2. **Experimental:**

The main objective of present work is to develop analytical methods for sensing components of nucleic acids such as nitrogen bases, nucleosides and nucleotides and also to understand molecular interaction with fluorescent drugs by using photoinduced electron transfer (PET) technique. The fluorescent probe and drugs satisfying PET criteria were obtained, tested for purity and used further in PET experiments. The experimental details, pertaining to source, purification, experimental set, equipment and operational procedure are given in this chapter.

2.1 **Fluorophors:**

The organic fluorescent materials namely, perylene (Merck-Schhuchardt 99%), 9-anthracene carboxylic acid (Merck-Schhuchardt 99%), proflavine hemisulphate (Himedia 99%) were used in present studies as probe.

2.2 **Quenchers (Some components of nucleic acids):**

The some of electron donor molecules like uracil and its derivatives, nucleosides and nucleotides obtained from s.d.Fine-Chem. Ltd.Mumbai 99% were used as quenchers in present study are given below.

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Nitrogen bases (Pyrimidines)</th>
<th>Nucleoside</th>
<th>Nucleotides</th>
</tr>
</thead>
<tbody>
<tr>
<td>01.</td>
<td>Uracil</td>
<td>Adenosine</td>
<td>Adenosine 5’monophosphate</td>
</tr>
<tr>
<td>02.</td>
<td>5-Methyl uracil</td>
<td>-</td>
<td>Guanosine 5’monophosphate</td>
</tr>
<tr>
<td>03.</td>
<td>5-Fluoro uracil</td>
<td>-</td>
<td>Uridine 5’monophosphate</td>
</tr>
</tbody>
</table>

2.3 **Purity Testing:**

The purity of the material used for experimental work was tested by determining physical constant and used for recording the fluorescence spectra at various excitation wavelengths. The observed values of melting points are
given in the Table 2.1 and compared with literature value which indicated the purity of materials.

Table 2.1: Physical constants of materials

<table>
<thead>
<tr>
<th>Sr. No</th>
<th>Name of Compound</th>
<th>Melting point °C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Observed</td>
</tr>
<tr>
<td>1.</td>
<td>9-Anthracene carboxylic acid</td>
<td>215 °C</td>
</tr>
<tr>
<td>2.</td>
<td>Perylene</td>
<td>276 °C</td>
</tr>
<tr>
<td>3.</td>
<td>Proflavine Hemisulphate</td>
<td>286 °C</td>
</tr>
<tr>
<td>4.</td>
<td>Uracil</td>
<td>336 °C</td>
</tr>
<tr>
<td>5.</td>
<td>5-Fluoro uracil</td>
<td>284 °C</td>
</tr>
<tr>
<td>6.</td>
<td>5-Methyl uracil</td>
<td>315 °C</td>
</tr>
<tr>
<td>7.</td>
<td>Adenosine</td>
<td>232 °C</td>
</tr>
<tr>
<td>8.</td>
<td>Adenosine 5’ monophosphate</td>
<td>180 °C</td>
</tr>
<tr>
<td>9.</td>
<td>Guanosine 5’ monophosphate</td>
<td>301 °C</td>
</tr>
<tr>
<td>10.</td>
<td>Uridine 5’ monophosphate</td>
<td>201 °C</td>
</tr>
</tbody>
</table>

2.3.1 Purity testing by fluorescence spectral data:

The materials were tested for fluorescence performance. The fluorescence spectra of the materials were recorded at different excitation wavelengths. The emission wavelength of the materials observed at different excitation wavelengths were noted and given in the Table 2.2. The emission wavelengths of the materials are constant even at different excitation wavelength. No additional emission peaks were found in the spectral range of measurement. The presence of fluorescence impurity exhibits its characteristic additional bands in the spectra. Therefore the absence of additional emission bands and the match between literature and experimental value of wavelength at maximum emission confirmed the spectral purity of materials. The uracils fluoresce weakly however nucleosides and nucleotides were found non fluorescent.
Table 2.2: Excitation and emission spectra of materials

<table>
<thead>
<tr>
<th>Sr. No</th>
<th>Name of Compound</th>
<th>Excitation Wavelength (nm)</th>
<th>Emission Wavelength (nm)</th>
<th>Observed</th>
<th>Literature</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>9-Anthracene carboxylic acid</td>
<td>333</td>
<td>413</td>
<td></td>
<td>412 (1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>345</td>
<td>413</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>363</td>
<td>413</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>383</td>
<td>413</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>Perylene</td>
<td>410</td>
<td>446</td>
<td></td>
<td>445 (2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>252</td>
<td>446</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>360</td>
<td>446</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>Proflavine Hemisulphate</td>
<td>444</td>
<td>511</td>
<td>510 (3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>300</td>
<td>511</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>490</td>
<td>511</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>Uracil</td>
<td>272</td>
<td>318</td>
<td>312 (4)</td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>5-Methyl uracil</td>
<td>270</td>
<td>315</td>
<td>320 (4)</td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td>5-Fluoro uracil</td>
<td>258</td>
<td>312</td>
<td>324 (4)</td>
<td></td>
</tr>
</tbody>
</table>

2.4 Experimental procedure for PET studies:

2.4.1 Preparation of solutions:

Stock solution of fluorescent materials and quenchers were prepared in water and in the micellar solution. Stock solutions of desired concentration were prepared by dissolving calculated quantity of the material in to the solvent media. The stock solutions were stirred overnight before further use.

2.4.2 Optimization of concentration and fluorescence intensity:

The fluorescence spectra of materials were recorded in the solution of known concentration prepared by diluting the stock solution. The fluorescence intensity at constant $\lambda_{em}$ and $\lambda_{ex}$ were noted at each dilution. Thus, experiments were performed to know what concentration of electron donor and acceptor are
more suitable in the PET experiments. The plot of fluorescence intensity vs. concentration as shown in Fig. 2.1 has been used to decide concentration suitable for the experiments. The model plot of the experiments is given below,

![Graph showing fluorescence intensity vs. concentration](image)

**Fig. 2.1: Optimization of 9-ANCA concentration in aqueous solution.**

### 2.4.3 PET experiments:

The concentration of electron acceptor was kept constant to a concentration at which the fluorescence intensity is maximum within the experimental range i.e. below 1000 a.u. The concentration of electron donor was varied in each set so that the intensity of fluorescence of acceptor changes appreciably. Care is taken so that the donor and acceptor should remain in the same environment and same experimental conditions. All solutions were degassed by passing nitrogen gas prior to fluorescence measurements.
2.5 Measurement of fluorescence spectra:

The fluorescence measurements were performed on Spectrofluorimeter available in the laboratory. The instrument details are given below,

Instrument: PC based spectrofluorometer
Make: JASCO, Japan
Model: FP-750
Light source: 150W xenon lamp with shielded lamphouse
Monochromator: Holographic grating with 1200 lines/mm
Wavelength range: 220 nm to 730 nm
Spectral bandwidth: 10, 20 nm on both Ex. and Em monochromator
Wavelength accuracy: ±3 nm
Wavelength threw speed: 30,000 nm/min
Wavelength scanning speed: 60,250,1000,4000 nm/min
Response: Fast, Medium, Slow, Auto
Sensitivity: Signal to noise ratio of Raman band of water is higher than 300:1
Photometric display: -999 to +999
Sample chamber: Single cell holder (standard)
Detector: Silicon photodiode for Ex. monochromator and Photomultiplier tube for Em. Monochromator

During recording of the fluorescence and fluorescence excitation spectra the parameters like spectral bandwidth (10 nm) data pitch (1 nm) and wavelength scanning speed (250 nm/min) were kept constant while other parameters were changed as per requirement of the experiment.
Spectrophotometer JASCO, Model – FP -750, Japan

The optical system of the instrument is given in Fig.2.2. The light from the source (Xenon lamp) is focused on to the entrance slit of the excitation monochromator by the ellipsoidal mirror Mo. The light from the slit is dispersed by the diffraction grating G₁ and monochromatic light is taken out by the exit slit. A part of the monochromatic light is led to the monitoring silicon photodiode (SP) by the beam splitter chamber by the plane mirror M₂ and ellipsoidal mirror M₃ where it is focused on the center of the sample cell.

The emission from the sample is focused on to the entrance slit of the emission (Em) monochromator by ellipsoidal mirror, M₄ and two plane mirrors, M₅ and M₆. The light dispersed by the diffraction grating of the emission monochromator going through the exit slit finally led to photometric photomultiplier tube PMT by the spherical mirror, M₇.
The schematic diagram for the FP-750 system is shown in Fig.2.3. The light incident on the monitoring detector (Silicon photodiode) and the emission detector (PMT) is converted into an electrical signal and then converted into digital signal by the A/D converter and is introduced to the microcomputer. The signal subjected to arithmetic operation by the microcomputer is outputted to the display unit as digital data or spectrum. Both wavelength as well as slit drives is controlled by the microcomputer.

The steps involved during recording of the fluorescence and fluorescence excitation spectra of luminophors are as follows;

1. Visual fluorescence color was observed by exciting the sample at 365nm (Hg line) excitation wavelength.
2. The emission monochromator was set at the approximate wavelength of visually observed color.
3. The excitation monochromator was scanned from 230 nm to a wavelength of emission monochromator.
4. The excitation spectrum was recorded and the $\lambda_{ex}$ was noted.
5. The excitation monochromator was set at $\lambda_{ex}$ observed in excitation spectrum.
6. The emission monochromator was allowed to scan in the range 300 nm to 750 nm.
7. The fluorescence emission spectrum was recorded and the $\lambda_{em}$ was noted.
8. The emission monochromator was then set at $\lambda_{em}$ and excitation spectrum monochromator was scanned and thus the excitation spectrum was recorded.
9. Finally the fluorescence spectrum was obtained by setting the excitation monochromator at $\lambda_{ex}$ obtained in above step. Similarly fluorescence excitation spectrum was obtained by setting $\lambda_{em}$ observed in the final emission spectrum.
Fig.2.2: Optical system of FP-750

LS : Light source (150 W Xenon lamp)  
M2, M5, M6 : Plane mirror  
M1 : Ellipsoidal mirror  
M3, M4 : Ellipsoidal entrance/exit slit  
S1 : Excitation entrance/exit slit  
S2 : Emission entrance/exit slit  
G1 : Excitation concave diffraction grating  
G2 : Emission concave diffraction grating (1200 lines/mm)  
SP : Silicon photodiode  
PMT : Photomultiplier tube  
BS : Beam splitter  
MO, M7 : Spherical mirror
Fig. 2.3: System Diagram

2.6 Calibration of the Spectrofluorimeter:

Calibration and sensitivity of the instrument is checked by measuring the Raman spectrum of water. The Quartz distilled water was used for calibration. The detailed procedure given in the Hardware manual of Spectrofluorometer was used. The parameters as given below were set on the parameter screen,

- **Measurement Mode**: Em
- **Excitation wavelength**: 350 nm
- **Excitation Slit bandwidth**: 10 nm
- **Emission Slit bandwidth**: 10 nm
- **Response**: Fast
- **Sensitivity**: High
- **Start Wavelength**: 350 nm
End Wavelength : 450 nm
Scanning Speed : 250 nm / min
Number. of cycles : 1
Display mode : Auto scale

1. Rectangular cell containing distilled water was set in the cell holder.
2. Em shutter at Ex was closed. Auto zero was pressed and allowed to execute.
3. Ex and Em shutters are opened and executed by pressing auto zero.
4. The spectra was scanned and presented in Fig.2.4.

The Raman spectrum of water given in the manual Fig.2.5 is identical. The maximum peak value at 397 nm is 726 a.u. According to the reports in the manual, if the intensity value is above 300 a.u. the sensitivity is normal and with the best performance of the instrument.

Fig. 2.4: Raman Spectra of water recorded under experimental condition
Fig. 2.5: Raman Spectra of water taken from the operational manual supplied by JASCO-JAPAN

2.7 Absorption Spectroscopy:

The absorption studies were performed to check the possibility of spectral change of acceptor in the presence of the donor. The absorption spectra of electron acceptor with and without electron donor in the given solvent media were recorded by using UV-VIS-NIR Spectrophotometer (UV-3600) Shimadzu (Japan) available in the laboratory. It is procured through FIST program sponsored by DST. The absorption spectra of the probe (electron acceptor) were recorded with and without electron donor by using absorption spectroscopy. The details of the Spectrophotometer are given below,
UV-VIS-NIR Spectrophotometer

Specification of Spectrophotometer

Wavelength range  185 to 3,300 nm
Wavelength Accuracy  
  Visible/Ultraviolet region  : ± 0.2 nm
  Near-infrared region       : ± 0.8 nm
Photometric method  Double-beam system
Monochromator  Grating/Grating-type double monochromater
Detector  
  UV/Visible range : Photomultiplier tube
  Near-infrared region : InGaAs-photodiode/cooled
  PbS photoconductive element
2.8 Electrochemical Measurements:

The oxidation and reduction potentials of the donor-acceptor pairs in aqueous solutions were measured by Cyclic-Voltammetry using a Princeton applied research Perkin Elmer Versa-Stat II model- 270, Interfaced with P-III-PC available in the Physics Department of our University. The experimental setup consists of Pt as a working electrode, graphite counter electrode and silver reference electrode. The experimental values of potential are converted against a saturated Calomel electrode (SCE) by subtracting 0.333 V from the measured potentials as 0.1 M KCl was used as supporting electrolytes.
2.9 References:


