1.1 Role of Cu in Biology:

Copper is the third most abundant metals in the earth’s crust and essential metallic element in the human body followed by iron and zinc [1]. Copper in complexes exists in oxidation state Cu (I) and Cu(II). Cu (III) is generally regarded as uncommon but now days it has received importance because of its involvement in many biological processes [2]. In human body more copper is found in the brain and heart than in any other tissue except for liver, where it is stored as copper thionein and released as ceruloplasmin or in the form of a complex with serum albumin. The high metabolic rate of the heart and brain requires relatively large amounts of copper metalloenzymes including tyrosinase, cytochrome C oxidase, dopamine-β-hydroxylase, pyridoxal – requiring monoamine oxidases, and Cu-Zn superoxide dismutase [3]. Copper deficiency leads to brain disease in infants, anaemia (since cytochrome oxidase is required for blood formation), and heart disease.

Copper also has antibacterial properties. Copper in trace quantities is required by all living organisms to maintain cellular function [4, 5, 6]. Excessive copper is extremely toxic due to its chemical reactivity. Cu in whole blood is distributed in several functions between the erythrocytes and plasma. Cu is transported through blood as complexes of ceruloplasmin (95%), albumin (5%) and low molecular weight copper complexes [7, 8]. It is predicted that Cu can be absorbed from the stomach and from the entire small intestine [9, 10]. The low molecular weight Cu complexes play an important role in facilitating the transport of copper across cell membranes in various tissues. Abnormally high Cu levels in the serum of rheumatoid arthritis patients have been recorded. For intracellular Cu, liver is the major store house.

In biology Cu is a micronutrient that plays an essential role in serving as a cofactor for many enzymes that include Cu-Zn superoxide dismutase, cytochrome C oxidase, ceruloplasmin and dopamine β- hydroxylase that modify neuropeptides, mobilize ions, coagulate blood, and crosslink connective tissue [11, 12]. Human and animal genetic diseases including Menkes disease and Wilson’s disease underscore critical roles for Cu absorption and distribution [13, 14]. In case of patients suffering from Menkes disease entrapment of Cu in intestinal cells leads to Cu deficiency as ascertained by defects in the activities of Cu containing enzymes. Patients of Wilson’s
disease accumulate Cu in liver, resulting in liver cirrhosis and neurodegeneration. Wilson’s disease results from genetically inherited metabolic defects in which copper can no longer be tolerated at normal levels. The clinical manifestations are liver disease, neurological damage, and brown or green rings in the cornea of the eyes. Patients suffering from Wilson’s disease have low levels of Cu storage protein ceruloplasmin; the gene and gene product responsible for altered metabolism have not yet been identified. But Wilson’s disease offers an excellent opportunity for modern methodologies to isolate and clone the gene responsible for this altered metabolism, ultimately providing a rational basis for treatment [15].

Cu is required for the normal functioning of plants, animals and most microorganisms. It is incorporated into variety of organics which perform specific metabolic function. As it is an essential metal, daily dietary requirement is necessary for all animals [16]. Moreover Cu is used in determining its biological availability, both in environment to control the growth of organism and in food.

In case of human being Cu exhibits considerable biochemical action either as an essential trace metal or as a constituent of various exogenously administered compounds. In its former role it is bound to ceruloplasmin, albumin, and other proteins, while in its latex it is bound to ligands of various types forming complexes that interact with biomolecules, mainly proteins and nucleic acid. The multifaceted role of Cu in biological systems is demonstrated by several studies. In particular the involvement of Cu in human diseases has been described from a medicinal, chemical and biochemical view [17, 18] focusing on the molecular physiology of Cu transport. A lot of function have been proposed to account for the homeostasis of inorganic noncomplex Cu in humans have been described [19,21] but only a limited number of review studies have focused on the multiple biochemical events which could be directly implicated in the use of Cu complexes in medicine.

Current interest in Cu complexes is due to their potential use as antimicrobial, antiviral, anti-inflammatory, antitumor agents, enzyme inhibitors, or chemical nucleases. Markedly, the biochemical action of Cu complexes with non steroidal anti inflammatory drugs (NSAIDs) has been studied [22]. Numerous Cu (II) complexes of NSAIDs showing enhanced anti inflammatory and antiulcerogenic activity, as well as reduced gastro intestinal toxicity compared to the uncomplexed drug, have been
prepared and structurally characterised [22]. They comprise a class of potential anti-inflammatory drugs with reduced side effects and their mode of action is attributed to their marked superoxide – dismutase (SOD) -mimetic activity. Other studies have concentrated on the potential chemotherapeutic properties of Cu based compounds [23, 24]. It was found that the infectivity of influenza A virus is reduced after exposure on copper surfaces [25]. In addition, the study and development of Cu complexes could be helpful in the design and production of antiviral and antibacterial materials, able to deactivate HIV or H1N1 viruses [26] and antibiotic resistant bacteria respectively.

Superoxide dismutase activating drugs have been used against inflammation for diseases such as rheumatoid, arthritis and Crohn's disease. Cu complexes with SOD activity can be used as cancer therapy. Cu appears to play an important role in immune responsiveness in animals, including humans [27] although the exact nature of that role is not yet fully understood [28]. Copper deficiency is reported to change regulatory mechanisms governing thrombosis and inflammation [29]. Certain copper-containing enzymes and organics like haemocyanin and Cu-DIPS [30] appear to act as an antioxidant defense system in animals [31, 32]. As reported earlier [33, 34] the role of copper in enzymes like cytochrome c oxidase is not fully understood although the metal may be essential for enzyme activity. Immune response may also be affected by direct or indirect action of copper on metabolism. Copper-deficiency may affect the physiological response of blood platelets to thrombin or correct lipid metabolism [35]. It may also be involved in the repair of components of nucleic acids [36] and affect the activity of a human plasma copper-binding growth factor [37]. Cu is required in the body for haemoglobin synthesis, growth, keratinisation, pigmentation, bone formation, reproduction, fertility, development and function of the central and peripheral nervous systems, cardiac function, cellular respiration, nerve function and mental and behavioural development, amongst others. The roles of Cu are based upon the requirement of coordinated copper at the active site of the Cu dependent enzymes. It is reported that copper complexes have the antitubercular activity and the complex Sodium3- (allylcuprothioureido)-1- benzoate is one of the more useful complex for this type of activity [38].

It is well known that Cu (II) complexes of inactive ligands and anti-inflammatory organic drugs are generally more active than the free ligands or organic
drugs themselves [39]. It has been suggested that the biological activity of acetylsalicylic acid (Aspirin) is due to its activity to form metal complexes and that the active form of this drug intact, a copper complex of aspirin \([\text{Cu}_2(\text{O}_2\text{C}_6\text{H}_4\text{OCOCH}_3)_4]\) [40] has been found to be more effective than aspirin itself as an anti-inflammatory agent. In addition, copper complex has also anticellular activity. \([\text{Cu}_2(\text{O}_2\text{C}_6\text{H}_4\text{OCOCH}_3)_4]\) has been found to be effective in the treatment of rheumatoid disorders, and it decreases tumour growth. The pyridine adduct \([\text{Cu}_2(\text{O}_2\text{CC}_6\text{H}_4\text{OCOCH}_3)_4(C_6\text{H}_5\text{N})_2]\) has also been found to be an effective anti-inflammatory, anticancer and anticonvulsant agent [41].

The potential of copper carboxylates as pharmacologic compound has prompted research into their physico-chemical properties. It is structurally characterised [42] that the mononuclear copper (II)bis(pyridine)acetylsalicylatocomplex \([\text{Cu}(\text{O}_2\text{CC}_6\text{H}_4\text{OCOCH}_3)_2(C_6\text{H}_5\text{N})_2]\) which has been found to be an effective anti-inflammatory, anticancer and anticonvulsant agent [41].

1.2 Mono and bi metallic Cu Proteins:

Cu proteins are proteins those contain one or more copper ions as prosthetic groups. Copper containing proteins perform a wide range of important functions in biological systems. Many are involved in the activation of dioxygen for the
functionalization of organic substrate. The metal centres in the Cu proteins can be classified into different ways.

**Class** I copper centres (C1Cu) are characterized by a single copper atom coordinated by two histidine residues and a cysteine residue in a trigonal planar structure, and a variable axial ligand. In one type of class I copper protein like amicyanin, plastocyanin and pseudoazurin the axial ligand is a methionine, whereas aminoacids other than methionine like glutamine give rise to type II C1Cu copper proteins. Amicyanin is a type of Cu protein that is the natural electron acceptor for the quinoprotein methylamine dehydrogenase (MADH). Azurins contain the third type of C1Cu centres: besides a methionine in one axial position, they contain a second axial ligand (a carbonyl group of a glycine residue). Azurin is widely studied as a model electron transfer protein (Cu (II) →Cu (I)), in particular with respect to the coordination of the Cu ion. In case of plastocyanin, the copper is situated in a flattened tetrahedron of essentially C$_{3v}$ symmetry, “half way” between the two idealized geometries [43]. This facilitates electron transfer compared to a system that might be at the tetrahedral extreme or at the square planner extreme.

C1Cu-containing proteins are usually called "cupredoxins", and show similar three-dimensional structures, they show strong absorption near 600 nm, which usually gives rise to a blue colour. Cupredoxins are therefore often called "blue copper proteins". When studied by EPR spectroscopy, C1Cu centres show small hyperfine splittings in the parallel region of the spectrum compared to common copper coordination compounds.

**Class** 2 copper centres (C2Cu) are binuclear centres consisting of two copper atoms, each coordinated by three histidine residues. These proteins exhibit no EPR signal due to strong antiferromagnetic coupling (i.e. spin pairing) between the two S = 1/2 metal ions due to their covalent overlap with a bridging ligand. These centres are present in some oxidases and oxygen-transporting proteins like hemocyanin and tyrosinase. Hemocyanins are respiratory proteins containing two copper atoms that reversibly bind a single oxygen molecule (O$_2$). The O$_2$ transporting protein hemocyanin (He) found in molluscs and arthropods [44], and the enzyme tyrosinase (Tyr) which catalyses the ortho hydroxylation of phenols to catechols and the further oxidation of these molecules to O-quinines [45]. Binuclear Copper centres are present in nitrous-
oxide reductase [46, 47] and cytochrome c oxidase [48-50]. The two copper atoms are coordinated by two histidines, one methionine, protein backbone carbonyl oxygen, and two bridging cysteine residues. Cytochrome c oxidase is one of the important proteins which act as the terminal enzymes of respiratory chains.

1.3 Importance of Cu (II) compounds as enzyme:

Enzymes are the catalysts of biological systems. Proteins and enzymes have binuclear metallocites in which the metals are essential for the biological activity [51-52]. Enzymes not only control the rate of reactions but, by favouring certain geometries in the transition state, can lower the activation energy for the formation of one product rather than another. The basic structure of enzymes is built of proteins.

The importance of Cu as an essential element can be estimated by the wide range of Cu proteins and enzymes playing different roles in biological systems [53]. Copper active sites play a major role in enzymatic activation of dioxygen. Cu forms a large number of metalloproteins and detailed understanding of their structure is the subject of considerable interest in recent years. In the last decades many bioinorganic studies were developed on mimetic complexes of Cu-dependent proteins, in order to verify the interrelations between structural and functional properties of active Cu centers [54]. Among the most studied Cu ion ligand, dimine compound have deserved special attention due to their flexibility, feasibility of preparation, and ability to stabilize both oxidation states of this metal [55].

The Cu is an essential micronutrient for feeding and a co-factor of several enzymes involved in oxidative metabolism like β-hydroxylases, quercetinase, ceruloplasmine, cytochrom oxidase, monoaminoxidase, superoxydismutase, ascorbic acid oxidase and tyrosinase [56, 57, 58]. The catalytical role of these enzymes is a two-step process, i.e., the reduction of Cu^{2+} ion to Cu^{+} and the fixation of molecular oxygen [59]. The rutine-Cu (II) complex shows the higher activity as antioxidants and antifree radical agents than free rutine [60, 61].
1.3.1 Mononuclear Cu complexes as models of enzymes:

Mono nuclear Cu enzymes play an important role in biology and their functionality is based on Cu (II)/ Cu (I) redox processes. Cu enzymes such as dopamine β-monooxy genase, peptidylglycine α-hydroxylating monooxygenase, galactose oxidase, and nitrate reductase present a mononuclear Cu ion buried in their active pocket opened to the external medium through a selective substrate access channel. Modelling a mononuclear site remains a challenge for a better understanding of its intrinsic reactivity [62]. A good chemical model for metelloenzymes is a key to the understanding of the fundamental mechanisms involved in the catalytic cycle and to the design of efficient and selective new tools for the synthetic chemist.

Latif Abuhijlesh et al. reported four mono nuclear Cu (II) complexes as catecholase –mimetic activity [63]. Those were prepared by allowing Cu(II) aspirinate to react with benzimidazole, 2-methylbenzimidazole, metronidazole or 2-methyl-5-nitrobenzimidazole ligands [63]. Harry Adams et al. synthesised a crystal of {2-[ bis(2-pyridylethyl) aminomethyl) phenolato} Cu(II) perchlorate towards a model for galactose oxidase [64]. S. Itoh reported active site models for galactose oxidase and related enzymes [65]. Mononuclear copper complexes of the type [Cu(L1)X][H(L1)]= 2-(bis(pyrid-2- ylmethyl)aminomethyl)-4-nitrophenol, X=Cl, NCS, CH₃COO, ClO₄ was reported by Mathuboothan Vaidyanthan and Mallayan Palaniandavar [66]. Tobias Kruse et al. reported few mono and dinuclear (o- thioetherphenolato)- copper (II) complexes as a structural model for galactose oxidase [67]. Mononuclear Cu (II) superoxo complexes using a tridentate ligand L(X), [1-(2p-X- phenethyl)-5- (2-pyridin-2-ylethyl)-1, 5- diazocyclooctane,X=CH, H, NO was also reported [68].

1.3.2 Binuclear Cu complexes as active centre analogous of Cu protein:

Proteins and enzymes have binuclear metallocites in which the metals are essential for the biological activity [69, 70]. The enzymes like hemocyanin, tyrosinase, and catecholase have binuclear copper centers in their active sites [71, 72]. Though these proteins have same binuclear copper centers, the functions of their centre atoms are different. The binuclear copper complexes have attracted immense attention with the
onset of the studies of metalloproteins. So far a number of binuclear Cu complexes have been synthesised and studied as a model of enzyme. S. Parimala et al. had reported a new series of binuclear Cu (II) complex of binucleating ligands L (L-, N'-R- bis (methyl-N- (2-pyridinyl) ketoacetamide) where R= ethylene, 1,3-propylene, o-phenylene [73]. The type of binuclear copper (II) complexes were [Cu₂L] where X=ClO₄⁻ and [Cu₂LX₂] where X=Cl⁻and Br⁻. Catecholase activities of these complexes were also observed. R. N. Patel et al. had synthesised binuclear Cu-Cu and Cu-Zn imidazolate- bridged complexes as an effective model for active site of SOD [74]. Few dinuclear Cu (II) complexes with variable endogenous and exogenous bridge having catecholase activity were reported by Jhumpa mukherjee et al. [75]. P. Akilan et al. had synthesised binuclear copper (II) complexes using new macrocycling tricompartmental unsymmetrical ligands to study the catecholase activity of the complexes [76]. R.N.Patel et al. also had reported novel Copper (II)- dien-imidazole/ imidazolate bridged Cu(II) complex [(dienCu(µ-im)CU(dien))(ClO₄)₃ as homonuclear model for the Cu (II) site of the CuZn-superoxide dismutase [77]. New macrocyclic binuclear Cu (II) complexes were synthesized by S. Anbu et al. to study the catalytic activity like catecholase activity and DNA binding and cleavage [78].

1.4 Superoxide dismutase (SOD) and active centre models of Superoxide dismutase:

1.4.1 General aspects:

In biological systems, Superoxide dismutase (SOD) is an antioxidant enzyme known to protect cells from the toxic effects of superoxide ion by its dismutation into dioxygen and hydrogen peroxide [79].

\[ 2O_2^- + 2H^+ \rightarrow O_2 + H_2O_2 \]

SOD is also called a metalloenzyme as it contains metal ions in addition to amino acids. The two families of metalloproteins i) CuZnSOD found in almost all eukaryotic cells and very few prokaryotes [80-82] and ii) the manganese and iron
superoxide dismutases, MnSOD and FeSOD, the former found in the mitochondria of eukaryotic cells and both found in many prokaryotes [83].

Recent studies of bacterial [84] and yeast [85] mutants that were engineered to contain no superoxide dismutase demonstrated that the cells were unusually sensitive to dioxygen and that the sensitivity to dioxygen was relieved when an SOD gene was reintroduced into the cells. These results indicate that the superoxide dismutase enzymes play a critical role in dioxygen metabolism, but they do not define the chemical agent responsible for dioxygen toxicity. In both living and nonliving organisms the reactive radical anion superoxide ion is formed by the one electron reduction of oxygen during numerous oxidation reactions under normal conditions. The oxygen is potentially dangerous for all cellular macromolecules because it is a very good reducing agent in the anionic form and a very good oxidizing agent in the protonated form, also it can generate other undesired reactive species. It has been suggested that superoxide and related free radical may contribute significantly to sustaining chronic inflammation by promoting connective tissue degradation. A number of studies that were done during recent years indicated the imbalance or deficiency of such antioxidant enzymes could lead to several disorders including diabetes, ischemia, cataract, Parkinson’s disease, cancers and many others.

Copper, Zinc superoxide dismutase (Cu, Zn-SOD) is believed to protect the cells against oxidative damage and inflammation due to toxic oxygen intermediates by the catalytic dismutation of superoxide radicals to oxygen molecule and hydrogen peroxide [86]. During the catalytic cycle the copper in the active site is alternately reduced and oxidised according to the scheme (1.1) below.
1.4.2 Structure:

The X-ray crystal structure of the oxidised form of CuZnSOD from bovine erythrocytes shows a protein consisting of two identical subunits held together almost entirely by hydrophobic interactions [80-82]. Each subunit consists of a flattened cylindrical barrel of β-pleated sheet from which three external loops of irregular structure extend (Fig.1.1) The metal-binding region of the protein binds Cu\textsuperscript{II} and Zn\textsuperscript{II} in close proximity to each other, bridged by the imidazolate ring of histidyl side chain (Fig.1.2). The Cu\textsuperscript{II} ion is coordinated to four histidyl imidazoles and water in a highly distorted square–pyramidal geometry with water at the apical position. The Zn\textsuperscript{II} ion is coordinated to three histidyl imidazoles (including the one shared with copper) and an aspartyl carboxylate group, forming a distorted tetrahedral geometry around the metal ion. One of the most unusual aspects of the structure of this enzyme is the occurrence of the bridging imidazolate ligands, which holds the copper and zinc ions 6Å apart.

The role of the Zn ion in CuZnSOD appears to be primarily structural. There is no evidence that water, anions, or other potential ligands can bind to zinc, so it is highly unlikely that superoxide could interact with that site. Moreover, removal of zinc under conditions where the copper ions remain bound to the Cu site does not significantly

\[
\text{Scheme 1.1}
\]

\[
\begin{align*}
\text{O}_2 & \quad \text{ECu}^{+} \quad \text{ECu}^{2+} \\
\text{ECu}^{2+} & \quad \text{O}_2^{-} + 2\text{H}^{+} \\
\text{O}_2^{-} & \quad \text{H}_2\text{O}_2
\end{align*}
\]
diminish the SOD activity of the enzyme.

The Cu site is the site of primary interaction of superoxide with the protein. The X-ray structure shows that the copper ion lies at the bottom of a narrow channel that is large enough to admit only water molecule, small anions and small ligands. In the lining of the channel is the positively charged side chain of an arginine residue, 5Å away from the copper ion and situated in a suitable position so that it could interact with superoxide anions when they bind to copper. The two positively charged lysine residues at the surface of the protein attract anions and guide them into the channel [87]. The SOD activity can be considerably reduced by changing glycine or arginine residues chemically.

Fig.1.1 Schematic drawing of the polypeptide backbone of one of the two subunits of bovine CuZnSOD
1.4.3 Enzymatic Activity:

It has been observed that several metal complexes catalyze superoxide disproportionation; in fact aqueous copper ion is, Cu$^{2+}$ in [Cu(H$_2$O)$_6$]$^{2+}$, is an excellent SOD catalyst, comparable in activity to CuZnSOD itself [88]. But free aqueous Cu$^{2+}$ is not suitable for use as an SOD in vivo because of its high toxicity and binding ability to a large variety of cellular components. Two mechanisms have been proposed for catalysis of superoxide disproportionate by metal complexes and metalloenzymes [88].

Fig.1.2 Structure of the active site of bovine CuZnSOD
Mechanism I:

\[ M^{n+} + O_2^- \rightarrow M^{(n-1)+} + O_2 \]
\[ M^{(n-1)+} + O_2 \rightarrow M^{n+}(O_2^{2-}) \xrightarrow{2H^+} M^{n+} + H_2O_2 \]

Mechanism II:

\[ M^{n+} + O_2^- \rightarrow M^{n+}(O_2^-) \]
\[ M^{n+}(O_2^-) + O_2^- \rightarrow M^{n+}(O_2^{2-}) \xrightarrow{2H^+} M^{n+} + H_2O_2 \]

In mechanism I, which is favoured for SOD enzymes and most redox active metal complexes with SOD activity, superoxide reduces the metal ion in the first step, and then the reduced metal ion is reoxidised by another superoxide, presumably via a metal–peroxo complex intermediate. In Mechanism II, which is proposed for nonredox metal complexes but may be operative in other situations as well, the metal ion is never reduced, but instead forms a superoxo complex, which is reduced to a peroxo complex by a second superoxide ion. In both mechanisms, the peroxo ligands are protonated and dissociate to give hydrogen peroxide.

1.4.4 Superoxide Dismutase (SOD) activity:

It is known that studies of metal–substituted derivative helped in the evaluation of mechanistic possibilities for the enzymatic reaction. Moreover studies of such derivatives have provided useful information about the environment of the metal–ion binding sites. Metal–ion substituted CuZnSOD have been prepared with Cu\textsuperscript{II}, Cu\textsuperscript{I},
Zn$^{II}$, Ag$^+$, Ni$^{II}$ or Co$^{II}$ bound to the native Cu site, and with Zn$^{II}$, Cu$^{II}$, Cu$^+$, Co$^{II}$, Hg$^{II}$, Cd$^{II}$, Ni$^{II}$, or Ag$^+$ bound to the native Zn site [80,81,89]. The derivatives with Cu in Cu site have high degree of SOD activity, whereas the nature of the metal ion in the zinc site or even its absence has little or no effect.

### 1.4.4.1 Assay of SOD activity:

Many assays for superoxide dismutase activity have been devised [90]. SOD - dependent inhibitor of the reduction of cytochrome C was the basis for the discovery of the enzyme and this assay is still widely used [91]. The specificity of this assay relies on the inhibition by SOD: superoxide independent cytochrome C reduction is also possible, but is unaffected by SOD.

Another widely used assay is based on the reduction of the chromogenic reagent nitro blue tetrazolium (NBT). NBT is yellow but upon reduction, it yields a pigment called formazon, which is purple blue coloured [92]. The pigment is insoluble; it precipitates and forms a stable coloured band in a polyacrylated gel. For detection of SOD, the chromogen is incubated with a superoxide generating system and enzyme activity is measured by the inhibition of colour formation [93]. In our research we mainly used NBT assay method for the measurement of SOD activity.

To directly assay the SOD activity, we need to initially generate high concentration of O$_2^-$ and follow its decay by measuring the absorbance change in the absence and in the presence of a tested compound. The measurement of SOD was carried out using NBT assay method where KO$_2$ –DMSO solution was the source of superoxide ions. The formation of formazon dye (MF$^+$) was indicated by an intense blue colour, which was immediately measured at 560 nm against an appropriate blank. The unit SOD activity (IC$_{50}$) was measured as the concentration of the test substance causing 50% inhibition of KO$_2$ –DMSO mediated formazon dye formation. The 100% of superoxide activity corresponds to any assay performed in the absence of complex. In order to determine the conc. of the complex required to yield 50% inhibition of the reaction, we plotted the percentage of inhibition against the molar concentration of the complex.
1.4.5 Importance of SOD models:

Study of a model compound of SOD is important when searching for the relationship between functions and structures of enzymes. Furthermore, SOD model compounds have potential for therapeutic usefulness. Although many SOD model compounds have been reported, their structures are quite different from those of the native enzyme. Unfortunately, many problems such as half-lifetime and antigenicity have not been overcome even though several copper (II) complexes are known to show SOD activity. Active oxygen species such as superoxide (O$_2^-$) from various components of the cellular electron transport chains, and provided during the respiratory burst of phagocytic cells, have been implicated both in the aging process and in degenerative diseases, including arthritis and cancer. Low molecular weight metal conjugates of SOD provide advantages over the natural enzymes in respect of their ability in crossing the cell membranes, offering no immunogenicity, possessing longer life-time of the active forms, possibility of oral administration and comparative low cost [94]. Since copper acts as the active center in CuZnSOD, a number of Cu complexes have been designed and synthesized as SOD model complexes to provide valuable insights into the structure and catalytic function of the active site. Since the dismutation of reaction involves the redox cycle of Cu(II) and Cu(I), it is reasonable to expect that the redox potential of Cu(II) /Cu(I) couple of the complex can influence the SOD -like activity, while the ligand of the complex determines the redox potential [95]. It is known that SOD cannot penetrate across cell membranes and it can be rapidly cleared by the kidney, the therapeutic use of SOD is limited. So, it is important to find SOD mimics, which have the activity of SOD and at the same time are stable, non toxic and capable of crossing cell membrane. Two kinds of SOD mimics are there- one is metal dependent and other is metal independent. Our work focuses on metal dependent mimics.

1.4.5.1 The synthetic SOD model compounds:

So, far a good number of Cu complexes have been synthesized as SOD model compounds in order to minimize the toxic effect of this native enzyme as well as to
study the structure and catalytic function of CuZnSOD active site.

Clandine Amar et al. had reported one histidine containing Cu (II) dipeptide complex with very high SOD activity [96]. Bhirud et al. had synthesised several adducts of Cu(II)₂(aspirinate)₄ of formula [Cu(II)(aspirinate)₂L₂], where L is pyridine, nicotinamide, 3-picoline, 4-picoline, imidazole, 1-methyl- imidazole, diethylamine and dimethyl sulfoxide and observed their SOD activity. They found that the pyridine adduct of Cu (II)( aspirinate)₄ had higher activity than other compound they prepared [97]. Z. Durackova and J. Labuda had reported superoxide dismutase mimetic activity of macrocyclic C(II) -tetrahydroaminobenzaldehyde (TAAB) complex. The IC₅₀ value of the synthesised complex was 0.144µM [98]. Atanu Barik et al. Reported one new copper(II) curcumin complex as superoxide dismutase mimic having IC₅₀ value 166 ±12 Nm [99]. Compounds having SOD mimics also include ketones of the type, [Cu(apy)L] (ClO₄), (where apy= 2-acetyl pyridine and L=N-ethylen(2-acetylpyridineimine)) and [Cu(appn)] (ClO₄)₂·H₂O and {[Cu(bppn)](ClO₄)₂}₂ (where appn=N,N_-propylenebis(2-actoylpyridineiminate) and bppn=N,N_-propylenebis(2-benzoypyridineiminato)) [100]. Tappi et al. had reported one of the most successful SOD mimic imidazole bridged copper complex, [Cu₂(bpzbiap)Cl₃] where (bpzbiap) is 1,5 bis (1-pyrazoly)-3-[bis(2-imidazolyl) methyl] azopentane. This compound has a close resemblance to native Cu–Zn–SOD [101]. Ohtsu et al. have recently described a series of imidazolatebridged Cu(II)–Zn(II) heterodinuclear and Cu(II)–Cu(II) homodinuclear complexes containing dinucleating ligands like 4,5-bis(di(2-pyridylmethyl)aminomethyl)imidazole, which have been found to possess higher SOD activities[102]. F. Saczewsk et al. have described the synthesis of four SOD active compounds,Dichloro[1-(4,5-dihydro-1H-imidazol-2-yl)-3,5-dimethyl-1H-pyrazole N,N]copper(II) ( IC₅₀ =.10µM), Dichloro[1-(4,5-dihydro-1H-imidazol-2-yl)-3,5-dimethyl-1H-pyrazole N,N]copper(II)(IC₅₀.,13µM), Dichloro[1-(4,5-dihydro-1H-imidazol-2-yl)-1Hbenzotriazole-N,N]copper(II)(IC₅₀ =.59µ M)and Dichloro[2-(4,5-dihydro-1H-imidazol-2-yl)-1Hbenzotriazole-N,N]copper(II)(IC₅₀=.06µM)[103] . Out of which the SOD activity of the fourth compound is comparable to those of the most potent, low molecular weight SOD mimics reported till date [104]. It is also reported that the Cu (II) complexes with Schiff base ligands of salicylaldehyde semicarbazone has SOD activity which could be tuned by heterocyclic bases pyridine and N-methyl
R. N. Patel introduced one Cu\textsuperscript{II}-Cu\textsuperscript{II} imidazolate bridged complex [(salala) Cu-Im-Cu(salala)]Na having IC\textsubscript{50} value 23µ mol dm\textsuperscript{-3} [106]. More recently, Li et al. have described Cu(II)–Zn(II) complex containing a macroyclic ligand with two hydroxyethyl pendant arms which can catalyze the dismutation of superoxide with high efficiency [107]. S. Dutta et al. have synthesized and characterized copper–oxaprozinate complex having IC\textsubscript{50} value 10.1x10\textsuperscript{-6} M [108]. 

S. Dutta et al. have recently synthesized three compound Cu\textsubscript{2}{bis(3,6 pyrazole-1-yl)pyridazine}{(cnge)OH(NO\textsubscript{3})\textsubscript{2}(H\textsubscript{2}O)}\textsubscript{2} .NO\textsubscript{3} (1) [Cu\textsubscript{2}{ bis(3,6 pyrazol-1-yl)pyridazine }{(cnge)OH (NO\textsubscript{3})\textsubscript{2}(H\textsubscript{2}O)\textsubscript{3} } .NO\textsubscript{3} (2) and [Cu\textsubscript{2}{bis(3,6 pyrazol-1-yl)pyridazine}{Cl\textsubscript{4}OH} Cl (3) (cnge=cyanooguanidine) which are very powerful superoxide radical scavengers and compound 3 is especially remarkable having the lowest IC\textsubscript{50} value (3.9 x10\textsuperscript{-7} M) [109].

### 1.5 Schiff bases as ligands for metal complexes:

The common structural feature of schiff base compounds is the azomethine group with the general formula RHC=N-R' where R and R' are alkyl, aryl, cyclo alkyl or heterocyclic groups which may be variously substituted. Presence of a lone pair of electrons in an sp\textsubscript{2} hybridised orbital of nitrogen atom of the azomethine group is of considerable chemical importance and impart excellent chelating ability especially when used in combination with one or more donor atoms close to the azomethine group. Schiff bases can be prepared by condensing carbonyl compounds [110] and amines in different conditions and in different solvents with the elimination of water molecules by the following reaction scheme:

\[
\text{R-NH}_2 + \text{R-CH} \rightarrow \text{R-N=CH-R} + \text{H}_2\text{O}
\]

Schiff bases of aliphatic aldehydes are relatively unstable and are readily polymerizable [111-113]. The presence of a dehydrating agent normally favours the formation of Schiff bases. Spectroscopically Schiff base ligands are characterized by the presence of C=Н stretching frequency at 1680 – 1603 cm\textsuperscript{-1}. The UV/visible spectra of Schiff base compounds containing unconjugated chromophores show bands due to n → \pi^* transitions in the range 235 – 272 nm. However conjugation with alkene or aryl groups causes a large change in spectra as the strong \pi→\pi^* transition covers the weak
$n\rightarrow\pi^*$ band. Salicylaldehyde is one of the most popular aldehyde used to form different Schiff base ligands. IR spectra display a rather broad peak at ca. 3500 cm$^{-1}$ due to free or weakly H-bonded hydroxyl groups. There is a very broad and strong absorption from 3500 to 2500 cm$^{-1}$ due to strongly H-bonded hydroxyl group. The electronic spectra shows two absorption at 415 nm due to $n\rightarrow\pi^*$ transition and at 320 nm due to $\pi\rightarrow\pi^*$ transition. The chelating ability of the Schiff bases combined with the ease of preparation and flexibility in varying the chemical environment about the C=\(\text{N}\) group makes it an interesting and ligand in coordination chemistry. Schiff bases easily form stable complexes with most transition metal ions and stabilize them in various oxidation states. Till date a number of copper complexes with Schiff base ligand have been prepared. M.E. Hosssain et al. reported some copper (II) complexes of the 2-benzylpyridine Schiff bases of S- methyl- and S-benzylthiocarbazate. [114]. Serdar Karabocek et al. reported mono and dinuclear copper (II) complexes of Schiff base ligand 4',5'-bis( salicylideneimino) benzo-15- crown-5 [115]. Synthesis and characterisation of Schiff base complexes derived from o-phenylenediamine and acetoacetanilide was reported by N. Raman et al. [116]. Arindam Mukherjee et al. reported a new Cu(II) complex of a mulidentate Schiff base N,N'- (2-hydroxypropane -1,3-diyl) bis (pyridine-2- aldimine) [117]. Synthesis and characterisation of copper (II) complexes derived from salicylaldehyde and glycylglycine was reported by Wen-Long Liu et al. [118]. An antiferromagnetically coupled hexanuclear copper (II) Schiff base complex containing phenoxo and dicyanamido bridges were also reported [119].

1.6 Electrochemical sensors:

Electrochemical sensors are devices that extract information about sample from measurement of some electrochemical parameter. The basic components of an electrochemical sensor are a working (or sensing) electrode, a counter electrode and usually a reference electrode. These electrodes are enclosed in the sensor housing in contact with a liquid electrolyte. Electrochemical sensor measurement can be made at steady-state or transient. The applied current or potential for electrochemical sensors may vary according to the mode of operation, and the selection of the mode which is often changed to enhance the sensitivity and selectivity of a particular sensor.
Electrochemical sensors are used mainly for environmental monitoring, clinical assays quality monitoring and medical application [120]. Electrochemical sensors have several advantages in terms of sensitivity, selectivity, fast response time and ease of preparation [121]. Compared to the mass spectrometric and chromatographic techniques, electrochemical sensing methods are generally preferred due to their simple setup and electronic requirements. Moreover they are easy to maintain and calibrate and their signals are obtained directly. Besides these kinds of advantages some limitations are observed such as long term instability, electrochemically active interfaces, and problematic electron transfer pathways.

In electrochemistry, electrochemical recognition of charged and neutral species is a newly studied challenging area [122-125]. The design and synthesis of electrochemically responsive ligand systems in which a redox-active centre is in close proximity to a host binding site have attracted considerable interest. Electrochemical sensors can be designed by linking a redox active group to a receptor. The receptor should be selective to the target molecule or the analyte and the binding process should be coupled to the redox reaction. A change in the redox properties of the receptor can be detected by an electrochemical technique such as cyclic voltametry (CV) or Square Wave Voltammetry (SWV). Changes in the cyclic voltamogram can therefore be used to sense the presence of this analyte. In general, an electrochemical sensor or detector functions by monitoring the properties of an electrical circuit when an analyte interferes with the normal profile of the circuit [126].
1.6.1 Principles of electrochemical sensors:

Electrochemistry implies the transfer of charge from an electrode to another phase, which can be either a solid or a liquid sample. During the electrochemical process, certain chemical reactions take place at the electrodes as a result of which the charge is conducted through the bulk of the sample phase. Both the electrode reactions and/or the charge transport can be monitored chemically and serve as the basis of the sensing process [127].

1.6.2 Types of electrochemical sensor

Electrochemical sensors generally can be categorized as conductivity/capacitance, potentiometric, amperometric, and voltammetric sensors. The amperometric and voltammetric sensors are characterized by their current-potential relationship with the electrochemical system and are less well-defined. Amperometric sensors can also be viewed as a subclass of voltammetric sensors.

1.6.2.1 Potentiometric sensors:

When a redox reaction, \( \text{Ox} + \text{Ze}^- = \text{Red} \), takes place at an electrode surface in an electrochemical cell, a potential may develop at the electrode-electrolyte interface. This potential is used to quantify the activity (on concentration) of the species involved in the reaction which is the basis of potentiometric sensors. Potentiometric sensors are classified depending on the nature of the electrode i.e. inert or active. These types of sensors generally work at thermodynamic equilibrium. At this equilibrium, the Nernst equation is applicable and can be expressed as:

\[
E = E^o + \frac{RT}{ZF} \ln \left( \frac{a_{\text{ox}}}{a_{\text{red}}} \right)
\]

Where \( E \) and \( E^o \) are the measured electrode potential and the electrode potential at standard state, respectively; \( a_{\text{ox}} \) and \( a_{\text{red}} \) are the activities of Ox (reactant in this case).
and Red (product in this case), respectively is the number of electrons transferred, $F$ the Faraday constant, $R$ the gas constant, and $T$ the temperature in the absolute scale. In the electrochemical cell, two half-cell reactions will take place simultaneously. However, for sensing purposes, only one of the two half-cell reactions should involve the species of interest, and the other half-cell reaction is preferably reversible and non interfering. If the number of electrons transferred, is one, at ambient temperature (25°C or 298°K) the slope is approximately 60 mV/decade. This slope value governs the sensitivity of the potentiometric sensor [128].

In the potentiometric sensors the recognition of hydrogen ion is done by the glass electrode, which is coupled with the reference electrode to complete the electrical circuit; and the sensor measures the potential difference between these two electrodes [129].

### 1.6.2.2 Amperometric sensors

In amperometric sensors, information is obtained from measurement of current, that the role of Ohm’s Law becomes immediately apparent. The electrode and its operation represent a resistance which at a given constant potential results in current. It is proportional to the concentration of the species, which are being electrochemically transformed (that is, reduced or oxidised) at the electrode. The redox process at the electrode ions that can proceed in either direction (oxidation or reduction) each with its own velocity. Those velocities depend on the potential applied and relate to the value of the charge transfer resistance. Two types of electrochemical reactions take place depending on their charge transfer resistance. If the charge transfer resistance is very low and reactions are very fast, we call such reactions “reversible”. On the other hand if reactions are very slow and their charge transfer resistance is very high, we call such electrode reactions “irreversible”. Thus, the relative speed of an electrochemical reaction can be related to its equivalent charge transfer resistance. At a more negative potential the reduction becomes faster and its charge transfer resistance becomes smaller.
1.6.2.3 Conductometric sensors:

Measurement of the conductivity of an electrochemical cell is the basis for a conductometric sensor. The conductance of a homogeneous solution is directly proportional to the cross-sectional area perpendicular to the electrical field and inversely proportional to the segment of solution along the electrical field. Thus, the conductance of this solution (electrolyte),

\[ G = \frac{\sigma A}{L} \]

Where \( A \) is the cross-sectional area (in cm\(^2\)), \( L \) is the segment of the solution along the electrical field (in cm), and \( \sigma \) (in \( \Omega^{-1}\) cm\(^{-1}\)) is the specific conductivity of the electrolyte and is related quantitatively to the concentration and the magnitude of the charges of the ionic species. For a practical conductivity sensor, \( A \) is the surface of the electrode, and \( L \) is the distance between the two electrodes. Equivalent and molar conductivities are commonly used to express the conductivity of the electrolyte. Equivalent conductance depends on the concentration of the solution.

Since the reciprocal of resistivity is conductivity, these sensors are interchangeably called conductometric sensors or chemiresistors. Some material, which can change its conductivity upon interaction with chemical species, is clamped between two contact electrodes and the resistance of the entire device is measured. Such arrangement is typical for chemiresistors, used for sensing in gases. In another way the chemically interactive layer is at the top of an electrode which is immersed in the solution of electrolyte. A suitable counter electrode completes the electrical circuit.

1.6.2.4 Voltammetric sensors:

The current-potential relationship of an electrochemical cell provides the basis for voltammetric sensors. In a voltammetric sensor, the current response is measured as function of applied potential. Voltammetric sensors examine the concentration effect of the detecting species on the current-potential characteristics of the reduction or oxidation reaction involved. So, it is quite essential that the species to be determined is electro active at the electrode material at a reasonable value of potential where solvent or electrolyte decomposition does not occur. Voltammetric sensors are either operated in
linear or cyclic sweep mode. The mass transfer rates of the detecting species in the reaction onto the electrode surface and the kinetics of the faradaic or charge transfer reaction at the electrode surface directly affect the current-potential characteristics. The electrode reaction kinetics and the mass transfer processes contribute to the rate of the Faradaic process in an electrochemical cell. This provides the basis for the operation of the voltammetric sensor. During our research we worked on i) voltammetric study of copper complex modified glassy carbon electrode for the simultaneous determination of dopamine and ascorbic acid, and ii) different micellar interaction on copper compounds-a cyclic voltammetric study.

1.6.2.4.1 Copper complexes as voltammetric sensors for Dopamine:

Dopamine (DA) is an important neuron-transmitter compound widely distributed in the brain of mammalian central nervous system. Deficiency of dopamine leads to neurological diseases like Parkinson’s disease, Alzheimer’s disease, schizophrenia and is also related to HIV infection [130,131]. Therefore determination of DA has attracted much attention from researchers. But it is very difficult to determine DA directly due to the interference from ascorbic acid (AA). To solve this problem chemically modified the interference of AA with the determination of DA [132-136]. Many mixed valent compounds such as oxides, complexes, or alloys of Copper [137-139], Cobalt [140-142] and ruthenium [143-145] have shown electro catalytic property. Copper complexes are probably the most studied metal complexes in a wide variety of fields such as catalysis, biomimetics, spectroscopy, magnetism, liquid crystal, basic coordination chemistry etc. But copper complexes as voltammetric sensor for detection of dopamine is very few reported due to the instability caused by the changed structure during redox process [141,146-148]. The group of L T Kubota reported that immobilization of cupric ions in poly (ethylene-co-vinyl acetate) film on electrode surface had excellent selective detection of DA over AA [149]. Square wave voltammetric detection of dopamine at a Copper – (3- mercaptopropyl) trimethyl silane complex modified electrode was reported by Mi –Sook Won et al. [150]. M. Wang et al. reported voltammetric studies of one novel bicopper complex, of the type $[L_2Cu_2biPy]$ where $L=2-[bis(2-amino-ethyl) amino]$ethanol modified electrode for the simultaneous
determination of DA and AA[151]. Amperometric sensor for DA using a Nafion membrane doped with copper dipyridyl complex was also reported [152].

1.7 Scope of the Thesis:

Modelling the active centre of metalloproteins, although not very new area of research in bioinorganic chemistry is still being carried out intensively to understand different structural and functional aspects of metalloproteins. Among the scores of metalloproteins, Cu-Zn superoxide dismutase is responsible for destroying the harmful superoxide ions (O$_2^-$) generated as by product in respiratory electron transfer reactions and leaks through cell membranes. Damage of DNA leading to early old age and cancer are the two most dangerous affect of superoxide ion on human health. In Cu-Zn superoxide dismutase the role of Zn$^{2+}$ ion is structural while superoxide scavenging is materialised by the Cu$^{2+}$ ion through a mechanism where it shuttles between oxidation states +1 and +2. Although Cu$^{2+}$ ion itself is an excellent scavenger of superoxide, its toxicity such as Wilson’s disease and damaging of vital organs (such as liver, lung and kidney) restricts its use as a substitute for Cu-Zn superoxide. Simple complexes of Cu$^{2+}$ ion having good superoxide scavenging activity might be a valuable substitute for Cu-Zn superoxide dismutase. The new simple Cu$^{2+}$ complexes reported in this thesis, except one, inherits good superoxide scavenging capacity and might be helpful for the research groups involved in developing metal based superoxide scavenging drugs.

Sensing molecules of biological interest by developing electrochemical sensors is another prominent field of research in chemistry. Voltammetry is a subclass of electrochemical sensors where the current generated due to the interaction of the analyte and the modified working electrode is mapped against applied potential. Although a variety of substances have been employed as electrode modifying agent only a scars of reports are available on Cu$^{2+}$ complexes as electrode modifying agent. One chapter of this thesis reports successful application of a new Cu$^{2+}$ complex encapsulated in the zeolite ZSM-5 in sensing the important neurotransmitter dopamine in presence of its main interfering ascorbic acid. This work is like to motivate researcher working in the field on voltammetric sensors based on metal complexes.
Hence the works resented in this thesis has tremendous scope in the field of model Cu$^{2+}$ ion complex based superoxide scavengers as well as voltammetric sensors.
1.8 References:


Chapter 1
Chapter 1


    b) R. L. Fletcher, I. M. Munda and A. Vukovic Bot. Mar. 31 (1988) 1


Chapter 1
30


Chapter 1