Chapter 2

Materials and Methods

Abstract: This chapter describes the materials and methods used for the study. The experimental details of the pulse radiolysis technique and the details of the linear accelerator used in the study are explained. The production of various free radicals used in the study is described. The laser source used for the femtosecond spectroscopic technique is also described in detail. The details of the fluorescence upconversion and time correlated single photon counting set ups are explained in this chapter. Also the various analytical techniques used in the study are explained in detail.
2.1 Materials

Commercially available high purity chemicals were used without further purification. 2-aminopurine (2AP), guanosine (G), adenosine (Ade), inosine (Ino), thymine (T), cytidine (Cyd), 2-aminopyrimidine (AP), 2-amino 4,6-dimethylpyrimidine (ADMP), 2-amino 4-methylpyrimidine (AMP), 2-amino 4-methoxy 6-methylpyrimidine (AMMP) and 2-amino 4-hydroxy 6-methylpyrimidine (AHMP), N,N,N',N'-tetramethyl-phenylenediamine (TMPD), methyl viologen (MV²⁺) and potassium peroxodisulphate (K₂S₂O₈) were purchased from Aldrich. The 2AP modified single strand DNA and their complimentary strands were purchased from Integrated DNA Technologies, USA and were used without further purification. The 15-mer sequences of the 2AP containing strands are defined below. The method of preparation will be detailed in the next section. All the solutions for the pulse radiolysis experiments were prepared in water purified by Millipore Milli-Q system.

![Molecules](image-url)
2-Amino 4,6-dimethyl pyrimidine (ADMP)

OCH₃

H₂N

2-Amino 4-methoxy 6-methyl pyrimidine (AMMP)

H₂N

CH₃

2-Amino 4-hydroxy 6-methyl pyrimidine (AHMP)

H₂N

CH₃

H₃C-N

N=CH₃

Methyl Viologen (MV²⁺)

H₃C-N

N=CH₃

N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD)

5'-GATTT[2AP]GTCTACCTC-3'

3'-CTAAATCAAATGGAG-5'

DNA Duplex 1 (D1)

5'-GATTT[2AP]AGTTACCTC-3'

3'-CTAAATCAAATGGAG-5'

DNA Duplex 2 (D2)

5'-GATTT[2AP]TGTTATCC-3'

3'-CTAAATCAAATAGG-5'

DNA Duplex 3 (D3)
2.1.1 Preparation of 2AP-Modified DNA Double Helix

Double helical DNA was prepared by conventional method. A 15-mer sequence was used to prepare the double stranded DNA. The 2AP-modified double helical DNA was prepared by hybridizing the 2AP modified single strand with its corresponding complimentary strand with thymine opposite to the 2AP residues. The properties of three 2AP modified single strands selected in this study are summarized in table 2.1. Both the single strands were dissolved in phosphate buffer (pH 7) and the duplex was prepared by mixing the 2AP containing strands with their complementary strands. It was heated to 90° C for about 5 minutes and cooled to room temperature by overnight. The double helical DNA, thus formed was stored at low temperature. To ensure that all 2AP-containing strands were hybridized, a 10% excess of the complimentary strand was added. The melting temperature of all the selected oligonucleotide was about 30-35° C.

Table 2.1 Properties of the 2AP modified single strands

<table>
<thead>
<tr>
<th>Properties</th>
<th>Sequence 1</th>
<th>Sequence 2</th>
<th>Sequence 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melting temp. (50mM NaCl)</td>
<td>35.1°C</td>
<td>30.7°C</td>
<td>32.3°C</td>
</tr>
<tr>
<td>GC content</td>
<td>35.7%</td>
<td>28.5%</td>
<td>28.5%</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>4,533.0</td>
<td>4,557.0</td>
<td>4,548.0</td>
</tr>
<tr>
<td>nmoles/OD260</td>
<td>7.9</td>
<td>7.4</td>
<td>7.8</td>
</tr>
<tr>
<td>ug/OD260</td>
<td>35.7</td>
<td>33.9</td>
<td>35.3</td>
</tr>
<tr>
<td>Ext. Coefficient L/(mol.cm)</td>
<td>128,800</td>
<td>134,600</td>
<td>128,700</td>
</tr>
</tbody>
</table>
2.2 Radiation Chemical Studies

Radiation chemical studies were performed by using the pulse radiolysis technique.

2.2.1. Pulse Radiolysis

Pulse radiolysis experiments were carried out at the Radiation and Photochemistry Division, Bhabaha Atomic Research Centre (BARC), Mumbai and the details of the experimental setup has been described elsewhere.¹

Pulse radiolysis consists of a linear accelerator delivering electron pulses of 7 MeV energy of 50 ns duration and the associated detection setup. The transient species formed on pulse radiolysis were monitored using a 450 W pulsed xenon lamp, a monochromator (Kratos GM-252) and a Hamamatsu R-955 photomultiplier as the detector. The photomultiplier output was digitized with a 100 MHz storage oscilloscope interfaced to a computer for kinetic analysis. A schematic diagram of the pulse radiolysis set up at BARC is given in figure 2.1.

Absorption spectroscopy is the commonly used technique to observe the transient characteristics in pulse radiolysis. Generally an absorption spectrophotometer comprises of a light source, optical train, monochromator, detector and a recorder. In pulse radiolysis the sample is irradiated with high-energy electrons and the transient recorder is triggered simultaneously to store the detector signal as a function of time.
If the transient species formed absorbs the light from the monitoring wavelength a deflection of the detector signal will track the transmittance changes ($\Delta I$) in the sample. This change in transmittance will be equivalent to the formation and kinetic decay behavior of the transient. By monitoring the intensity of incident light ($I_0$) and change in transmittance of the transient species, we can obtain the absorbance as a function of time. Beer-Lambert law is the basic principle of optical detection system.
Figure 2.1 Pulse radiolysis setup at BARC, Mumbai
2.2.2 Dosimetry

Dosimetry is used to determine the amount of energy transferred from the radiation field to the absorbing material and to find the distribution of the absorbed energy within the material. In radiation chemical studies, absorbed dose is the generally used term and it is the amount of energy absorbed per unit mass of irradiated sample. The SI unit of absorbed dose is joules per kilogram (J kg⁻¹), which is called gray (Gy). Absorbed dose rate is defined as the absorbed dose per unit time and it has the unit of gray per unit time (Gy s⁻¹). The absorbed dose is a direct measurement of the energy transferred to the irradiated material, which is capable of producing chemical or physical changes.

In pulse radiolysis, the dosimetry is different from those used with continuous radiation sources. One of the reasons for this is the much higher dose rate involved in pulse radiolysis (~ $10^6$ to $10^{10}$ Gy s⁻¹ compared with 0.1 to 10 Gy s⁻¹ from $^{60}$Co γ-sources). It is also noteworthy that the very high concentrations of radicals produced by pulse radiolysis lead to increased radical-radical reaction at the expense of radical-solute reaction. Hence, the radiation chemical yields determined at lower dose rates are not applicable to pulse radiolysis experiment. Therefore thiocyanate dosimeter is the generally used dosimetry in pulse radiolysis experiment.² The thiocyanate dosimeter monitors the transient species formed on irradiation. It consists of a neutral solution of potassium
thiocyanate (10 mol dm$^{-3}$) saturated with either oxygen or nitrous oxide.

In N$_2$O saturated aqueous solution, 'OH is the main reactive species. Therefore, on irradiation, hydroxyl radical react with SCN$^-$ to form (SCN)$_2$'$^-$, which absorbs around 500 nm. The absorption is measured immediately following the radiation pulse and can be corrected for the decay of the transient.$^2$

\[ \cdot \text{OH} + \text{SCN}^- \rightarrow \text{HOSCN}^- \quad (k = 1.1 	imes 10^{10} \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1})^3 \] (2.1)

\[ \cdot \text{OH} + \text{SCN}^- \rightarrow \text{SCN}' + \text{OH}^- \] (2.2)

\[ \text{SCN}' + \text{SCN}^- \rightarrow (\text{SCN})_2'^- \quad (k = 6.9 \times 10^{9} \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1})^4 \] (2.3)

The dose per pulse is calculated by using the following equation

\[
\text{Dose rate (Gy)} = \frac{(A \times 6.023 \times 10^{23}) \times 100}{(\varepsilon \times G \times 6.24 \times 10^{13} \times 1000)}
\] (2.4)

where $A$ is the absorbance of (SCN)$_2'^-$, $\varepsilon$ is the molar extinction coefficient (in N$_2$O saturated solution $G \times \varepsilon_{252 nm} = 5.2 \times 10^{-4} \text{ m}^2 \text{ J}^{-1}$).

In our study we employed thiocyanate dosimetry to measure the absorbed dose. An aerated aqueous solution of KSCN ($1 \times 10^{-2}$ mol dm$^{-3}$) was used to monitor the dose per pulse with $G \times \varepsilon_{500} = 21520 \text{ dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$ ($G$ is the G-value which is defined as the number of molecules produced or destroyed by the absorption of 100 eV radiation energy and this
quantity can be expressed in SI unit by multiplying with $1.036 \times 10^{-7}$ mol J$^{-1}$, and $\epsilon_i$ is the molar extinction coefficient of (SCN)$_2^-$ resulting from the reaction of \('OH\) with SCN$^-$ and was normally kept at 13-15 Gy. A low dose per pulse of around 6 Gy was used for the investigation of the electron transfer reaction between the electron adducts and methyl viologen (MV$^{2+}$) and also the electron transfer reaction between \('OH\) adducts and N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD).

2.2.3 Production of Free Radicals
Varicous free radicals can be selectively produced by suitably altering the experimental condition. The details of the production of different free radicals are described below.

(i) Hydrated Electron
The hydrated electron ($e_{aq}^-$) is produced by the radiolysis of water in N$_2$ saturated solution containing 2-methyl-2-propanol (0.2 mol dm$^{-3}$). 2-Methyl-2-propanol acts as an \('OH\) scavenger and the yield of $e_{aq}^-$ is expected to be 2.7 mol J$^{-1}$ at neutral pH.$^5$

\[
\begin{align*}
\text{H}_2\text{O} & \xrightarrow{\text{radiolysis}} e_{aq}^-, \text{H}^+, \text{'OH}, \text{H}_2, \text{H}_2\text{O}_2, \text{H}_3\text{O}^+ \quad (2.5) \\
\text{(CH}_3\text{)}_3\text{COH} + \text{'OH} & \xrightarrow{\text{} \text{radiolysis}} \text{'CH}_2\text{(CH}_3\text{)}_2\text{COH} + \text{H}_2\text{O} \quad (2.6)
\end{align*}
\]

(ii) Sulfate Radical Anion
Sulfate radical anion (SO$_4^{2-}$) is produced by the radiolysis of N$_2$ saturated aqueous solution containing 2-methyl-2-propanol (0.2 mol dm$^{-3}$)
and $S_2O_8^{2-}$ ($5 \times 10^{-2}$ mol dm$^{-3}$). Persulfate reacts with hydrated electron and forms $SO_4^{2-}$ (reaction 2.7 and 2.8).

\[
e_{aq}^{-} + S_2O_8^{2-} \rightarrow SO_4^{2-} + SO_4^{2-} + \text{H}^+ \quad (2.7)
\]
\[
^*H + S_2O_8^{2-} \rightarrow SO_4^{2-} + SO_4^{2-} + \text{H}^+ \quad (2.8)
\]

(iii) **Hydroxyl Radical**

The reaction of hydroxyl radical ($^*\text{OH}$) was carried out in $N_2O$ saturated solutions where $e_{aq}^{-}$ is quantitatively converted to $^*\text{OH}$ (reaction 2.9). In this condition the main reactive species is hydroxyl radical and the yield is expected to be 5.3-6.8 mol J$^{-1}$ depending upon the concentration of the solute as well as its reaction rate constant.

\[
N_2O + e_{aq}^{-} \rightarrow ^*\text{OH} + \text{OH}^- + N_2 \quad (2.9)
\]

(iv) **Oxide Radical Anion**

The reaction of oxide radical anion ($O^{2-}$) radicals was investigated in $N_2O$ saturated aqueous solution at pH $=14$ since $^*\text{OH}$ is in equilibrium with its basic form $O^{2-}$ at highly basic medium. The $pK_a$ of the $^*\text{OH}$ is 11.9 (reaction 2.10).

\[
^*\text{OH} + \text{OH}^- \rightarrow O^{2-} + H_2O \quad (2.10)
\]

(v) **Hydrogen Radical**

The reaction of hydrogen radical ($H^*$) was investigated at pH 1 in $N_2O$ saturated solutions in the presence of 2-methyl-2-propanol (0.2 mol dm$^{-3}$) as
'OH scavenger. At pH 1 the yield of \(^*H\) is 3.3 mol J\(^{-1}\) since hydrated electron is also converted to \(^*H\) (\(G(\(^*H\)) = 3.3\)).

\[
e_{aq}^{-} + \text{H}^+ \rightarrow \text{H} + \text{H}_2\text{O} \quad (2.11)
\]

2.3 Femtosecond Spectroscopy

Femtosecond spectroscopic experiments were carried out at the Femtosecond Spectroscopy Laboratory, Department of Chemistry, Pohang University of Science and Technology (POSTECH), South Korea.

2.3.1 Laser Source

Ti:sapphire laser is used as the femtosecond light source. All experiments were carried out with the output from a homemade cavity-dumped femtosecond optical parametric oscillator (OPO) based on periodically poled lithium niobate. The details of the OPO have been described elsewhere.\(^7\) The OPO was synchronously pumped by a commercial Ti:sapphire laser (Tsunami, Spectra-Physics) that generate 800 mW at 800 nm at a repetition rate of 81.5 MHz. The Ti:sapphire laser was pumped by 5 W output of a frequency doubled diode pumped Nd:YVO\(_4\) laser (Millennia, Spectra-Physics). The OPO is assembled in a vacuum tight box to remove oxygen and water vapor. Efficient cavity dumping was achieved by using a Bragg cell made of TeO\(_2\). Cavity dumper driver (NEOS) was synchronized to the 81.5/2 MHz radio frequency signal derived from the Ti:sapphire pump laser. Output of the cavity dumper driver was amplified by an RF amplifier (Ophir RF) before
feeding it to the Bragg cell. The cavity dumped pulse energy at 1.2 μm was 90 nJ at the repetition rates up to 500 KHz and 75 nJ at 1 MHz. The pulse duration was 56 fs when a Gaussian pulse shape is assumed. Tunable femtosecond pulses in the visible region can be obtained from the second harmonic generation in an LBO crystal. A schematic representation of the optical parametric oscillator is shown in figure 2.2.

![Figure 2.2 Schematic representations of the cavity-dumped near infrared optical parametric oscillator (OPO). BC-TeO2 Bragg cell, P-SF11 equilateral prism, L-plano-convex lens, M1-M6-spherical mirrors, PZT, piezoelectric actuator, PD-photodiode.](image)

The advantage of this cavity dumped femtosecond OPO is the wide tunability, variable repetition rate, high pulse energy, short pulse duration and high stability. An image of the OPO is shown in figure 2.3.
2.3.2 Fluorescence Upconversion Setup

The femtosecond fluorescence upconversion setup has been described elsewhere.\textsuperscript{6} Fluorescence upconversion is the fastest fluorescence detection technique known so far. The femtosecond light source was based on a home made cavity dumped optical parametric oscillator which generates about 90 nJ pulse at a repetition rate of 1 MHz and is broadly tunable from 1.0 to 1.5 μm with a pulse duration of <60 fs at 1.2 μm. Figure 2.4 shows a schematic representation of upconversion setup used in the study.

The fourth harmonic of the fundamental was generated by using a 3 mm LBO and 500 μm BBO crystals. This pump beam was centered at 320 nm and was used to selectively excite 2-aminopurine incorporated in
the oligonucleotide as well as the free 2'AP. The energy of the pump beam used was attenuated to 0.7 nJ per pulse. The residual second harmonic (640 nm) was separated by using a beam splitter and was used as a gate pulse. The energy of the gate pulse was set to be 10 nJ per pulse. Two pairs of prisms were used to compensate group velocity dispersions of pump and gate beams. The Berek compensator was used to rotate the polarization of the pump beam and it was set at the magic angle (54.7°). A 5 cm focal length lens was used to focus the pump beam to a 100 μm quartz cell containing sample solution. The sample cell was continuously moved to avoid the photo damage. The fluorescence from the sample was collected and focused it into a 500 μm BBO crystal by using a reflective microscope objective lens. The upconverted fluorescence signal was generated by frequency mixing of this fluorescence with the gate pulse in BBO crystal in the phase matching direction. Phase matching condition is the main criteria to generate the sum frequency signal (upconverted signal). The upconverted light was focused into a monochromator (Acton, Spectrapro 300) and detected with a photomultiplier tube (Hamamatsu, R1527) connected to a gated photomultiplier counter (Stanford Research SR 400) interfaced to a computer for data acquisition. The time resolution was achieved by delaying the gate pulse relative to the pump pulse using a linear encoder stage. The full width at half maximum (fwhm) of the cross-correlation function was 200 fs.
Figure 2.4 Schematic representation of Femtosecond fluorescence upconversion setup. L-Lens; M-Mirror; P-Prism; BS-Beam splitter; S-Sample; Mono-Monochromator

In fluorescence upconversion experiment the data acquisition was carried out by using a program written by using Lab view (a graphical programming language by National Instruments). Here we used the PC plug-in Data Acquisition (DAQ) boards. A typical DAQ system consist of transducers, signal conditioning hardware, plug-in DAQ boards, and Lab view application software.
2.3.3 **Time Correlated Single Photon Counting (TCSPC) Setup**

In time correlated single photon counting (TCSPC) experiment, the same laser source as in the upconversion experiment is used. This is one of the best methods to measure the time dependantant fluorescence in the nanosecond to picosecond time range. A schematic diagram of the TCSPC setup is shown in figure 2.5.

![Figure 2.5 Schematic representation of Time correlated single photon counting setup. L-Lens; M-Mirror; P-Prism; PM-Parabolic mirror; S-Sample](image)

In this case also, the polarization of the pump beam was set at the magic angle. A parabolic mirror was used to direct the excitation beam to sample and the fluorescence was collected using a 20 cm focal length lens (UVFS 200FL BBAR). The emission was focused into a
monochromator and was detected with a thermoelectrically cooled MCP-PMT (Hamamatsu R3809U-51). The signal was amplified by a wide-band amplifier (Philip Scientific), sent to a Quad constant fraction discriminator (Tennelec), a time-to-amplitude converter (Tennelec), and a counter (Ortec), a multichannel analyzer (Tennelec/Nucleus), and stored in a computer for data processing. The instrumental response function was \( \sim 50 \) ps in fwhm. The histogram in the multichannel analyzer (MCA) represents the fluorescence decay curve of the sample. The fluorescence lifetime of the sample can be obtained by the analysis of the histogram obtained in the MCA channels because the multichannel analyzer is calibrated with time.

The data analysis was performed based on the following principle.

The observed decay curve \( (I(t)) \) is a convolution of the true decay curve, \( G(t) \) and the effective time profile of the excitation pulse, \( P(t) \). Assuming the excitation pulse to be a combination of a large number of \( \delta \)-pulses, the function \( I(t) \), \( G(t) \) and \( P(t) \) are related by the following convolution integral,

\[
I(t) = \int_{0}^{t} P(t') G(t-t') \, dt'
\]

(2.12)

\( I(t) \) and \( P(t) \) can be obtained experimentally and during the data analysis a decay function \( G(t) \) is assumed for the sample and it is convoluted with the observed \( P(t) \) to obtain a calculated curve \( Y(t) \). The curve \( Y(t) \) is then
compared with \( l(t) \) and the variable in the function \( G(t) \) is iteratively changed till a best fit between the \( Y(t) \) and \( l(t) \) is obtained.

2.4 Steady State Absorption and Fluorescence Measurements

Absorption spectra were measured using UV-S3100 (Scinco) spectrophotometer. The wavelength scanning range was from 190 to 1100 nm with a resolution of 0.95 nm. The detector used in this spectrophotometer is 1024 channel PDA. The wavelength accuracy is \( \pm 0.5 \) nm with a reproducibility of \( \pm 0.02 \) nm.

Fluorescence spectra were recorded by using QM-6/2005 (Photon Technology International) dual emission spectrofluorimeter with a signal to noise ratio of 6000:1. Excitation and emission wavelength can be selected by means of auto-calibrated, computer-controlled, scanning QuadraScopic monochromators (grating 1200 G/mm) with variable slits. The light source used was 75W Xenon lamp. The wavelength range of illumination is from 200-650 nm. The emission was focused into a monochromator and detected with a photomultiplier tube (185-900 nm).
References


