CHAPTER 1

INTRODUCTION:
BIOCONJUGATES OF
FERROCENE AND THEIR
APPLICATIONS
INTRODUCTION

The work presented in this thesis consists of studies on synthesis and structural conjugates of ferrocene with nucleobases and nucleic acids. This chapter gives an overview on background literature for undertaking the research work and gives an account of its recent advancements in the chemistry of ferrocene conjugates of nucleobases, nucleosides, nucleotides, amino acids and peptides, proteins with emphasis on their synthesis and applications. A brief introduction to nucleic acids structure and bonding, synthesis, physical and chemical properties of ferrocene is presented in next section.

1.1 Nucleic Acids: Chemical Structure


Nucleic acids (DNA, RNA) are the most important of all biopolymers. DNA is the basic hereditary material in all cells and contains all information necessary to make proteins. It is present in the nucleus of organisms and contains the genetic instructions
specifying the biological development of all cellular forms of life and many viruses.\(^1\) It has been 55 years since Watson and Crick proposed the double-helical structure for duplex DNA (Figure 1.1).\(^2\) The molecular architecture of DNA consists of a double-stranded helix, of uniform diameter, with right handed twist. DNA is a polymer made up of nucleotide units. Each nucleotide unit consists of a nitrogenous base, a deoxyribose sugar, and a phosphate. The main chemical constituents of DNA are the sugar-phosphate unit present on the outer side of the helix which constitutes the backbone of each strand and the nitrogenous bases adenine (A), thymine (T), guanine (G) and cytosine (C) which are pointed towards the center of the helix. Each base is connected to a sugar via a \(\beta\)-glycosyl linkage (Figure 1.2). The adjacent nucleoside units (base + sugar) are connected via the O3' of one nucleoside to O5' atoms of other forming phosphodiester linkages. Hydrogen bonds between complementary base pairs (A:T; G:C) hold the two strands together (Figure 1.1). RNA contains ribose rather than deoxyribose sugars and the base composition are: adenine (A), uracil (U), guanine (G) and cytosine (C).

![Figure 1.2](image)

**Figure 1.2** (A) Chemical structure of DNA; Watson-Crick hydrogen-bonding scheme for (B) A:T and (C) G:C base pairs.

The double helix of DNA is nature’s simple and elegant solution to the problem of storing, retrieving, and communicating the genetic information of living organism.\(^3\) The specificity and the reversibility of the hydrogen bond formation between the complementary nucleobases is one of the most important characteristic features, which
allow the strands of the double helix to be unwound and then rewound in exactly the same configuration. The construction of DNA and design of its analogues for use in the recognition of specific DNA and RNA sequences has emerged as intellectual and practical assignment for synthetic and structural organic chemists. The recognition of DNA and RNA sequences by complementary oligonucleotides is a central feature of biotechnology and is vital for hybridization based biological applications. The study of such complementary recognition is possible with the widely used experimental techniques and diagnostic protocols.

1.1.1 Shapes of nucleotides

The molecular geometry of an individual nucleotide is very closely related to that of the corresponding nucleotide units in oligomers and nucleic acid helical structure. The details of the conformational structure of nucleotides are accurately defined by the torsional angles $\alpha$, $\beta$, $\gamma$, $\delta$, $\epsilon$ and $\zeta$ in the phosphate backbone, $\theta_0$ to $\theta_4$, in the furanose ring, and $\chi$ for the glycosidic bond (Figure 1.3). Because many of these torsional angles are interdependent, one can simply describe the shapes of nucleotides in terms of four parameters: the sugar pucker, the syn-anti conformation of the glycosidic bond, the orientation of C4'-C5' bond and the phosphodiester backbone.

**Figure 1.3** Structures of A) C3'-endo, B) C2'-endo preferred sugar puckers and C) torsion angle notation for polynucleotide chain
1.1.2 Sugar pucker in nucleosides

The pentose sugar rings in nucleosides are twisted or puckered in order to minimize non-bonded interactions between their substituents. This ‘puckering’ is described by identifying the major displacement of carbon C2’ and C3’ from the median plane of C1’-O4’-C4’. Thus, if the endo-displacement of C2’ is greater than the exo-displacement of C3’, the conformation is called C2’-endo and so on for other atoms of the ring (Figure 1.3A and 1.3B). The endo-face of the furanose is on the same side as C5’ and the base; the exo-face is on the opposite face to the base. The sugar pucker is located in the north (N) and south (S) domains of the pseudorotation cycle of the furanose ring. In solution, N and S conformations are in rapid equilibrium and are separated by a low energy barrier. The average position of the equilibrium is influenced by (i) the preference of the electronegative substituents at C2’ and C3’ for axial orientation, (as in the case of RNA the C2’-OH interact with backbone phosphate, C3’-endo is preferable conformation), (ii) the orientation of the base (syn goes with C2’-endo), and (iii) the formation of an intra-strand hydrogen-bond from O2’ in one RNA residue to O4’ in the next, which favors C3’-endo-pucker. The rise in the sugar phosphate backbone for each monomeric unit is 5.9 Å in case of RNA and 7.0 Å for DNA.

1.1.3 Base pairing via hydrogen bonding

The mutual recognition of A by T and of C by G uses hydrogen bonds to establish the fidelity of DNA transcription and translation. The N-H groups of the bases are potent hydrogen donors (d), while the sp²-hybridized electron pairs on the oxygens of the base C=O groups and on the ring nitrogens are much better hydrogen bonding acceptors (a) than are the oxygens of either the phosphate or the pentose. The acceptors:donors (a:d) hydrogen bonds so formed are largely ionic in character.

Watson and Crick recognized the complementarities in hydrogen-bonding capability of A:T and G:C base pairs during DNA model-building studies in 1952. This base pairing pattern known as Watson-Crick pairing (Figure 1.4) consists of two hydrogen bonds in an A:T base pair and three hydrogen bonds in a C:G base pair.2
While Watson-Crick base pairing is the dominant pattern between the nucleobases, other significant pairings are Hoogsteen (HG)\(^5\) and Wobble base pairs.\(^6\) A Hoogsteen A:T base pair (Figure 1.5) has the N7 position of the purine base as a hydrogen bond acceptor and C6 amino group as a donor, binding to the Watson-Crick (N3-O4) face of the pyrimidine base. Hoogsteen pairs have quite different properties from Watson-Crick base pairs. The angle between the two glycosylic bonds (ca. 80° in the A:T pair) is larger and the C1'-Cl' distance (ca. 8.6 Å) is smaller than in the regular geometry. In reversed Hoogsteen base pairs, one base is rotated through 180° with respect to the other.

Hoogsteen base-pairing allows binding of pyrimidine third strand in the major groove (Section 1.1.5) of Watson-Crick purine: pyrimidine duplexes to form triple-helical structures in 1:2 stoichiometry of (poly(dA):2poly(dT)) and (poly(rG):2poly(rC)). The triplexes do occur in DNA and RNA in polypurine:polypyrimidine structures.
In Wobble base pairing (Figure 1.6), a single purine base is able to recognize different pyrimidines (e.g. I:U and I:A where I = inosine, U = uracil, A = adenine) and have importance in the interaction of messenger RNA (m-RNA) with transfer RNA (t-RNA) on the ribosome during protein synthesis (codon-anticodon interactions). Several mismatched base pairs and anomalous hydrogen bonding patterns have been seen in X-ray studies of synthetic oligodeoxynucleotides.

![Figure 1.6 Wobble base pairings for A) I:U, B) A:U, C) I:A, D) G:U and E) I:C. I = Inosine.](image)

1.1.4 DNA secondary structures

Three DNA conformations are normally found in nature, A-DNA, B-DNA, and Z-DNA (Figure 1.7). The "B" type described by Watson and Crick predominates in cells. B-DNA is a right-handed double helix with a wide major-groove and a narrow minor-groove, where the bases are perpendicular to the helical axis. It is 23.7 Å wide and extends in a helical form over 34 Å per 10 bp of sequence. A-DNA and Z-DNA differ significantly in their geometry and dimensions from B-DNA, although still forming helical structures. At low humidity and high salt, the favored conformation is highly crystalline A-DNA while at high humidity and low salt, the dominant conformation is B-
DNA. In both A-DNA and B-DNA the Watson-Crick base pairing is maintained by \textit{anti} glycosidic conformation of the nucleobases. The sugar conformation however, is different in both conformations with the B-DNA showing C2'-\textit{endo} puckered sugar and the A-DNA exhibiting C3'-\textit{endo} sugar-pucker.

A very unusual conformation of DNA-duplex is the left-handed Z-DNA.\textsuperscript{10} This conformation of DNA is stabilized by high concentrations of MgCl\textsubscript{2}, NaCl and ethanol, and is favored for alternating purine:pyrimidine sequences of high G:C content. In Z-DNA the Watson-Crick base pairing is achieved by purines adopting \textit{syn} glycosidic conformation with C3'-\textit{endo} sugar-pucker\textsuperscript{x} leading to characteristic zig-zag phosphate backbone. The characteristic features of major DNA conformations are summarized in Table 1.1.

RNA can also form double stranded duplexes. The most important structural feature of RNA that distinguishes it from DNA is the presence of a hydroxyl group at the 2'-position of the ribose sugar. The presence of this functional group enforces C3'-\textit{endo} sugar conformation (as opposed to the C2'-\textit{endo} conformation of the deoxyribose sugar in DNA) causing the helix to adopt the A-type geometry rather than the B-type which is
most commonly observed in DNA. In A-DNA the major groove is very deep and narrow and the minor groove is shallow and wide.

<table>
<thead>
<tr>
<th>Table 1.1</th>
<th>Salient features of the three major forms of DNA and RNA strands</th>
</tr>
</thead>
<tbody>
<tr>
<td>Geometry attribute</td>
<td>A-DNA</td>
</tr>
<tr>
<td>Helix sense</td>
<td>right-handed</td>
</tr>
<tr>
<td>Repeating unit</td>
<td>1 bp</td>
</tr>
<tr>
<td>Rotation/bp</td>
<td>33.6°</td>
</tr>
<tr>
<td>Mean bp/turn</td>
<td>11</td>
</tr>
<tr>
<td>Inclination of bp to axis</td>
<td>+19°</td>
</tr>
<tr>
<td>Rise.bp along axis</td>
<td>2.3 Å</td>
</tr>
<tr>
<td>Pitch/turn of helix</td>
<td>24.6 Å</td>
</tr>
<tr>
<td>Mean propeller twist</td>
<td>+18°</td>
</tr>
<tr>
<td>Glycosyl angle</td>
<td>anti</td>
</tr>
<tr>
<td>Sugar pucker</td>
<td>C3'-endo</td>
</tr>
<tr>
<td>Diameter</td>
<td>25.5 Å</td>
</tr>
<tr>
<td>Conditions</td>
<td>Low humidity &amp; High salt</td>
</tr>
<tr>
<td>Major groove</td>
<td>Narrow &amp; deep</td>
</tr>
<tr>
<td>Minor groove</td>
<td>Wide</td>
</tr>
</tbody>
</table>

Other DNA conformations such as C-DNA, D-DNA, E-DNA, L-DNA, P-DNA, and S-DNA are variations of A or B-type of DNA and have not been observed in naturally occurring biological systems.

1.1.5 Molecular recognition in the major and minor grooves of duplex DNA

The major and the minor grooves of DNA differ significantly in their electrostatic potential, steric effects, hydration and dielectric strength. This is because of differing exposure of H-bonding sites in grooves. A:T and T:A base pairs can accept additional hydrogen bonds from ligands bound in the major groove via the C4 carbonyl of T and N7 of A, while in the minor groove hydrogen bonding occurs through the C2 carbonyl of T and N3 of A (Figure 1.8). The only hydrogen bond donor in the major groove for the
A:T base pair is the N6 amino group of A, while none exists in the minor groove. For C:G and G:C duplexes, the H-bond acceptors in the major groove are N7 and O6 for G and in the minor groove are O2 of C and N3 of G. The hydrogen bond donor in the major groove for C:G is the N4 amino of C and in the minor groove, the N2 amino of G.

Figure 1.8. Hydrogen bond donor and acceptor sites in the major groove of duplex DNA at T:A, A:T, C:G and G:C base pairs. Arrows pointing to the atoms indicate acceptor sites; arrows pointing away from the atoms indicate donor sites.

The salient outcome of this pattern of hydrogen bond donors and acceptors in the grooves is that the molecules binding in groove can discriminate the A:T base pair from C:G efficiently from the major groove side but not so well in the minor groove. Two additional features of molecular discriminations are also noteworthy. In A:T and T:A base pairs, the C5 methyl of T offers substantial hydrophobic recognition in the major groove which is absent for C:G and G:C base pairs. However, in the C:G and G:C duplexes, the N2 amino group of G presents a steric block to hydrogen bond formation at
N3 of G and the C2 carbonyl of C in the minor groove. It is possible to distinguish A:T from T:A and G:C from C:G in the major groove since the horizontally ordered array of hydrogen bonding sites and hydrophobic centers differ among the four pairs (Figure 1.8). The negative electrostatic potential due to phosphate charges is greater in the A:T minor groove than in G:C-rich regions of DNA and this provides an additional important source for A:T-specific minor groove recognition.\footnote{4a}

1.1.6 Bioorganometallic chemistry of DNA

The preceding sections of this chapter gave an overview on a brief introduction to structure and bonding of nucleic acids.

The redox active units linked to self-base pairing nucleobases or DNA/RNA could be useful building blocks in supramolecular chemistry coupling molecular recognition with electrochemistry, leading to novel applications for the electrochemical recognition of a large variety of DNA/RNA binding substrates. The main interest in redox labeled oligonucleotide derivatives is for electrochemical DNA sensors and is also important in studies over DNA mediated electron transfer processes, which has large potential for biomedical application, which have resulted in the synthesis of numerous transition metal (Ru, Os, Fe, Rh and Cu complexes) modified oligonucleotides.

In this context, ferrocene has received considerable attention due to its good stability, enabling convenient synthetic chemistry. Further, ferrocene and its derivatives have received a lot of importance in molecular recognition research, due to their redox characteristics. As a part on this chapter the following section gives a brief introduction to synthetic approaches, physical and chemical properties of ferrocene as well as recent advancements in the chemistry of ferrocene conjugates of nucleobases, nucleotides, nucleosides, amino acids, peptides and proteins.

1.2 Ferrocene: A Sandwich Compound

In 1951, a new compound containing iron and two cyclopentadienide ligands was reported. Two independent groups of chemists Kealy and Pauson\textsuperscript{13} and Miller \textit{et al.}\textsuperscript{14} almost simultaneously arrived at the same conclusions, albeit accidentally. Both groups noted that the new compound was insoluble in water, air-stable and sublimable with excellent solubility in organic solvents. Although even the first reports noted its high and
unexpected stability, the correct structure was only soon afterward suggested independently by Wilkinson and Fischer. To elucidate the correct structure of dicyclopentadienyl iron, Wilkinson used chemical, physical and spectroscopic methods while Fischer used X-ray crystallography to characterize the compound.

Wilkinson proposed a possible structure where the iron atom was placed between the two cyclopentadienyl (Cp) ligands. This reveals very strong bonding due to excellent overlap of the metal $d$ orbitals and the $\pi$-electrons in the $p$ orbitals of the Cp ligands. Wilkinson, Woodward and co-workers began to characterize the material and concluded that the $\pi$-complexed sandwich structure had to be correct\textsuperscript{15} (Figure 1.9.A). Wilkinson discovered that the iron centre of the compound could be readily oxidized from +2 to +3 and formed a number of [(C$_5$H$_5$)$_2$Fe]$^+$X$^-$ derivatives from the blue cation. Fischer’s X-ray diffraction studies gave indisputable evidence of the ‘sandwich’ structure and postulated a ‘double-cone’ shape (Figure 1.9.B).

![Figure 1.9 (A) ‘Sandwich’ structure and (B) ‘double cone’ shape of dicyclopentadienyl-iron](image)

Woodward discovered that the aromatic nature of cyclopentadienyl rings were similar to benzene to carry out electrophilic aromatic substitution reactions. This, and a number of other aromatic similarities of the Cp rings to the benzene moiety, led one of Woodward’s postdoctoral fellows, Mark Whiting, to coin the name ‘ferrocene’\textsuperscript{16}. The entire class of transition metal dicyclopentadienyl compounds became rapidly known as the ‘metallocenes’. Wilkinson and Fischer shared the Nobel Prize for Chemistry in 1973 for pioneering work in organometallic chemistry. The term “sandwich compound” for this compound is today universally accepted for a much wider class of compounds. The discovery and recognition of this new type of bonding between metals and organic unsaturated molecules gave organometallic chemistry a whole new lease of life.
1.2.1 The methods of synthesis of ferrocene

There are two main routes that are normally employed in the formation of ferrocene.\textsuperscript{17}

1.2.1.A Using a metal salt and cyclopentadienyl reagents

The synthesis starts with the ‘cracking’ of dicyclopentadiene through a retro Diels-Alder reaction to produce the monomeric cyclopentadiene (C\textsubscript{5}H\textsubscript{6}). Cyclopentadiene, a weak acid with pK\textsubscript{a} = 15, can be deprotonated by strong bases or alkali metals. Sodium cyclopentadienide (NaCp) upon reaction with ferrous chloride yields ferrocene (Figure 1.10).

\[ \text{FeCl}_2 + 2\text{NaC}_5\text{H}_6 \xrightarrow{180 \, ^\circ \text{C}} \text{NaCl} \rightarrow \text{[(C}_5\text{H}_5)_2\text{Fe}] \]

Figure 1.10 The ‘cracking’ of dicyclopentadiene and synthesis of ferrocene.

1.2.1.B Using a metal salt and cyclopentadiene

If the salt anion has poor basicity and cannot deprotonate cyclopentadiene, an auxiliary base can be utilized to generate the cyclopentadienyl anions \textit{in situ} which can sometimes be more convenient.

\[ \text{FeCl}_2 + 2\text{C}_5\text{H}_6 + 2\text{C}_2\text{H}_5\text{NH} \rightarrow \text{[(C}_5\text{H}_5)_2\text{Fe}] + 2[(\text{C}_5\text{H}_5)_2\text{NH}]\text{Cl} \]

1.2.2 Structure and physical properties of ferrocene

Ferrocene sublimes readily above 100 °C. It is soluble in most of the organic solvents. It is insoluble in, and apparently unattacked by, water, 10 % caustic soda and concentrated hydrochloric acid even at the boiling point. It dissolves in dilute nitric or concentrated sulphuric acid.
Table 1.2 Physical properties of ferrocene

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IUPAC name</td>
<td>bis(η^5-cyclopentadienyl)iron(II)</td>
</tr>
<tr>
<td>Molecular formula</td>
<td>C_{10}H_{10}Fe</td>
</tr>
<tr>
<td>Molar mass</td>
<td>186.04 g/mol</td>
</tr>
<tr>
<td>Appearance</td>
<td>light orange powder</td>
</tr>
<tr>
<td>Density</td>
<td>2.69 g/cm^3 (20 °C)</td>
</tr>
<tr>
<td>Melting point</td>
<td>174 °C</td>
</tr>
<tr>
<td>Boiling point</td>
<td>249 °C</td>
</tr>
<tr>
<td>UV-λ_{max}</td>
<td></td>
</tr>
<tr>
<td></td>
<td>326 nm (ε = 50)</td>
</tr>
<tr>
<td></td>
<td>440 nm (ε = 87)</td>
</tr>
</tbody>
</table>

Ferrocene can be crystallized from solution in the monoclinic, triclinic, and orthorhombic crystal modifications, depending on the temperature. Original X-ray diffraction studies indicated a staggered configuration of the rings with a molecular centre of symmetry (D_{sh}). However, gas-phase electron diffraction observations projected a D_{sh} geometry with C-C = 1.440(2) Å and Fe-C = 2.064(3) Å. It is known in literature that below 164 K, the triclinic form persists and show virtual D_s symmetry, the rings being rotated by about 9° from the eclipsed orientation. The room temperature monoclinic crystalline form is a disordered species, indicating a staggered conformation (D_{sh}), whilst in the orthorhombic form the rings are fully eclipsed (D_{sh}). Although the orthorhombic form is thermodynamically stable up to 242 K, crystallization of this phase from solution occurred in only at much lower temperatures (≤ 110 K).

It is readily oxidized to a blue cation [Fe(C₅H₅)₂]^+ (called ‘ferrocenium’), which is blue or green in dilute solution or blood red when concentrated. Upon oxidation there is a slight increase in Fe-C bond lengths and also within the rings, as is to be expected for removal of a bonding electron.
1.2.3 Chemical properties of ferrocene

The cyclopentadienyl rings are aromatic and, in general, most of the chemistry of ferrocene and its derivatives may be predicted on this basis. It is possible to carry out a variety of transformations on the cyclopentadienyl ligands due to its great stability and the ability to maintain the ligand-metal bonding under harsh condition.

1.2.3A General electrophilic substitution

Ferrocene is more reactive towards electrophilic substitution than benzene indicating that more electrons are readily available. In fact compared to benzene, ferrocene reacts $3 \times 10^6$ faster. It has been proposed that the electrophilic substitution takes place via three possible mechanisms. The electrophilic substituents ($E^+$) interact first with the weakly bonding electrons of the iron atom and then transfer to the Cp ring with $E$ in the *endo* (i.e. metal side) position which upon proton elimination gives substituted ferrocene (Route I) (Figure 1.12). Alternatively, it is thought that electrophilic attack takes places on the ring to the less hindered *exo* face of the ligand, and does not involve direct participation of the metal, which then loses a proton to give the product (Route II) (Figure 1.12). There is a third possible mechanism which involves addition of the electrophile to the *endo* face of the ligand but not via any metal interaction (Route III) (Figure 1.12).

![Figure 1.12 Proposed mechanisms for electrophilic substitution of ferrocene.](image)

In fact, each route is plausible and probably all do occur, but it has been suggested that the stereochemistry of the product of electrophilic substitution and the
kinetic features of the reaction are governed by the nature of the electrophile. In the
proton-exchange reaction, it seems that the \textit{exo} and \textit{endo} pathways are equally likely.

\textbf{1.2.3B Friedel-Crafts reaction}

Ferrocene readily undergoes Friedel-Crafts acylation reaction on one ring and
less readily on both the rings (Figure 1.13). It gives only one 1,1'-disubstituted
compound due to free rotation of two Cp rings. Ferrocene also undergoes Friedel-Crafts
alkylation on treatment with alkyl halide or alkenes. However, such reactions are not
synthetically useful because of side reactions and poor yields.

![Figure 1.13: Friedel-Crafts acylation of ferrocene.](image)

\textbf{1.2.3C Mannich reaction}

Ferrocene undergoes Mannich reaction as it demonstrates the reactivity of its
rings and resembles the reactive thiophene and phenol species rather than benzene which
does not undergo Mannich condensations.

![Figure 1.14: Mannich reaction of ferrocene.](image)

\textbf{1.3 Bioorganometallic Chemistry of Ferrocene}

The discovery of ferrocene and elucidation of its remarkable structure is arguably
the starting point for modern organometallic chemistry. Currently \textit{bioorganometallic
chemistry} is growing rapidly, networking classical organometallic chemistry to biology,
medicine, and molecular biotechnology.\textsuperscript{20-22} Ferrocene and its derivatives have numerous
medicinal applications. Ferrocene itself shows properties as an anti-aenemic or cytotoxic agent.\textsuperscript{23,24} It was found, by Neuse \textit{et al.}, that there is a great enhancement in activity of cytotoxic metal compounds including ferrocene when these were bound to polymers as pro-drugs.\textsuperscript{25} Conjugates of ferrocene with well-known drugs, antibiotics such as penicillins and cephalosporins, were reported.\textsuperscript{26} In addition, structural variations of established drugs with the ferrocenyl moiety were reported, such as the anti-malarial drugs chloroquine (termed ferroquine), quinine, mefloquine, artemisinin,\textsuperscript{27} ferrocenyl aspirin,\textsuperscript{28} and the anti-cancer drug tamoxifen to give ferrocifen.\textsuperscript{29}

The work presented in this thesis is mainly focused on the synthesis and characterization of conjugates in which ferrocene is covalently bonded to the biomolecule. The stability of the ferrocenyl group in aqueous, aerobic media, the accessibility of a large variety of derivatives, and its favorable electrochemical properties have made ferrocene and its derivatives very popular molecules for biological applications and for conjugation with biomolecules. Bioconjugates of ferrocene with amino acids and peptides, proteins, DNA, RNA, PNA, carbohydrates, and hormones have received significant attention. An attractive feature of ferrocene is that the two cyclopentadienyl (Cp) rings can rotate around the Fe atom, which can act as a ball bearing.\textsuperscript{30} Further, the vertical distance between the two Cp rings in ferrocene is 0.35 nm, which is similar to the distance between the stacked base pairs in DNA\textsuperscript{31,32} (Figure 1.15).

![Figure 1.15: (A) Distance between the stacked base pairs in DNA double helix (B) Interring spacing in ferrocene](image-url)
Bioconjugates of ferrocene with amino acid and peptides received considerable attention. Amino acids and peptides often assemble, by hydrogen bonding between individual molecules,\textsuperscript{33,34} into extended supramolecular three-dimensional structures.

The properties of such extended peptide networks are related to the molecular arrangement of the individual molecules and offer fascinating array of structures. The utilization of a ferrocene unit as a molecular scaffold is considered to be one approach to study the hydrogen bonding ability of various peptide strands (Figure 1.16).\textsuperscript{35} It's so because the inter-ring spacing of ferrocene is suitable for hydrogen bonding of the attached peptide strands. Hydrogen bonds play a crucial role in regulating the three-dimensional structure and function of biological systems. The utilization of self-assembling properties of short peptides, which possess chiral centers and hydrogen bonding sites, is considered to be a relevant approach to highly ordered molecular assemblies.

Conformational enantiomers based on the torsional twist about the Cp(centroid)-Fe-Cp(centroid) axis are possible in the case of the 1,1'-disubstituted ferrocene (Figure 1.17).\textsuperscript{36} These conformational enantiomers are easily interconvertable because of the low energy barrier of Cp ring twisting. The incorporation of peptide chains into a ferrocene scaffold is envisaged to induce conformational enantiomerization by restriction of the torsional twist through the intramolecular hydrogen bonding. This bioorganometallic chemistry is envisioned to provide not only a peptidomimetic base for protein folding, but also pharmacologically useful compounds, artificial receptors, asymmetric catalysts, and new materials with functional properties.\textsuperscript{37} Recently, significant efforts have been aimed at equipping non-covalent supramolecular peptide assemblies with redox active groups and giving them specific electric properties that may be exploited as biomolecular wires.

**Figure 1.16:** Schematic representation of ordered structure of peptides with molecular scaffolds.\textsuperscript{35}
In past, significant efforts have been directed at the side-chain-selective and covalent labeling of proteins with organometallic complexes. Various conjugates of ferrocene with proteins are known and such redox labeled proteins can be used as biosensors. The rationale behind the derivatization was to make direct electrochemical communication possible between protein and the electrode. The glucose sensor is one of the most important biosensors for glucose detection. This is vital not only in the field of biotechnology for fermentation process control but also in medical services.\textsuperscript{38}

Glucose oxidase (GOD) is a dimeric glycoprotein which oxidizes $\beta$-D-glucose to $\delta$-D-glucolactone via two-electron process which is sensitive and specific. The current generated from the biochemical reaction between the enzyme and glucose can be computed by measuring the consumed oxygen or hydrogen peroxide produced in the reaction.

\[
\begin{align*}
\text{GOD(FAD)} + \beta\text{-D-glucose} & \rightarrow \text{GOD(FADH}_2\text{)} + \delta\text{-D-glucolact} \\
\text{GOD(FADH}_2\text{)} + \text{O}_2 & \rightarrow \text{GOD(FAD)} + \text{H}_2\text{O}_2 \\
\text{GOD(FADH}_2\text{)} + 2\text{Fc-R}^+ & \rightarrow \text{GOD(FAD)} + 2\text{Fc-R} + 2\text{H}^+
\end{align*}
\]

However, the direct electrochemical measurement of the amount of oxidized product is hindered since the enzyme does not react with electrode surfaces directly and as there can be dissolved oxygen in the samples. A redox-active mediator can be used to facilitate the quantitative oxidation of glucose under catalytic conditions. The glucose oxidase enzyme, labeled with ferrocene derivatives (Figure 1.18), is commonly used in biosensors to detect levels of glucose by keeping track of the number of electrons passing through the enzyme by connecting it to an electrode and measuring the resulting charge.\textsuperscript{39,40} The use of ferrocene or its derivatives, as a mediator, avoid the effect of any dissolved oxygen (Figure 1.19).
D-Amino acid oxidase (DAAO) is a flavoprotein which catalyzes the oxidative deamination of D-amino acids. DAAO, isolated from two different sources, pig kidney and *Rhodoturula gracilis* (yeasts), were labeled with ferrocene derivatives. The addition of D-alanine to either conjugate resulted in an electrochemical response. A linear relationship was observed between the D-alanine concentration and the current, thus making the use of the modified enzymes in amperometric D-amino acid sensors possible.$^{41,42}$ Bovine serum albumin (BSA) is a protein which was first labeled with ferrocene derivative in 1969. Ferrocenyl-BSA conjugates were applied as efficient diffusing macromolecular mediators between the enzyme glucose oxidase and the electrode. The tetrameric protein avidin (isolated from egg white) is remarkable for its ability to bind up to four molecules of biotin with high affinity. These ferrocenyl avidin conjugates were used to construct an enzyme-based biotin sensor by immobilizing them on an electrode.

1.3.1 Ferrocene-nucleobase conjugates

Ferrocene-nucleobase conjugate is a relatively new and less explored field in bioorganometallic chemistry as compared to the ferrocene conjugates with amino acids.
and proteins described in previous section. Studies of interaction of ferrocene compounds with nucleobases can be categorized as (1) those involving Metal-Carbon bonds derived from carbon atoms of the biomolecule,\textsuperscript{43-45} (2) ferrocene fragments co-ordinated at N- and O- donor sites of the nucleobases,\textsuperscript{46} and (3) conjugates in which ferrocene is covalently bonded to the biomolecules with no direct interaction between biomolecules and metal ion. This last group of compounds is of interest as they represent a novel class of redox-active ligands with potential co-ordination chemistry.

Gautheron \textit{et al.} have reported the first synthesis of ferrocene derivatives of nucleosides in 1991.\textsuperscript{47} They used a variety of different Pd-catalyzed C-C coupling reactions to synthesize ferrocene derivatives of uridine, 2'-deoxyuridine, and adenosine derivatives\textsuperscript{47} (Figure 1.20). They carried out a reaction of TMS-protected 5-iodouridine, 5-iodo-2'-deoxyuridine, and 8-bromoadenosine with zirconylated compound Cp$_2$Zr(Cl)CH=CH-Fc (derived from ethynylferrocene and Cp$_2$Zr(Cl)H (Schwartz’s reagent)) and (C$_6$H$_5$CN)$_2$PdCl$_2$ as a catalyst. A reaction was also carried out with ethynylferrocene and 5-iodouracil, 5-iodouridine, 5-iodo-2'-deoxyuridine, and bromoadenosine giving 5-ethynylferrocenyluracil, 5-ethynylferrocenyluridine, 5-ethynylferrocenyldeoxyuridine, and ethynylferrocenyladenosine via Sonogashira coupling\textsuperscript{48} (Figure 1.21).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.20.png}
\caption{Ferrocenylated nucleosides with uridine (A), 2'-deoxyuridine (B), and adenosine (C).\textsuperscript{47}}
\end{figure}
Figure 1.21: (A) 5-ethynylferrocenyluracil, (B) 5-ethynylferrocenyluridine, (C) 5-ethynylferrocenyldeoxyuridine, (D) ethynylferrocenyladenosine, and (E) cyclized ferrocenyl derivative obtained via Sonogashira coupling.

Houlton \textit{et al.} have published another approach for synthesis of metal nucleobases derivatives.\textsuperscript{49} Trimethyl(ferrocenylmethyl)ammonium iodide \textbf{I} (Figure 1.22) was used as a convenient source of the ferrocenylmethyl cation in the reaction with various nucleobases and nucleobase derivatives. Complete range of cytosine, thymine, uracil, guanine, and adenine derivatives have been synthesized and characterized by X-ray analysis. X-ray single crystal analysis shows interesting intermolecular hydrogen bonding pattern in solid state for some of the compounds. Houlton \textit{et al.} synthesized 9-ferrocenylmethyladenine \textbf{II}.\textsuperscript{49a} The first synthesis of this compound was reported by Chen in 1980 who obtained a mixture of isomeric compounds, \textit{N}\textsuperscript{6}-ferrocenylmethyladenine and 7-ferrocenylmethyladenine.\textsuperscript{50} The X-ray single-crystal structure analysis of \textbf{II} reveals that the hydrogen bonding between the adenine rings are formed by combination of Watson-Crick (two hydrogen bonds, central) and Hoogsteen sites (peripheral, one hydrogen bond each) (Figure 1.23).

Figure 1.22: Synthesis of 9-ferrocenylmethyladenine\textsuperscript{49a}
An interesting application of hydrogen bonding involving nucleobases was reported by Inouye and Takase who synthesized ferrocene-modified artificial receptors and showed the strong binding of these designed receptors for dinucleotides. Thymidylyl$(3'\rightarrow5')$thymidine (TpT) was synthesized as a target dinucleotide. The molecular design of ferrocene-modified artificial receptor III (Figure 1.24) for TpT was based on the inter-ring spacing between two cyclopentadienyl (Cp) rings in ferrocene ($0.35 \text{ nm}$), which is almost the same as the distance between stacked base pairs in DNA. The receptor III (Figure 1.24) was synthesized by Sonogashira coupling of 1,1'-diiodoferrocene IV (Figure 1.24) with two equivalent of 2,6-diamido-4-ethynylpyridine V. An induced CD signal was observed when ferrocene moiety was associated with TpT. This induced CD signal can only be explained by a twisted orientation of the two Cp rings as consequence of strong hydrogen bonding to the chiral TpT molecule because the receptor III (Figure 1.24) is achiral. This is similar to the work of Hirao and Moriuchi, where hydrogen bonds between adjacent amino acids and peptides were
restricting the torsional twist of Cp rings on ferrocene, thus introducing an element of helical chirality.\textsuperscript{35} This is useful for highly selective extraction of the dinucleotides into nonpolar solvents by using the receptors. Although it is not clear how this principle can be extended to longer oligonucleotides, such selective receptors may certainly play a role in identification and purification of oligonucleotides in the future.

After our initial publication on X-ray crystal structure studies of ferrocene-nucleobase conjugates,\textsuperscript{52a} Verma \textit{et al.} reported the synthesis and crystal structure of an adenine-ferrocene conjugate\textsuperscript{52b} (Figure 1.25). The nucleobase forms a ribbon-like motif with the help of intermolecular hydrogen bonds. Hydrogen bonding, $\pi-\pi$ stacking and CH-$\pi$ interactions play an important role in the stability of the homoadenine tetrad structure, which facilitates ferrocenyl moieties to adopt an interesting spatio-temporal arrangement in the lattice superstructure.

![Figure 1.25: View of ferrocenylated adenine ribbons.\textsuperscript{52b}](image)

In view of the above literature, the work presented in this thesis involves synthesis and characterization of conjugates in which redox active ferrocene is covalently bonded to the self pairing nucleobases or DNA/RNA with a designed spacer. These could be useful building blocks in supramolecular chemistry wherein molecular recognition is coupled with electrochemistry, and may also lead to novel applications for the electrochemical recognition of DNA/RNA binding substrates.

1.3.2 Ferrocene-oligonucleotide conjugates

The main interest in ferrocene oligonucleotide derivatives is for electrochemical DNA sensors and is also important in studies on DNA mediated electron transfer. The
detection of specific DNA sequences using real-time methods has received increasing attention for applications in clinical diagnostics, food quality control, environmental protection and forensic science. Among the several detection techniques in literature, those based on radioactive isotopes is sensitive but unsafe due to their hazardous nature and short shelf life. Non-radioactive detection methods such as luminescence, fluorescence\textsuperscript{53} or quartz crystal microbalance measurements\textsuperscript{54} have been reported. The most common method of detection today is fluorescence, due to its high sensitivity and ease of handling both chemically and spectroscopically. Electrochemical detection offers an alternative means of detection due to their high sensitivity and the simplicity of the detection apparatus. Compared to fluorescence spectroscopy, electrochemical detection is probably more robust and less prone to errors. Therefore electrochemical methods have received particular attention in the development of inexpensive and compact devices. Electrochemical sensors can be built much smaller in size than fluorescence spectrometer. For all these reasons, there is a high interest in electrochemical DNA sensors.\textsuperscript{55} One of the approaches of screening the DNA hybridization with a complementary strand is based on the change of the electrochemical response of labeling DNA with redox active compounds. In this context, ferrocene has received particular attention due to its high stability in aqueous aerobic media and facilitating convenient synthetic chemistry.

There are several methods for synthesis of ferrocene-oligonucleotide conjugates. In one instance, Mitsunobu reaction of ferrocenyl methanol with 5'-\textit{O}-(4,4'-dimethoxytriphenylmethyl)-3'\textit{-O}-acetylthymidine gives product \textbf{VI}, which was readily converted to the phosphoramidite \textbf{VII} (Figure 1.26) which was used as a monomer in automated oligonucleotide synthesis of DNA.\textsuperscript{56}

In other work, Gautheron and coworkers used a variety of different Pd-catalyzed C-C coupling reactions to synthesize ferrocene derivatives of uridine, 2'-desoxyuridine, and adenosine derivatives\textsuperscript{47} (Figure 1.20). Sonogashira coupling of ethynylferrocene or related ferrocene derivatives with an ethynyl groups to iodouridin or bromoadenosine yields ferrocenyalted nucleosides\textsuperscript{48} (Figure 1.21), which in turn can be easily transformed into phosphoramidites and subjected to automated oligomer synthesis.
Yu et al. \(^5\) synthesized a new type of ferrocene-containing phosphoramidite where a ferrocenyl group was linked to the 2'-position of ribose ring such as VIII and IX (Figure 1.27). These compounds were the first ferrocenyl-RNA derivatives that may incorporated into an oligonucleotide strand at any position with possibilities for multiple ferrocenyl incorporation into the oligomer.

Ferrocenylated nucleotides were synthesized by direct coupling of active ester of ferrocenecaboxylic acid to an end tagged primary amine on the DNA oligonucleotide in solution\(^6\) (Figure 1.28). In all cases reported so far, the ferrocene label has been introduced at the monomer stage by metal catalyzed reaction (Sonogashira), and then employed in an automated solid-phase DNA synthesizer using phosphoramidite chemistry. Alternatively these may be introduced after the assembly of the oligomers sequence at the 5'-end.
Anne et al. have presented an interesting alternative for the labeling at the 3'-end of an oligonucleotide through a transformable 5'-monophosphate on a monomer.\(^9\) The phosphate group at 5'-end is of importance since it can be profitably exploited for diverse bioconjugations and extension modifications.\(^6\) Dideoxynucleotide triphosphate Fc-ddUTP \(X\) (Figure 1.21) was used as a building block to extend the 3' terminus of an oligonucleotide resulting in enzymatically 3'-end-labeling by presynthesized 5'-phosphorylated oligonucleotides. This leaves the 5'-end of oligonucleotides intact.\(^9\) (Figure 1.29).
Brisset \textit{et al.} have synthesized monofunctional ferrocene containing phosphoramidite group XI and new type of bifunctional ferrocene derivatives XII containing phosphoramidite and demithoxytrityl (DMT) groups (Figure 1.30). These ferrocenyl-phosphoramidites have been directly employed in an automated solid-phase DNA synthesizer using phosphoramidite chemistry for synthesis of ferrocene-labeled oligonucleotides, as potent markers of DNA hybridization, without the need for nucleotide chemistry. The advantage of this method is that it allows a non-specialist in nucleotide chemistry to access labeled oligonucleotides. Ferrocene labeled oligonucleotides, modified at the 3' and/or 5' extremities, with ferrocenyl moiety in the main backbone rather than in side chain was synthesized. It was demonstrated, that a ferrocenyl group at the 3' or 5' position has no drastic effect on the thermal stability of oligonucleotides. The 5' position appears to be more important due to the increase in the electrochemical signal for ferrocene arising only due to binding with the exact target, and hence important for applications in DNA chip technology.

\textbf{Figure 1.29}: (A) Ferrocenylated dideoxynucleotide triphosphate (Fc-ddUTP) (B) the enzymatic incorporation reaction of the Fc-ddUTP at the 3'-terminus of the oligonucleotide.\textsuperscript{59}

\textbf{Figure 1.30}: Structure of ferrocene phosphoramidites.\textsuperscript{61}
1.3.3 Applications of ferrocene-labeled DNA oligomers as gene sensors

Ihara et al.\textsuperscript{63} have demonstrated the detection of DNA and RNA at femtomole levels using HPLC, equipped with an ordinary electrochemical detector (ECD). Ferrocene-oligonucleotide \textbf{XIII} was synthesized by coupling of amino-terminated oligonucleotide with an activated ester of ferrocene-carboxylic acid\textsuperscript{54} (Figure 1.31). The electrochemically active probe DNA \textbf{XIII} was hybridized to the complementary DNA, and the conjugate was identified electrochemically by HPLC-ECD.\textsuperscript{63-65} In more practical case, the same probe was hybridized with a plasmid containing a choline transporter gene (CTG) fragment of 3693 bp in which the promoter of CTG fragment region contained one A\textsubscript{13} sequence, enabling the detection at concentration as low as 20 fmol.\textsuperscript{63}

![Figure 1.31 Structure of a ferrocene-labeled (dT)\textsubscript{12} oligonucleotide.\textsuperscript{63}](image)

The development of chip technology has modernized the genetic analysis. On a single chip, thousands of oligonucleotides of different sequences can be immobilized in a spatially addressable manner. A fluorescent signal is generated when complementary DNA or RNA binds to these oligonucleotides. For detecting hybridization process in a chip format with electrochemical techniques, the surface of the electrode should serve as a chip on which the oligonucleotides are immobilized. Letsinger et al.\textsuperscript{66} immobilized the ferrocenyalted oligonucleotides in a self-assembled redox-active monolayer on a Au surface. Ferrocenyalted thymidine monomer \textbf{XIV} (Figure 1.32) with 3'-thiol group was adsorbed on the surface of Au electrodes as a monolayer. The system exhibits a reversible redox signal due to the ferrocene label, which allowed the characterization of the modified surface.

Anne et al.\textsuperscript{59} have shown that single stranded DNA molecules on gold surfaces are very flexible and become significantly stiffened by hybridization of a complementary strand to form duplex DNA.\textsuperscript{59} The conformational changes lead to changes in the mobility of the ferrocenyl reporter group, which in turn changes its electron-transfer properties.\textsuperscript{67}
Ihara et al. have published a slightly different approach to an immobilized electrochemical gene sensor, using three oligonucleotide systems, in a “sandwich assay” (Figure 1.33). First, an oligonucleotide capture probe, possessing five successive thiophosphate groups was immobilized on an Au electrode surface. The target DNA strand was then hybridized to the immobilized capture probe through the presence of the complementary sequence. This was followed by the hybridization of a ferrocene-oligonucleotide to a complementary sequence on the single strand DNA target to result in a ternary complex. During this process the ferrocene was brought close to the electrode which resulted in a detectable electrochemical signal.

The amperometric detection of DNA was examined by Ihara et al. using redox-labeled nucleic acids as probes, e.g. ferrocene-labeled nucleic acids. Willner et al. extended this work through a bioelectrocatalytic amplification of the DNA detection process. The scheme for the generation of the redox-active DNA replicas and the amperometric amplified bioelectrocatalytic analysis of the gene is outlined in Figure 1.33.

Figure 1.32: 5’-Ferrocenyalted thymine monomer with a 3’-thiol group for immobilization on a Au surface.

Figure 1.33: Schematic illustration of principle of the “sandwich assay” for electrochemical DNA sensors.
1.34. Viral DNA interacts with a thiolated oligonucleotide assembled on an Au electrode. The thiolated monolayer of oligonucleotide is complementary to the cyclic viral DNA (M13Φ) and thus the viral DNA binds to the DNA film. The partially double-stranded assembly was then allowed to interact with a nucleotide mixture that includes the synthetic ferrocene tethered dUTP as a redox-label. In the presence of polymerase, Klenow fragment I, the viral DNA was replicated incorporating the ferrocene-modified DNA. This was confirmed by quartz crystal microbalance. With ferrocene unit as electron-transfer mediators between the electrode surface and glucose oxidase to convert glucose to gluconic acid, a bioelectrocatalytic amplification of the DNA detection process was achieved. This system provides a new aspect in DNA bioelectronics with an ability to form redox-active DNA replicas that may be activated through a bioelectrocatalytic cascades.

![Figure 1.34](image)

**Figure 1.34:** Polymerase induced generation of a redox-active nucleic acid replica for amplified detection of viral DNA and the bioelectrocatalyzed oxidation of glucose to gluconic acid by glucose oxidase (Gox).
1.4 Present Work

The preceding sections of this chapter give an overview on bioorganometallic chemistry of ferrocene with emphasis on bioconjugates of ferrocene with amino acids and peptides, proteins, DNA, and RNA. Ferrocene-nucleobase conjugates (conjugates in which ferrocene is covalently bonded to the nucleobases with no direct interaction between nucleobases and metal ion) is a relatively new and less explored field of bioorganometallic chemistry. These conjugates represent a novel class of redox-active ligands which shows an interesting intermolecular hydrogen bonding.

Strategies for synthesis of ferrocenyl-modified oligonucleotides have been discussed. The ferrocene-oligonucleotide conjugates have applications as gene sensors with electrochemical detection.

The work presented in this thesis involves the design, synthesis, characterization and X-ray crystallographic analyses of the redox active ferrocene-nucleobase conjugates with designed spacers. This is followed by synthesis of ferrocenyl-phosphoramidite monomer, its incorporation into oligonucleotides, characterization and biophysical evaluation of ferrocenyl-modified oligonucleotides. Electrochemical investigation of ferrocene-nucleobase conjugates and ferrocene-oligonucleotide conjugates have been done using cyclic voltammetry.

Chapter 2 describes the construction of a series of new ferrocene-linked mono and bis-nucleobase conjugates with different spacer. A complete series of thymine, uracil, 5-bromouracil and adenine derivatives (mono and bis), with n-butyl and methylene spacer was synthesized (Figure 1.35) and comprehensively characterized, by X-ray analysis.

![Figure 1.35: Ferrocene-linked mono and bis-nucleobase derivatives. T = thymine, U = uracil, (Br)U = 5-bromouracil, A = adenine](image-url)
The synthesis of chimeric mixed base ferrocene derivatives (Figure 1.35) and X-ray crystallographic analyses are presented. The newly designed ferrocene-linked mono and bis-nucleobase conjugates show different supramolecular assemblies mediated via centrosymmetric reverse Watson-Crick (rWC) base pairings.

Chapter 3 describes the synthesis and characterization of bisfuncional ferrocene containing phosphoramidite and dimethoxytrityl (DMT) groups XV (Figure 1.36) useful for incorporation in an automated solid-phase DNA synthesizer and for synthesis of 3’ and/or 5’ modified oligonucleotides. Electrophoretic gel shift assay was used to establish the binding of different ferrocenyl modified oligonucleotides to the complementary DNA. The thermal stability of double strand oligonucleotides was studied by its melting temperature ($T_m$).

Chapter 4 deals with the electrochemical studies of ferrocene of mono and bisfunctional ferrocene-nucleobase conjugates using cyclic voltammetry in non-aqueous media. The redox behavior of the systems is understood in terms of the quantitative functional parameters derived from experimental electrochemical data, which is
correlated with their chemical structure. This is followed by electrochemical investigation of ferrocene-oligonucleotide conjugates using steady state voltammetry. The electrochemical properties of these ferrocene-oligonucleotide conjugates were analyzed before and after hybridization with different oligonucleotides. The ability of using these ferrocenyl-labeled oligonucleotides in DNA sensors has been demonstrated.

1.5 References


