

Materials and techniques used

This chapter lists the different materials used in the spectroscopic studies, and brief descriptions of the various spectroscopic techniques and softwares.

4.1. Materials

The fluorophores DMTCO, MDDCO, TDCO, KTHC-67 and MOTHCA have been obtained from the same stock that was synthesized and purified by Saha *et. al.*⁸³. NAN, R-BINOL, S-BINOL and anthracene have been purchased from Sigma and used after recrystallization. HSA and β -cyclodextrin has also been purchased from Sigma and used as received. Spectroscopic or HPLC grade solvents HEPT, Bz, Tol, DOX, MTBE, DIPE, DME, EtAc, THF, ACN, DMF, DMSO, MeOH, EtOH and BuOH have been purchased from Spectrochem Pvt. Ltd. and used without further purification. HxOH, OcOH, DcOH and DdOH have been purchased from Loba Chemie and used after repeated distillations. DMA, DEA, TEA, TBA and DIEA have been purchased from Spectrochem Pvt. Ltd. and used after repeated distillation. Ultra-pure water from the Millipore water purification system has been used. All the solvents have been additionally checked for impurities using absorption and fluorescence spectroscopy.

The solutions have been prepared freshly before the experiments most of the time, or stored in a cold and dry place. None of those show any sort of degradation throughout the experimental period. Prior to each transient absorption measurements, the solutions were de-aerated for 20 min by passing pure argon gas.

4.2. Instrumental techniques

1.0 cm path length quartz cuvettes have been used for the measurements of absorption spectroscopy, steady-state fluorescence spectroscopy and TCSPC. Quartz cells of path length 1 mm have been used for circular dichroism spectroscopy. All the cuvettes have been purchased from Hellma Analytics.

4.2.1. UV- visible absorption Spectroscopy

UV-vis absorption spectra were recorded by JASCO V-650 spectrophotometer. Prior to recording any absorption data, baseline correction was done in all cases using the corresponding solvent. For the ground state complex formation between two interacting species, firstly the absorption spectrum of one sample had been taken in a sample cuvette along with the same volume of buffer in the reference cuvette, and then the changes in absorption spectrum of this sample were recorded with sequential addition of another interacting sample over the particular range of concentration.

Beer-Lambert law expresses the probability of light absorption⁴. It states that, the fraction of incident radiation absorbed by a transparent medium is independent of the intensity of incident radiation but depends on the concentration of the absorbing species and that each successive layer of the medium absorbs an equal fraction of incident radiation. The efficiency of light absorption at a wavelength λ by an absorbing medium is described by the absorbance A or the transmittance T and according to the Beer–Lambert Law,

$$A = -\log T = \varepsilon(\lambda)lc \quad (4.1),$$

where $\varepsilon(\lambda)$ is the molar absorption coefficient, c is the molar concentration of the absorbent and l is the absorption path length in cm.

4.2.2. Steady-state Fluorescence Spectroscopy

The steady-state fluorescence emission spectra, fluorescence excitation spectra, synchronous fluorescence spectra were recorded using Spex Fluoromax 3 (Jobin Yvon Horiba, Japan). The fluorescence anisotropy measurements were done in Hitachi F-7000 spectrofluorimeter.

For samples of high optical density at the excitation wavelength, approximate correction for the fluorescence emission intensity has been done using equation 3.9.

4.2.3. Circular dichroism spectroscopy

Circular dichroism measurements were measured in Chirascan (Applied Photophysics, UK). An accumulation of three scans with a scan speed of 20 nm per minute and response time of 2 s was

performed to collect data. Each spectrum were corrected by subtracting appropriate blank runs on protein-free solutions and subjected to noise reduction.

4.2.4. Fluorescence decay measurements

Fluorescence lifetimes were measured using a time-correlated-single-photon counting (TCSPC) technique^{7,30}, in the picoseconds-resolved TCSPC spectrometer Jobin Yvon Horiba. In our setup samples are excited using a picosecond diode laser (IBH Nanoleds). We have used 295 nm and 340 nm excitation diode lasers of pulse width ~75 ps with a repetition rate ~1 MHz. Appropriate band pass filters are used to block the exciting light during the collection of fluorescence from the sample. The liquid samples are kept in a quartz cell of 1 cm path length. The sample fluorescence, collected at 90 degree to the excitation beam, is dispersed by a monochromators (IBH, model MCG-910 IB) and detected using a cooled micro-channel plate photomultiplier tube (Hamamatsu, 5000-U-09). The polarization (parallel, perpendicular and magic angle) of the fluorescence is selected by an analyser, as shown in figure 4.1.

In reverse mode TCSPC, fluorescence induced electronic start pulse triggers the time to amplitude converter (TAC. Tennelec TC 863), through a constant function discriminator (CFD), to build up a voltage which is linear with time. Another trigger pulse from the diode laser driver synchronous with excitation laser pulse is guided to the TAC as electronic stop pulse. A delay (Tennelec TC 412 A) is used in the path of the stop pulse in prior to entering into the TAC. The voltage developed at TAC determines in which channel of the multichannel analyser (MCA, PCA3card, Oxford Corporation, U.K.) a count is registered. The voltage at the stop time is converted into a time difference between the excitation of the sample and the detected photon of the fluorescence.

After a large number of excitation and detection events, a histogram is constructed, where the x-axis is the time difference between the excitation pulse and the observed photon and the y-axis the number of photons detected for this time difference. The histogram of pulse heights resembles the waveform of fluorescence intensity decay, if much less than 1 photon is detected per excitation pulse. The histogram is biased to shorter times, when the count rate is higher, because the TAC will take into account only the first fluorescence pulse (pileup effect). It is

worthy to mention that, multiple photons per pulse can be measured for decay times near a microsecond or longer but not when the lifetimes are in the nanosecond range.

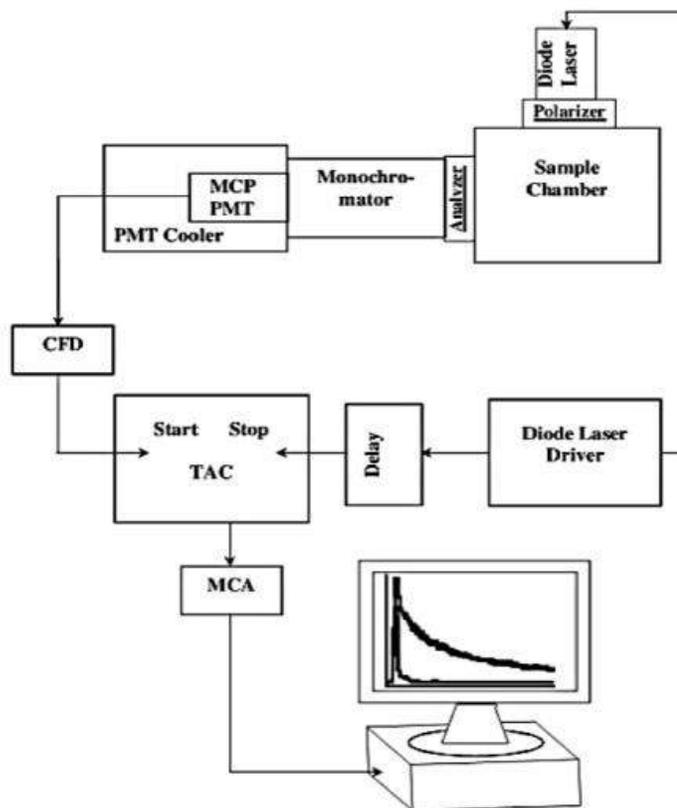


Figure 4.1. Schematic diagram of the TCSPC setup.

The global lifetime analysis software, IBH DAS6 is used for decay processing, where the deconvolution technique is used to calculate the decay of the fluorophore using the measured decay data and the instrument response function (IRF). IRF is the response of the instrument to a zero lifetime sample and is also representative of the time profile of the exciting pulse. It is recorded under the same conditions by replacing the sample with a dilute scattering solution of Ludox. The analysis software presents the goodness of fit of the lifetime data with χ^2 .

4.2.5. Transient absorption spectroscopy

The transient absorption spectroscopy for the detection of transient, non-fluorescent species has been measured using the nanosecond laser flash photolysis setup from Applied Photophysics, containing a Q-switched, Neodymium doped Yttrium Aluminium Garnet [Nd:YAG] laser

[Lab series, Model Lab 150, Spectra Physics]. The active medium is Nd^{3+} that generates a fundamental emission at 1064 nm. In all of our experiments, the sample solutions were excited at the third harmonic, 355 nm, which has been produced by frequency conversion in the non-linear crystal of potassium dideuterium phosphate. The excitation pulse has 60 mJ of energy and pulse widths of 6-7 ns. Transient species in solution were monitored through absorption of light from a pulsed xenon lamp [150 W] at right angle to the laser beam. The AK-347 control unit triggers the laser within the flat portion of the Xe lamp profile so that the transients are excited by absorbing an intense and steady source of light. The wavelength from the probe beam was dispersed with a monochromator and detected with R928 photomultiplier detector. The photomultiplier output was fed into an Agilent Infiniium oscilloscope [600 MHz, 4 Gs/s, DSO8064A], and the data were transferred to the Laser software running in an Iyonix range of ARM-based RISC OS computer. A schematic diagram of the laser flash photolysis set-up is shown in figure 4.2. The changes in transmittance at various wavelengths are converted to absorbance and the transient absorption spectra are obtained by plotting OD vs wavelength at a particular time delay after the laser flash.

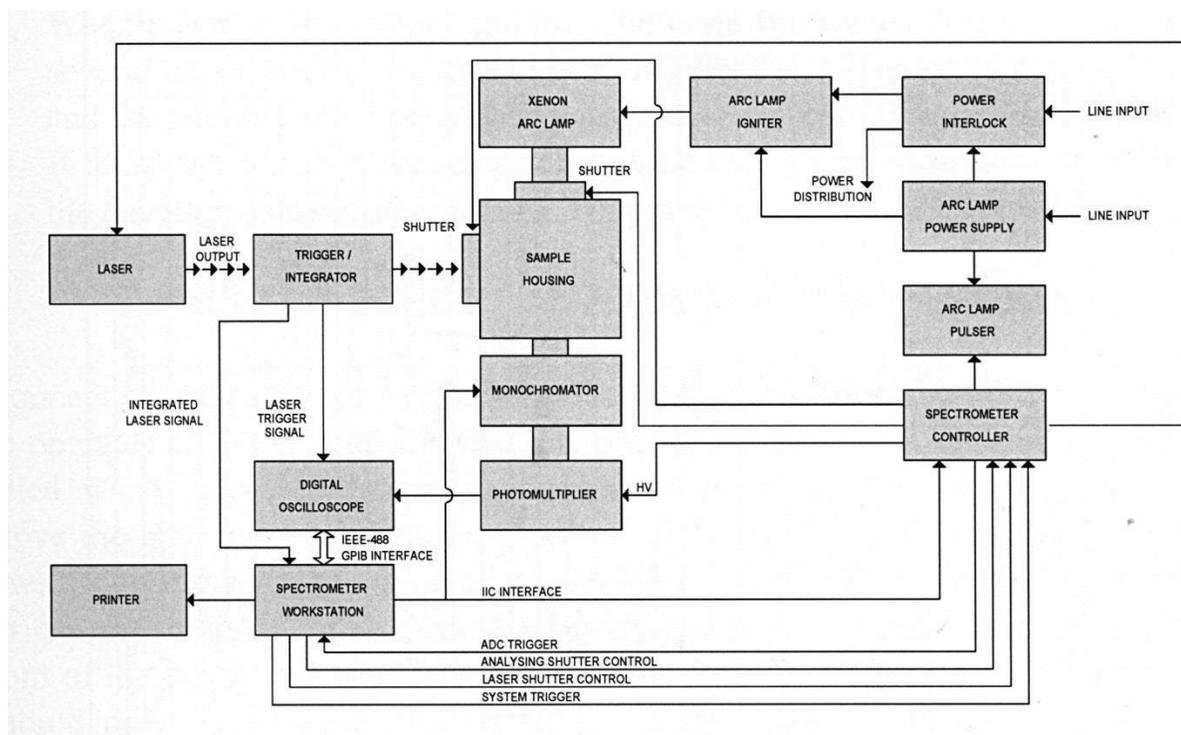


Figure 4.2. Schematic diagram of the laser flash photolysis setup.

4.3. Softwares

Various softwares have been used for drawing and presentation of experimental as well as theoretical results, and for theoretical calculations.

4.3.1. DichroWeb

It is possible to calculate the percentages of alpha-helix, beta-sheet, beta-turns and other types of secondary structures from CD spectral data using various algorithms and reference databases. However, it is difficult to use and compare the results from the various available algorithms. The input spectral formats differ from one algorithm to another, and from the types of outputs of most commercial CD instruments, the input formats require different scaling factors or expression in different types of units. Moreover, the use of different reference databases, and the lack of comparability of different results due to the wide range of different statistical or goodness-of-fit parameters calculated by the different programs, worsens the situation. It is, therefore, suggested to use DichroWeb, a website established to facilitate such on-line analyses of protein secondary structures from CD spectra²⁵⁴⁻²⁵⁶. It is available via a password-accessible server, which accepts a variety of input and output formats and units, and allows all the methods to use a wide range of different reference databases. Data analyses can be done using a number of the most popular secondary structure determination algorithms, including SELCON3, CONTIN, VARSLC, CDSSTR and K2D. DICHROWEB permits facile comparisons between the results from different methods using graphics outputs, and a consistent parameter to measure the comparability of experimental and calculated spectra in all cases.

4.3.2. Avogadro

The energy minimized structures of molecules required for theoretical calculations were obtained in *Avogadro software* via steepest descent and conjugate gradient algorithms in UFF force field until it converges ($\Delta E = 1 \times 10^{-7}$ KJ mol⁻¹)²⁵⁷. This cross-platform software is extensively used in computational chemistry, molecular modelling, bioinformatics, materials science, and related areas, for building molecular structures, formatting input files, and analyzing output of a wide variety of computational chemistry packages.

4.3.3. AutoDock4

The AutoDock4 software, developed by The Scripps Research Institute, La Jolla, CA, is capable of quickly and accurately predicting bound conformations and binding energies of ligands with macromolecular targets ²⁵⁸⁻²⁶¹. It uses a grid-based method to allow rapid evaluation of the binding energy of trial conformations, in which the target protein is embedded in a grid and then a probe atom is sequentially placed at each grid point. The interaction energy between the probe and the target is computed for each grid point and is stored for later use as a lookup table during the docking simulation. Thereafter, a Lamarckian genetic algorithm is used for conformational searching, where in the successive generations, each individuals from the population of trial conformations mutate, exchange conformational parameters, and compete in a manner analogous to biological evolution, and ultimately those with the lowest binding energy gets selected. Each individual conformation is capable of searching their local conformational space, finding local minima, and then passing on this information to later generations. The software uses a semiempirical free energy force field, based on a comprehensive thermodynamic model, to predict binding free energies of small molecules to macromolecular targets. The force field incorporates intramolecular energies into the predicted free energy of binding, and also a new charge-based desolvation method that uses a typical set of atom types and charges.

4.3.4. OriginPro 8.5

OriginPro 8.5 from OriginLab has been used for the data plotting, fitting and presentations. In this software, the adjusted R-Square values represent how well data fit a statistical model.

4.3.5. ChemBioDraw Ultra 12.0

ChemBioDraw Ultra 12.0 from PerkinElmer Informatics has been used for drawing molecular structures and schemes.

4.3.6. LigPlot+

LigPlot+ program has been used for automatic generation of 2D ligand-protein interaction diagrams ²⁶².