** reference dataset is selected

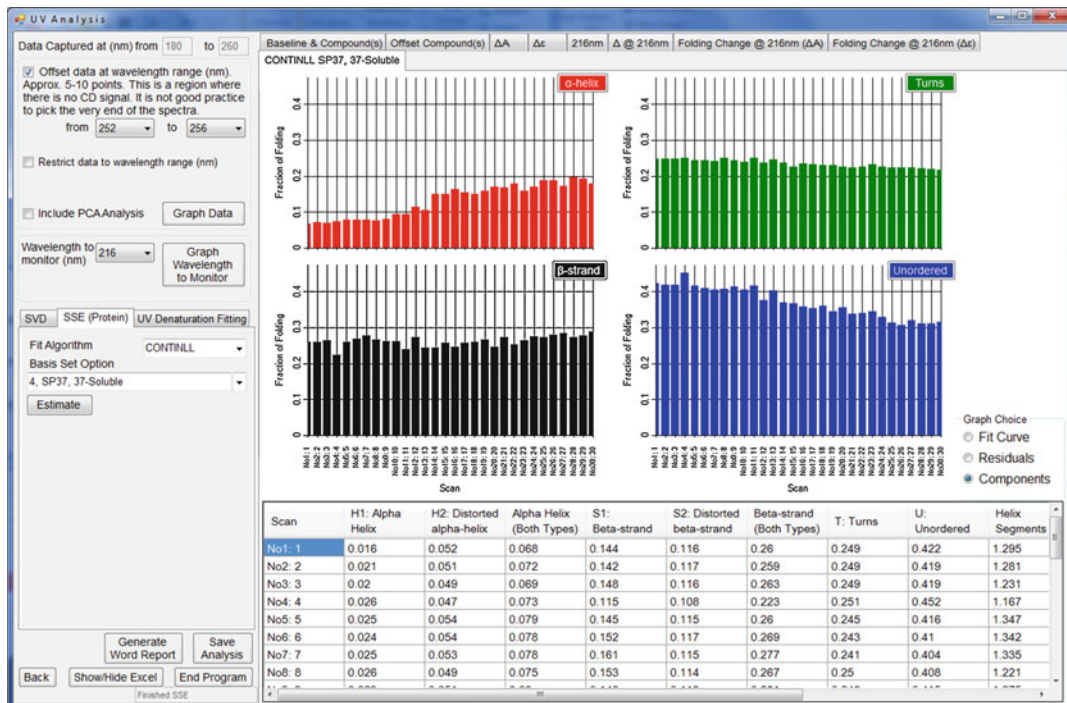


**Fig. 4** Program appearance during UV denaturation analysis. Selecting the wavelength to monitor UV denaturation and clicking “Graph Wavelength to Monitor” button produces a graph which can be fitted to determine the kinetics of the denaturation

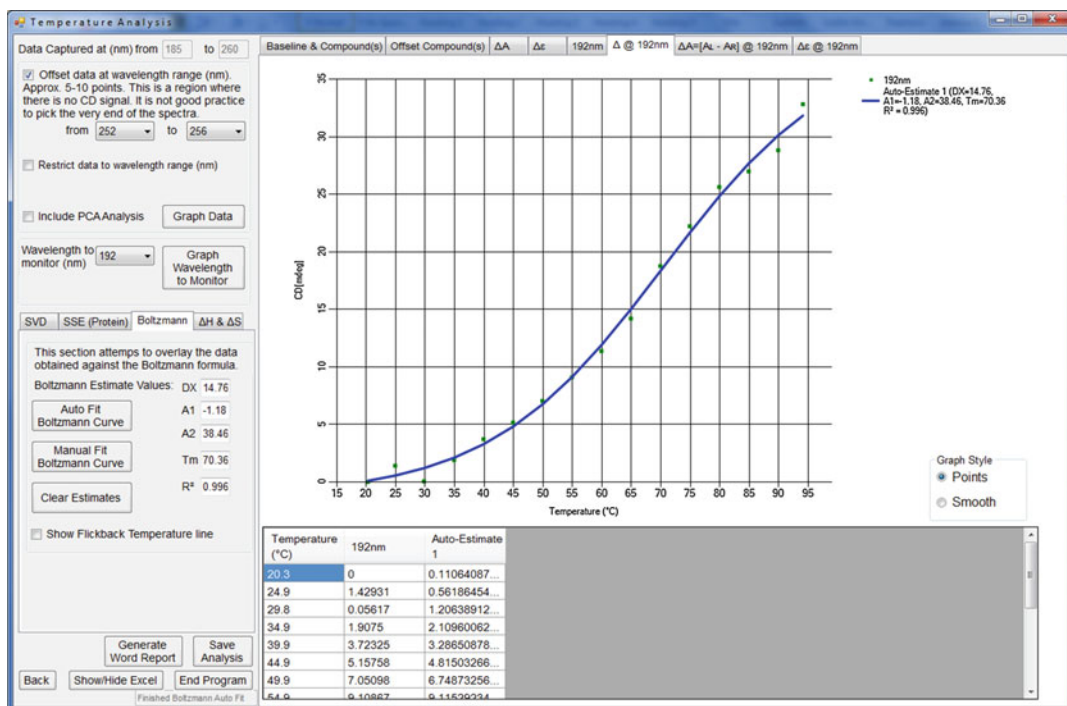
Thermal Melt Analysis

1. Select Thermal Melt option from drop-down menu for “Scan type.”
2. Highlight (click to make line blue) the Sample and Baseline sample lines.  
 Note: Unlike the 1 to *n* analysis option, only one sample can be analyzed at a time.
3. Click “Analyse Experiment Data.”
4. Set the off-set region for the data (where no CD signal is observed).
5. Select the wavelength to monitor for thermal denaturation by either clicking on the region of the observed signal in CDApps or selecting the wavelength from the drop-down menu.
6. Click “Graph Wavelength to Monitor” button.
7. Under the Boltzmann tab in CDApps, select the “Auto Fit Boltzmann Curve” button or, if the kinetics are known for the system and the analysis serves as a check to fit the details that can be manually inputted, then click “Manual Fit Boltzmann Curve” button (Fig. 6).

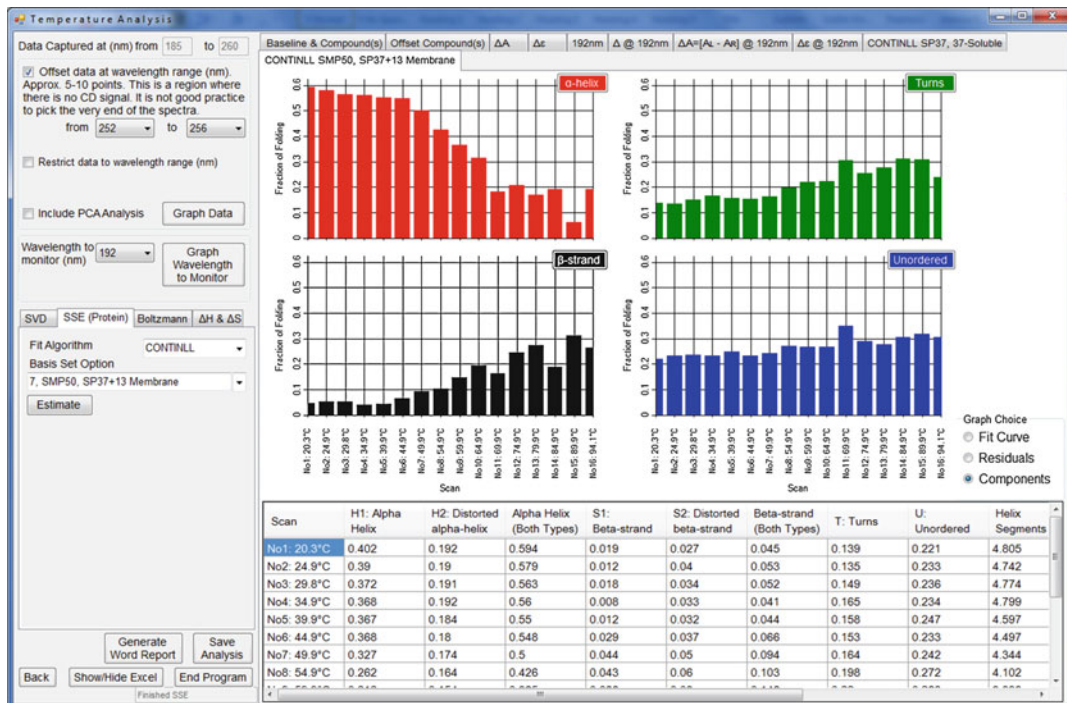




**Fig. 5** SSE for each measurement of the denaturation file. SSE can be performed for each scan in the file using the options under the “SSE (protein)” tab



**Fig. 6** Analysis window for Thermal Melt analysis. Change in CD can be monitored at a specific wavelength for calculation of denaturation kinetics. Kinetics calculated using the Boltzmann tab

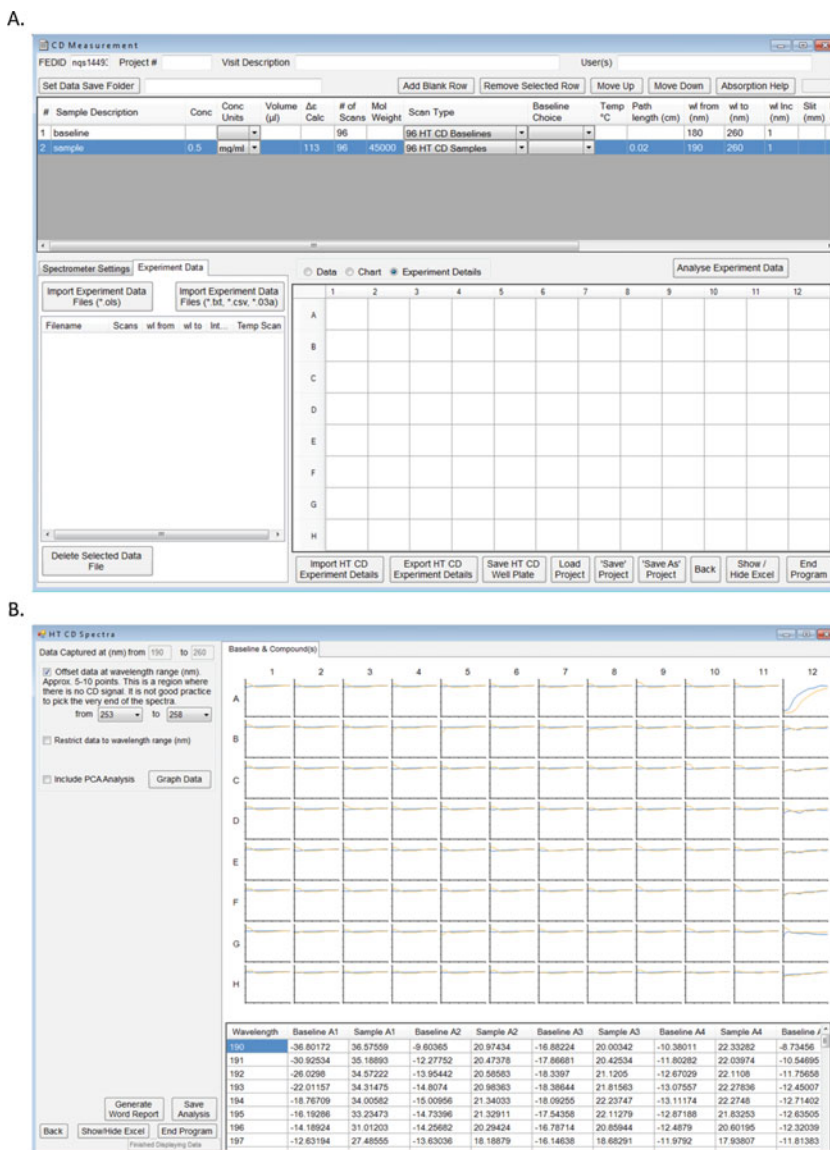


**Fig. 7** SSE for scans taken at each temperature of the denaturation. SSE is performed by selecting the parameters under the “SSE (Protein)” tab

8. Alternatively, copy the data from the “Folding Change” tab of the Excel workbook for analysis using alternative software.
9. Under the SSE (Protein) tab, select appropriate algorithm for protein secondary structure estimation (Fig. 7).

96-Well Analysis

1. Allocate baseline and sample labels to the datasets by selecting the appropriate scan type from drop-down menu (Fig. 8a):
  - (a) 96 HTCD Baselines for baseline dataset.
  - (b) 96 HTCD Samples for sample dataset.
2. Input experimental parameters into the workbook (see Note 17).
3. Insert the sample description for each sample in the plate by either clicking a cell to highlight and type in details directly, alternatively complete from the “Description” section of the “Sample Details” tab. Do this from the “Experiment details” section for each sample line when highlighted. A dialog box informing the User of the number of experimental details entered will appear when the overall sample line is changed, or if the “Analyse Experimental Data” button is selected. Click OK to continue analysis (see Notes 18–20).
4. Select the Sample and Baseline sample lines by clicking the desired line (the line becomes blue).



**Fig. 8** Windows during the analysis for HTCD data. (a) Initial window where experimental details are input for the analysis under the “Experimental Details” section of the “Experiment Data” tab (b) off-set region for the data where no CD signal is observed

5. Click “Analyse Experiment Data” (*see Note 17*).
6. Set the off-set region for the data (where no CD signal is observed). Click “Graph Data” button (Fig. 8b).
7. If a particular wavelength is to be monitored across the plate for a change in signal, this can be selected from the “Wavelength to monitor (nm)” section of CDApps. Data for this

analysis will be available in the “Folding Change” tab of the Excel Workbook (Fig 8b)”.

8. For SSE, under the SSE (protein) tab, choose the algorithm (CONTINLL, SECLON3, CDSSTR) and the database (SP29, SP23, SP1, SP37, SP2, SP42, SMP50, SP43, SDP48, SMP56, CLSTR) most appropriate for the sample measured. Click “Estimate.” Data from this analysis is available in the Excel workbook. The easiest way to access is via the Index page of the Excel workbook, click the cell containing the Sheet number reference next to the appropriate title which generally contains reference to the algorithm and database used followed by “SSE component Data.”

### 3.4.3 Analysis Using the “CD Titration” Option

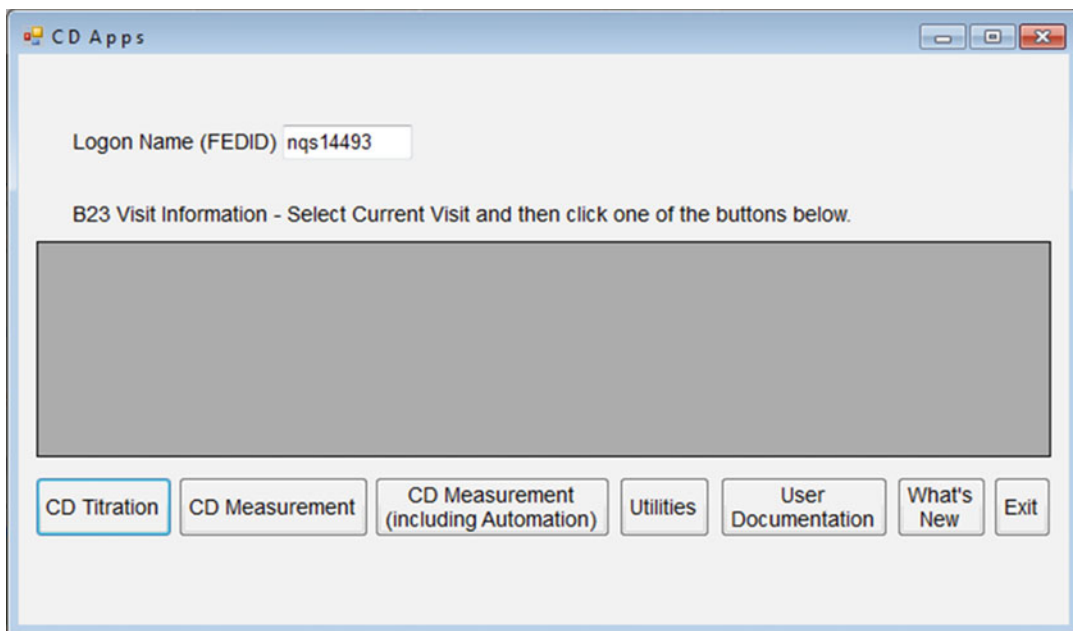
A dedicated suite of programs, called CDApps [16], have been developed at B23 to process data files collected at the beamline. Different analysis options are available within the program (1 to  $n$  spectra, titration, UV denaturation, thermal melt, and 96-well format), enabling the User to select the most appropriate type of analysis for their data type.

The 1 to  $n$  spectra option allows the User to obtain averaged, baseline subtracted, and normalized spectra of multiple files in one workbook, provided that the wavelength ranges are the same for sample and baseline. UV denaturation experiments are analyzed by subtraction of baseline from each scan in the file, treating each scan individually. The changes in signal at a specific wavelength can be monitored to determine the kinetics of the change as a function of scan number. Thermal melt similarly analyzes each scan individually, instead as a function of temperature. Change of signal at a specific wavelength for each temperature can be fitted with a Gibbs-Helmholtz equation [38, 47], a derivative of the Boltzmann equation. For files collected using the 96-well format, files are analyzed, so each of the 96 scans are individually treated as single spectrum (Fig. 3a). Through CDApps, the data can be then analyzed through the program in terms of protein secondary structure content using either or three of publicly available algorithms CONTINLL [19–21], CDSSTR, and SECLON3 [20] (Fig. 3b).

1. Load CDApps and click the “CD Titration” button on the option window (Fig. 9).

### Setting Up the Analysis Workbook of “Fixed” Volume Titrations in CDApps

For the fixed volume titration option, calculations will be made for each titration step to determine the volume of each component to combine with a defined fixed volume. Each titration step will be a new, individual experiment. Therefore, this option is best for cases where an abundance of material is available as it can be costly in material determined on the total volume for each titration step and the number of molar ratio points to be investigated. Downstream analysis does not require a dilution factor because all titrations are performed in the same volume; therefore, points after ligand

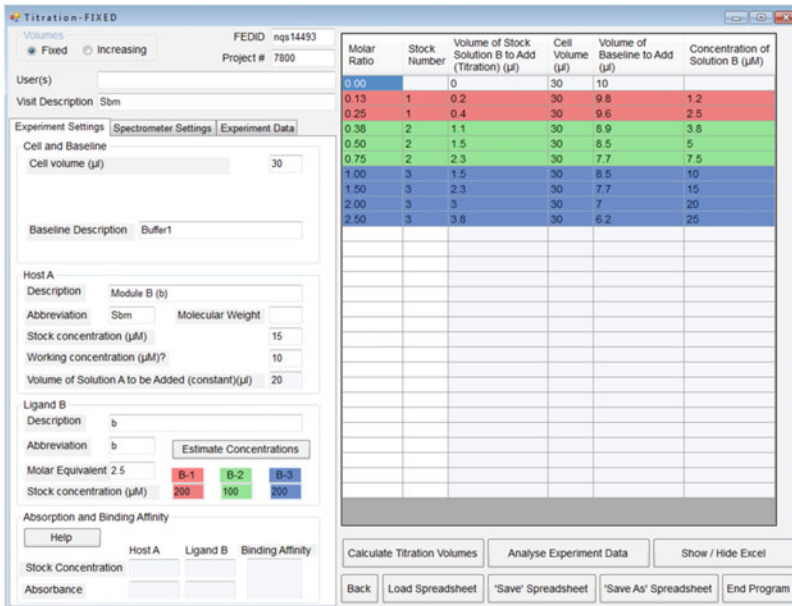


**Fig. 9** Option window in CDApps

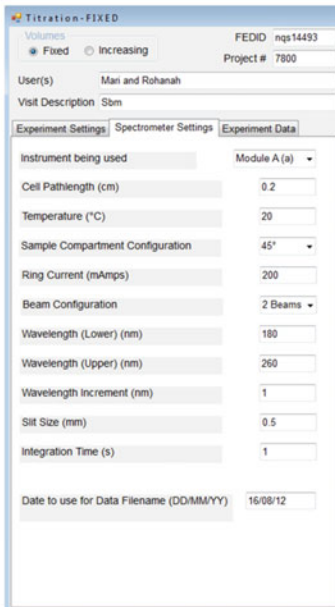
subtraction and zeroing can be taken directly for analysis of the binding constants.

1. Select the “Fixed” volume option at the top of the workbook.
2. Under the “Experiment Settings” tab, complete the details for the cell parameters, Host and Ligand. The information provided here will be used to determine the volumes required at each step of the titration (Fig. 10a).
3. To determine the Ligand stock concentration to be used and the volumes to be used at each titration point, in the spreadsheet section of the workbook enter the titration points under the “Molar Ratio” column (Fig. 10a).
4. Add the number of the Ligand stock (1, 2, or 3) which will be used at each point (Fig. 10a) to the “Stock Number” column. Ensure aliquot volumes are feasible within the error of the pipettes. For ease of the User, each Ligand stock is color coded to quickly identify which stock to use.
5. Complete experimental parameters under the “Spectrometer Settings” tab (Fig. 10b).
6. Import data files into the workbook under the “Experiment Data” tab by selecting the appropriate button for the data to be imported (either .ols or .csv/.txt files) and locate the files. When multiple files are to be imported, highlight all appropriate files (Ctrl + Click/Shift + Click). Click Open. The files will be imported into CDApps in the “Imported Data Files” column (Fig. 10c).

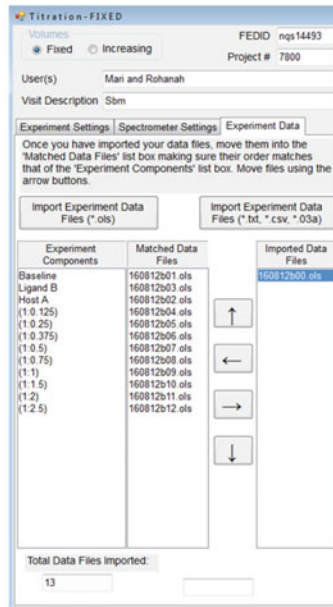
A.



B.



C.



**Fig. 10** Analysis workbook windows in CDAapps for titrations in fixed volume. (a) Experiment setting, (b) Spectrometer setting, (c) Experiment data

7. Match the appropriate files to the “Experiment Component.” This can be done one by one or in bulk. Four arrows are used to control the order and matching of files (Fig. 10c).

(a) ↑ and ↓ control the position of the file in the “Imported Data Files” column.

- (b)  $\leftarrow$  and  $\rightarrow$  control the transfer of files between the “Imported Data Files” and “Matched Data Files” columns.
- Only when in the “Imported Data Files” column can the order of the files be changed. Once they have been moved to the “Matched Data Files” column they cannot be reordered. To rearrange the order, files will have to be moved to the “Imported Data Files” column, ordered, and then transferred to the “Matched Data Files” column.
  - Files can be transferred between columns either individually by selecting individual files or in bulk by highlighting multiple files (Ctrl + Click/Shift + Click) before using  $\leftarrow$  and  $\rightarrow$ .
  - Once the workbook has been completed, save the spreadsheet by clicking “Save As” Spreadsheet.” This will enable the spreadsheet to be loaded at another time to continue the analysis and to ensure that a copy has been saved in case of any downstream erroneous analysis.
  - The Workbook is saved as an Excel workbook, and the experimental plan can be printed from the “Titration” tab of the workbook for the convenience of the User to perform the experiment. The plan details the volumes required at each step, and ligand stocks are color coded for quick identification by the User (Fig. 11).
  - Instruction on how to perform the analysis is provided in Subheading 3.4.3.3.

Project Number:	7900	Starting Cell Volume (µl):	30	Solution A Description:	Module B	Solution B Description:	Module A (a)	Wavelength (lower)(nm):	180
User(s):	Mari and Rohanah	Max. Volume that can be Added(µl):		Solution A Abbreviation:	Stom	Solution B Abbreviation:	Stom	Wavelength (upper)(nm):	260
Experiment:	Stom	Maximum Volume Added(µl):		Stock Con of Solution A (µM):	15	Stock Con of Solution B - 1:	200	Temperature (°C):	20
Volumes:	Fixed	Buffer Description:	Buffer1	Working Con of Solution A (µM):	30	Stock Con of Solution B - 2:	100	Sample Compartment:	45°
				Volume of Solution A to be Added (constant):	20	Stock Con of Solution B - 3:	200	Ring Current (mAmps):	200
				Solution A Molecular Weight:		Molar Equivalent Solution B:	2.5	Beam Configuration:	2 Beams

Oils Filename	Repetition #	Sample Description / Titration Point	Cell Volume (µl)	Stock # Number	Solution # to Add at Titration Point (µl)	Volume of Buffer to Add (µl)	Concentration of Solution B (µM)
1408121001.xls		Surfer					
1408121002.xls		Ligand B (0.2.5)		1			3.8
1408121003.xls		Host A (1.0)		1			1.0
1408121004.xls		Stom + B (1.0.2.5)	30	1	8.2	9.8	2.5
1408121005.xls		Stom + B (1.0.2.5)	30	1	8.4	9.4	2.5
1408121006.xls		Stom + B (1.0.3.75)	30	2	1.3	8.7	3.8
1408121007.xls		Stom + B (1.0.5)	30	2	3.5	8.5	5
1408121008.xls		Stom + B (1.0.75)	30	2	2.8	7.2	6.2
1408121009.xls		Stom + B (1.1)	30	1	1.4	6.5	10
1408121010.xls		Stom + B (1.2.5)	30	1	2.3	7.7	10
1408121011.xls		Stom + B (1.25)	30	1	1	7	10
1408121012.xls		Stom + B (1.2.5)	30	1	1.8	8.2	10

**Fig. 11** Titration tab of the workbook detailing the experiment plan can be printed for a convenient plan to use for the experiment in the lab

Setting Up the Analysis  
Workbook of “Increasing”  
Volume Titrations  
in CDApps

For titrations performed in an increasing volume, a single sample of the Host is made, and aliquots of Ligand stock are added at each titration step so that a cumulative concentration of ligand is achieved at each step. For these types of experiments, during the analysis a dilution factor will have to be applied to the different spectra of the Host at each titration point to account for the reduced signal intensity resulting from the dilution caused by the addition of each ligand aliquot.

1. Select the “Increasing” volume option at the top of the workbook.
2. Under the “Experiment Settings” tab, complete the details for the cell parameters, Host and Ligand. The information provided here will be used to determine the volumes required at each step of the titration (Fig. 12a). CDApps will automatically calculate the maximum volume of ligand which can be added within acceptable dilution limits.
3. To determine the Ligand stock concentration to be used and the volumes to be used at each titration point, in the spreadsheet section of the workbook enter the titration points under the “Molar Ratio” column (Fig. 12a).
4. Add the number of the Ligand stock (1, 2, or 3) which will be used at each point (Fig. 12a) to the “Stock Number” column. CDApps will notify you if the maximum volume of added ligand is exceeded. If this happens, adjust the concentrations of the ligand stocks to reduce the total added volume, using a maximum of three stocks using CDApps. Ensure aliquot volumes are feasible within the error of the pipettes. For ease of the User, each Ligand stock is color coded to quickly identify which stock to use.
5. Complete experimental parameters under the “Spectrometer Settings” tab (Fig. 12b).
6. Import data files into the workbook under the “Experiment Data” tab by selecting the appropriate button for the data to be imported (either .ols or .csv/.txt files) and locate the files. When multiple files are to be imported, highlight all appropriate files (Ctrl + Click/Shift + Click). Click Open. The files will be imported into CDApps in the “Imported Data Files” column (Fig. 12c).
7. Match the appropriate files to the “Experiment Component.” This can be done one by one or in bulk. Four arrows are used to control the order and matching of files (Fig. 12c).
  - (a) ↑ and ↓ control the position of the file in the “Imported Data Files” column.
  - (b) ← and → control the transfer of files between the “Imported Data Files” and “Matched Data Files” columns.



A.

**Titration-INCREASING**

Volumes: Fixed  Increasing  FEDID nqs14493  
Project # EDS1

User(s) EDS  
Visit Description EDS1

Experiment Settings | Spectrometer Settings | Experiment Data

Cell and Baseline

Starting Cell volume ( $\mu\text{l}$ ) 2000  
Max. volume that can be added ( $\mu\text{l}$ ) 300  
Maximum volume added ( $\mu\text{l}$ ) 220  
Baseline Description Buff1  
Volume of Baseline to Add 1900

Host A

Description SolA  
Abbreviation SolA Molecular Weight  
Stock concentration ( $\mu\text{M}$ ) 300  
Working concentration ( $\mu\text{M}$ )? 15  
Volume of Solution A to be Added (constant)( $\mu\text{l}$ ) 100

Ligand B

Description SolB  
Abbreviation SolB Estimate Concentrations  
Molar Equivalent 5 B-1 B-2 B-3  
Stock concentration ( $\mu\text{M}$ ) 1000 2000 3000

Absorption and Binding Affinity

Help Host A Ligand B Binding Affinity  
Stock Concentration Absorbance

Titration Point (A:B Molar Ratio)	Stock Number	Volume of Stock Solution to Add at Titration Point ( $\mu\text{l}$ )	Total Cell Volume ( $\mu\text{l}$ )	Dilution Factor (This must be kept below a max. of 15% of the Total)	Total Volume of Stock Solution B to Add (Titration) ( $\mu\text{l}$ )
0.00			2000	1	0
0.33	1	9.9	2009.9	1.005	9.9
0.66	1	9.9	2019.8	1.01	19.8
1.00	1	10.2	2030	1.015	30
2.00	2	15	2045	1.022	45
3.00	2	15	2060	1.03	60
5.00	2	30	2090	1.045	90
7.00	3	20	2110	1.055	110
10.00	3	30	2140	1.07	140
16.00	3	80	2220	1.11	220

Calculate Titration Volumes Analyse Experiment Data Show / Hide Excel  
Back Load Spreadsheet 'Save' Spreadsheet 'Save As' Spreadsheet End Program

B.

**Titration-INCREASING**

Volumes: Fixed  Increasing  FEDID nqs14493  
Project # EDS1

User(s) EDS  
Visit Description EDS1

Experiment Settings | Spectrometer Settings | Experiment Data

Instrument being used Module A (a)

Cell Pathlength (cm) 10  
Temperature ( $^{\circ}\text{C}$ ) 20  
Sample Compartment Configuration  
Ring Current (mAmps)  
Beam Configuration  
Wavelength (Lower) (nm) 190  
Wavelength (Upper) (nm) 260  
Wavelength increment (nm) 0.875  
Slit Size (mm) 1  
Integration Time (s) 1  
Date to use for Data Filename (DD/MM/YY) 17/10/11

C.

**Titration-INCREASING**

Volumes: Fixed  Increasing  FEDID nqs14493  
Project # EDS1

User(s) EDS  
Visit Description EDS1

Experiment Settings | Spectrometer Settings | Experiment Data

Once you have imported your data files, move them into the 'Matched Data Files' list box making sure their order matches that of the 'Experiment Components' list box. Move files using the arrow buttons.

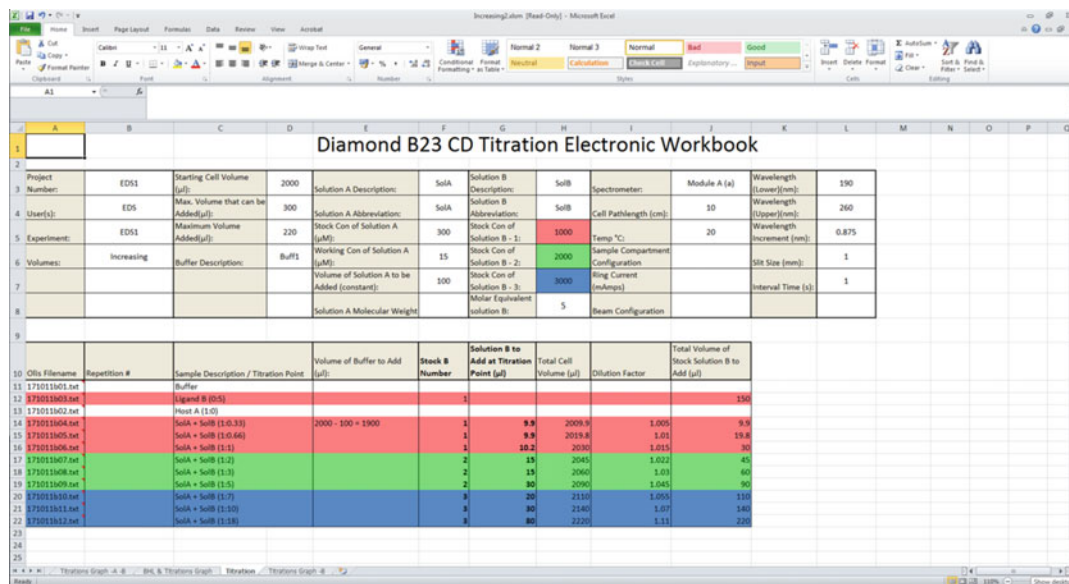
Import Experiment Data Files (\*.ols) Import Experiment Data Files (\*.txt, \*.csv, \*.03a)

Experiment Components	Matched Data Files	Imported Data Files
Baseline	171011b01.txt	171011b00.txt
Ligand B	171011b03.txt	
Host A	171011b02.txt	
(1:0.33)	171011b04.txt	
(1:0.66)	171011b05.txt	
(1:1)	171011b06.txt	
(1:2)	171011b07.txt	
(1:3)	171011b08.txt	
(1:5)	171011b09.txt	
(1:7)	171011b10.txt	
(1:10)	171011b11.txt	
(1:18)	171011b12.txt	

Total Data Files Imported: 13

**Fig. 12** Analysis workbook windows in CDApps for titrations in increasing volume. (a) Experiment setting, (b) Spectrometer setting, (c) Experiment data

8. Only when in the “Imported Data Files” column can the order of the files be changed. Once they have been moved to the “Matched Data Files” column they cannot be reordered. To rearrange the order, files will have to be moved to the



**Fig. 13** Titration tab of the workbook detailing the experiment plan. Can be printed for a convenient plan to use for the experiment in the lab

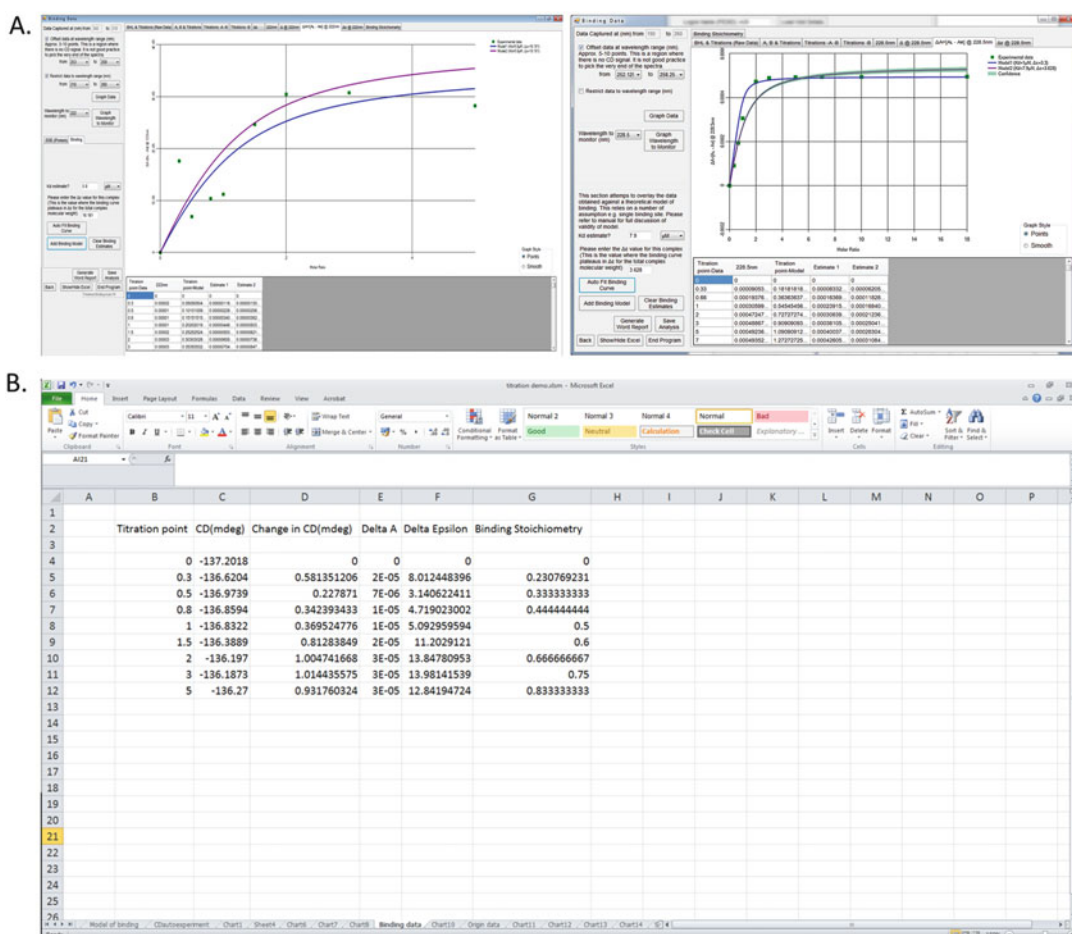
“Imported Data Files” column, ordered, and then transferred to the “Matched Data Files” column.

9. Files can be transferred between columns either individually by selecting individual files or in bulk by highlighting multiple files (Ctrl + Click/Shift + Click) before using ← and →.
10. Once the workbook has been completed, save the spreadsheet by clicking “Save As” Spreadsheet.” This will enable the spreadsheet to be loaded at another time to continue the analysis and to ensure that a copy has been saved in case of any downstream erroneous analysis.
11. The Workbook is saved as an Excel workbook, and the experimental plan can be printed from the “Titration” tab of the workbook for the convenience of the User to perform the experiment. The plan details the volumes required at each step, and ligand stocks are color coded for quick identification by the User (Fig. 13).
12. Instruction on how to perform the analysis is provided in Subheading 3.4.3.3.

Determination of the Binding Constants

1. Load CDApps, select CD Titration button, and load the appropriate Spreadsheet. All details and
2. Click “Analyse Experiment Data” button.
3. Off set data to a region where no CD signal is detected.

4. If necessary, restrict the wavelength range of the data to remove noise. For measurements in the far-UV region requiring SSE, ensure restriction does not compromise the wavelength ranges required by the algorithms and datasets, e.g., 190 nm and above.
5. From the “Titrations –A –B” tab or the “Wavelength to monitor (nm)” section, select the wavelength from which binding constant calculations will be made. This is usually the region on the spectra which shows the biggest change in magnitude over the course of the titration. Click “Graph Wavelength to monitor” button (Fig. 14).



**Fig. 14** Analysis of the binding constants from titration data. (a) Analysis window in CDApps. (b) Binding Data tab of the analysis workbook. Data can be exported to alternative programs for further analysis and display. The example in Fig. 14a left has been chosen deliberately to show the results of a preliminary CD titration. The experimental data appear to be a bit scattered in the plot intensity versus ligand concentration indicating that for the membrane protein, the incubation time might require to be increased and also that extra intermediate points might confirm that subtle change between the first two point and also between the 5th and 6th point

6. A “Binding” tab will then appear. From here fitting can be performed for the points to determine the  $K_d$  of the binding interaction. Fitting can either be automatically fitted through CDApps using the “Auto Fit Binding Curve” button, or manually by entering  $\Delta\epsilon$  values and clicking “Add Binding Model” button.

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## 4 Notes

1. Conduct measurements in the “most UV-transparent” buffer possible, achieved using buffering systems at their lowest working concentration for the system of interest. Phosphate buffer is the most appropriate buffer system of choice.
2. Minimize or eradicate the concentration of chloride anions present. Substitute with fluoride salts where possible, and adjust the pH of buffers using non-chloride acids including nitric and phosphoric acids. This is especially important when measuring low-concentration protein solutions (<1 mg/mL) in the far-UV region.
3. Use only very pure products for measurements (>95% pure), and chemicals which are at least analytical grade purity.
4. Ensure no bubbles are present in the solutions before measurements. Degas buffers beforehand, and when cuvettes are filled, ensure no bubbles are present by tapping gently.
5. Pathlengths of cuvettes used for measurements can be changed depending on the concentration of the protein solution being measured in accordance to the Beer-Lambert law [35].
6. For samples with weak signals, it is recommended to check the absorbance of the sample. If it is within the ideal limits, then an alternative is to increase the number of scans collected. The underlying reason for increasing the number of scans is because

$$\text{signal} \propto \frac{1}{n}$$

Therefore, the average spectrum from more scans (4, 9, or 16) or increasing the integration time will improve the signal:noise ratio.

7. When cleaning cuvettes, especially when using protein solutions and after thermal melt or long-term UV denaturation experiments, be sure to use concentrated nitric acid to clean thoroughly wet cells from wash of 96% ethanol. Also thoroughly rinse and dry the cells before reuse to remove residual acid which can change the pH affecting the protein folding.
8. Remove all debris and solid particulates by centrifugation beforehand and using only the supernatant, or filtering the solution through a 0.2  $\mu\text{m}$  filter.

9. Be sure that the HV of the PMT detector does not exceed the recommended voltage from the CD instrument manufacturer (for B23 is about 600 V, which is common with other benchtop instruments using the PMT detector) as this is a sign of over-saturation of the detector. If this occurs, remove the portion of spectra for which this occurs before analysis or decrease the sample concentration, or reduce the cell pathlength accordingly.
10. Observe the absorbance and discount measurements which are above 1.5. Reduce the concentration to reduce the absorbance, or alternatively decrease the cell pathlength. Also consider the absorbance contribution from the ligand as this should be factored in for ligand-binding studies, especially titration experiments.
11. In cases where the ligand absorbs at 280 nm, a lower starting protein concentration can be used (0.4 mg/mL) to allow for the absorbance contribution of the ligand throughout the titration.
12. When conducting titration experiments in increasing volume, ensure the total added ligand volume does not exceed 20% of the original volume (CDApps will warn when a 15% dilution has been reached). This ensures any changes observed are solely as a result of ligand binding rather than any potential “concentration effects” influencing the proteins conformation or local tertiary structure. To aid this, use ligand stocks at the highest possible concentration (usually in the region of 10–20 times the final concentration of ligand required for the titration).
13. When conducting ligand-binding studies ensure thorough and gentle mixing in the solution before measurement, avoiding the formation of bubbles (*see Note 4*). Check for a homogeneous appearance. Look for signs of insufficient mixing, e.g., streaking of solutions, and rectify before measurement. This is incredibly important when conducting titration experiment, and care should be taken to thoroughly mix after the addition of each aliquot.
14. Determine the incubation time required for the system to stabilize after the addition of ligand by determining the time required for consecutively measured spectra to overlap and no longer change. The determined time can be used as standard for subsequent measurements.
15. Care should be taken to monitor for precipitation during the titration, a sign of unsuitable conditions for the protein (e.g., though changes to the pH of the solution upon addition of the ligand) and the possibility of reduced protein concentration which should be accounted for during the analysis for accuracy

especially for measurements in the far-UV region and SSE. Solid particulates should be removed to prevent light scattering.

16. The extent of UV denaturation is specific to a protein. Use experience of the proteins behavior and stability to judge the number of scans to collect.
17. When using CDApps for analyses of data sets more complicated than standard 1 to  $n$  measurements (UV denaturation, Thermal Melt, 96 HT format), only one dataset can be analyzed at a time.
18. When analyzing data for the 96-HT format, be sure to complete the experimental details and parameters for all of the scans in the file.
19. Ensure the number of scans in the workbook match the number of scans in the uploaded data files.
20. After selection of the HTCD analysis options, experimental parameters (e.g., concentration and units,  $\Delta\epsilon$ , molecular weight, and pathlength) will have to be re-entered. A dialog box will ask if you want the value to be applied to all of the scans in the file, click OK if this is the case, otherwise they can be manually inserted for each individual scan under the “Experiment details” section in CDApps.

---

## Acknowledgement

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## References

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