Chapter 6

Understanding the binding of Copper(II) with DNA in Chromatin
Introduction

Chromatin is the complex of protein-associated form of DNA in the eukaryotic cell and has been extensively characterized. The primary functions of chromatin are to package DNA into a smaller volume to fit in the cell, to strengthen the DNA to allow mitosis and meiosis and prevent DNA damage (Polo and Jackson et al, 2011). It also helps to control gene expression, DNA replication and DNA repair. The structure of chromatin depends on several factors such as genes present in the DNA during the interphase, any alteration in the methylation, acetylation of DNA and histone modification. An alteration in the chromatin organization may lead to the neuronal cell death and loss of regulation of DNA methylation, leading to altered gene expression as observed in many neurodegenerative disorders (Kornberg, 1974; Kornberg, 1977; gladrossi et al, 2007). The chromatin structure is susceptible to change in ionic strength, pH, temperature and interaction with divalent metal ions with DNA (Scarpa et al, 2003). DNA molecules are prone to damage upon interaction with metals, mainly copper. The interaction of copper ions with DNA has been of particular interest because of the involvement of copper ions in regular activities of cells. At the same time copper can induce the production of highly reactive ROS, which causes oxidative DNA damage that may probably leads to various pathological changes in living organisms (Sagripanti et al, 1991, Gina Manda et al, 2009).

Copper is an essential component of chromatin to maintain regular activities such as cellular respiration and neurotransmitter biosynthesis etc. Copper also works as cofactor for numerous enzymes and plays an important
role in central nervous system development. However, excessive levels or perturbation of copper metabolism can lead to an accumulation of copper preferentially in heterochromatin regions of the chromosomes cause intracellular toxicity (Weller and walkimoto, 1995). Copper is a redox-active metal that are constantly participate in the production of (ROS) reactive oxygen species via a Fenton reaction that causes catastrophic damage to lipids, proteins and DNA by altering their structure and function (Halliwell and Gutteridge, 1984). Altered structural and functional modifications are involved in chromatin condensation may be accompanied by a decrease in the DNA repair capacity. The imbalance of DNA damage and repair produces a buildup of unrepaired genetic damage that can initiate neurodegenerative pathology. For this reason elevated levels of copper in the brain is one of the strongly suspected etiological factors in brain disorders such as, Alzheimer’s disease, Parkinson’s disease, Huntington disease and familial amyotrophic lateral sclerosis (Hartter and Barnea,1988; Strausak et al, 2001; Rouault, 2001; Brewer, 2007; Desai and Kaler, 2008; Vasudevaraju et al, 2010). Copper can function as double edged sword in the brain by both helping for many enzymes that prevents the deleterious consequences of genome damage, and also helping in generating large numbers of the DNA-attacking ROS. Hence, it is critical to maintain homeostatic concentrations of copper. In the present study, the properties of chromatin with respect to conformational changes and damage as a consequence of interaction with copper were carried out. Structural and conformational aspects of the interaction of copper with chromatin are studied by a variety of spectroscopic techniques.
including UV-visible spectrophotometry, optical melting studies, circular dichroism, CD melting studies and fluorescence spectroscopy. Temperature studies provide the basis to understand the factors that dictate the stability and structure of chromatin in the presence of copper. The role of copper in DNA damage and pathogenesis in relation to neurodegenerative disease is discussed.

Materials and Methods

Isolation of the nuclei from the brain samples: Nuclei were isolated from the cortex region of the human brain (JSS Medical College, Brain bank) according to the method described by Rao et al., (1983). Briefly, brain tissue was weighed (5 g) and perfused with normal saline to remove the any blood in the tissue. Brain tissue was cut into small pieces and minced thoroughly. The minced brain tissue was homogenized in 0.34M sucrose in buffer (50 Mm Tris–HCl, pH 7.5, 25 mM KCl, 5 mM MgCl₂, 0.5 mM PMSF) using homogenizer. The homogenate was filtered through 2 layers of cheese cloth and the filtrate was centrifuged at 1000g (3500 rpm) for 10 min at 4°C. The supernatant was decanted carefully and the pellet was re-suspended in 1M sucrose in buffer A. The suspended pellet was homogenized using hand held homogenizer and centrifuged at 100,000g (42,000 rpm) in ultracentrifuge for 1 h. The pellet obtained was washed with 1M sucrose in buffer A and then washed again with 0.34 M sucrose with 0.1% Triton X- 100. The pellet is dissolved in Tris-HCl buffer. The concentration of the nuclear suspension was determined by taking absorbance at A₂₆₀ in 1 ml of 2M NaCl/5M urea.
**Preparation of nuclei and soluble chromatin:** Soluble chromatin was prepared from the isolated nuclei by limited digestion of nuclei with micrococcal nuclease (Korenberg et al, 1989). Nuclear suspension (100 μL) was mixed with 0.1M CaCl₂ and incubated at 37°C for 2 min. After incubation, the nuclear suspension was digested with micrococcal nuclease (50 units) by incubating at 37°C for 1 min. The reaction was stopped by 0.25 M EDTA and centrifuged at 5000 rpm for 5 min. The pellet obtained was suspended in 10mM NaHSO₃, pH 7.5, 1mM EDTA. The soluble chromatin was used for the copper interaction studies.

**Copper chloride** (CuCl₂.2H₂O): Copper chloride dihydrate was purchased from Merck Schuchard, and was used without purification. 50mM stock solution was prepared by dissolving 8.5 mg of CuCl₂ 2H₂O in 990 ml of milliQ water. The required dilutions were prepared when necessary for the experiments.

**Ethidium bromide** (C₂₁H₂₀BrN₃): It was purchased from Amersham life sciences and used the samples without further purification. 5mg/ml stock solution of EtBr was prepared by dissolving EtBr in milliQ water and stored at 4°C in the dark. Required dilutions were prepared when necessary for the experiments.

**Tris buffer:** Tris was purchased from Sigma-Aldrich, USA. The stock solution was prepared by dissolving 2.4 g of Tris in 200ml of milliQwater. The pH of the buffer was set by the addition of μl aliquots of dilute HCl. The pH of the solution was measured directly using digital pH meter with a combined glass electrode (EUTECH Instruments).
**UV/Vis absorption studies:** The electronic absorption studies of chromatin and its ability to bind to copper chloride were investigated using a Jasco V-530 spectrophotometer equipped with a peltier temperature controller. Chromatin samples were prepared in Tris-HCl buffer (5 mM, pH 7.4) in the absence and the presence of CuCl2 (50 µM and 100 µM) (total quantity of sample used for UV/Vis absorbance spectrum was 400 µl). Absorbance spectrum was measured at wavelength between 210nm and 320nm with a matched set of 1-cm pathlength quartz cuvettes. Buffer baseline was subtracted with the jasco software and the resultant spectrum was recorded.

**Circular dichroism studies:** CD spectroscopy is one of the most sensitive techniques available for monitoring conformational properties of DNA in solution. The copper induced conformational change of chromatin were measured on a Jasco J-715 spectropolarimeter at 25°C, using a path length of 1 mm quartz cuvette at 1 nm intervals in the wavelength between 200 and 320 nm. Spectra were recorded as an average of four repetitive scans using a scan speed of 20 nm /min. Chromatin sample prepared in Tris-HCl buffer (5 mM, pH 7.4) (total sample was 300 µl) in the absence and presence of different concentrations of the CuCl2. 2H2O (50,100 and 500 µM). Buffer background was subtracted by using the built-in feature of jasco software and the resultant spectrum was recorded.

**UV Thermal denaturation studies:** UV- thermal denaturation studies of chromatin in the absence and presence of CuCl2 were measured with jasco V-
530 spectrophotometer equipped with Jasco ETC-505T temperature controller and cell holder that permits temperature control using the temperature control programme.

**Circular dichroism (T_m) studies:** Melting studies of chromatin in the presence and absence of CuCl_2 were measured on a Jasco J-715 spectropolarimeter connecting with Model PTC-348WI, peltier type temperature control system. Samples were recorded in the wavelength between 200 nm and 320 nm by varying the temperature of 20°C to 100°C at 10°C intervals.

**Fluorescence studies:** Fluorescence spectroscopy is an important technique for probing the structure and dynamics of nucleic acids. The utility of fluorescence techniques stems from the ability of fluorophores to reflect changes in their molecular environment through measurable alterations in emission properties. Fluorescence emission studies were carried out using equimolar concentrations of chromatin and EtBr (1:1). The EtBr binding pattern of chromatin and the effect of different concentrations of CuCl_2 (100 µM to 500 µM) on the EtBr fluorescence were analyzed. DNA/EtBr solutions were excited at 530 nm and emission spectra were recorded from 550 nm to 650 nm using Jasco J-600 spectrofluorimeter.

**Results**

**CD Studies:** Circular dichroism is a useful technique for studying conformational changes and the degree of asymmetry of bases of DNA in chromatin in solution
(Adler et al, 1974). Structural transition of chromatin in the absence of copper, exhibits a characteristic positive peak at 275 nm due to base stacking and a negative peak at 210 nm due to the presence of protein (Fig 2a). CD spectra of chromatin between 250-300 nm are dominated by DNA, proteins contribute very little to CD spectra of this region (Sissoeff et al, 1976; Baserqa and Nicolini, 1976; Rajarshi et al, 2008). Upon addition of CuCl$_2$.2H$_2$O to chromatin, a decrease in the magnitude of both the positive and the negative bands, due to complex formation between Cu$^{2+}$ and the bases of DNA was noticed. This was accompanied by a conformational change with a cross over point at 245 nm as illustrated in Fig 2a. These data indicated that cationic copper binds to the anionic phosphate ions of the DNA back bone and consequently, the lengthening of DNA and induced the loss of conformation. The binding of copper to bases of DNA depends on the accessibility of DNA in chromatin. Copper competes with hydrogen bonds and disrupt interactions between DNA bases and weaken base stacking, causes a decrease in the intensities of the CD bands due to unwinding of the helix and induced loss of conformation from B-DNA to altered B-DNA conformation (Dugoid et al, 1993). The altered DNA structures will significantly differentially alters gene expression and in the normal process of DNA replication and transcription and inhibit RNA polymerase activity. These results provide the evidence of copper induced DNA damage in the chromatin organization and may lead to the neuronal cell death and its crucial role is implicated in many neurological disorders.
**UV absorption studies:** To understand the nature of the conformational changes of chromatin in CD on binding with copper, spectrophotometric binding studies of the copper with chromatin were performed at pH 7.4. The absorbance spectra of chromatin showed the absorption maximum at 265 nm (Fig 1a). Upon addition of CuCl$_2$.2H$_2$O (50 µm & 100 µM), there was an increase in the absorbance with the blue shift to 260 nm. On further addition of copper, no change in the intensity was noticed indicating saturation. This wavelength region (Far UV) of the absorption spectra is sensitive to π–π* transitions of the electrons of the purine and pyrimidine rings, due to the increased positive base pair tilting of conformational change in DNA (Mergny et al, 2005). The hyperchroism and blue shift (hypsochromic shift) is because of binding the copper to the bases of DNA in chromatin, resulting in disruption of hydrogen bonds by the process of partial unwinding, thus inducing structural changes such as loosening of base-base interaction and base tilting and destabilization of the DNA double helix leading to DNA denaturation. The destabilization is due to most probable binding sites in DNA such as Cu$^{2+}$ to N$_7$ of guanine and N$_3$ of cytosine in line with our present results and with the predictions by other workers (Eichhorn and Clark, 1965; Zimmer et al, 1971).

**Thermal melting studies:** The thermal behavior of chromatin is monitored by using UV/Vis absorbance spectroscopy, which provides information about the binding affinity of copper with DNA in chromatin and conformational changes. It is known that double stranded DNA gradually dissociates to single strands
enhanced with increasing temperature (Mandel and Fasman, 1974). $T_m$ is strictly related to the stability of the double helix and the interaction of copper with DNA may alter the $T_m$ by stabilizing or destabilizing the complex. The melting temperature of DNA enhanced with the increase in temperature and stabilized the DNA in chromatin as shown in Fig 1b and 1c. Identical melting curves are produced for both chromatin and chromatin with 50 µM copper. Melting profile of chromatin clearly reveals two transitions, the first transition at 58.8 ºC and the second $T_m$ at 70.27 ºC (Fig 1d). With 100 µM copper, $T_m$ shifts to 60ºC and 72.5 ºC. The small increase in $T_m$ indicates that copper interact with DNA in chromatin and changes the conformation of chromatin structure (Rosetto and Nieboer, 1994).

Thermal denaturation of chromatin in the absence of copper by varying temperatures from 20ºC to 105ºC was monitored using circular dichroism (Fig 2b). Addition of 50µM copper is shown in Fig 2c, and 100µM copper is shown in Fig 2d. There is an increase in intensity of both positive and negative bands at the isodichroic point at 227 nm for chromatin and 229 nm with copper indicating that the CD transition is at two states, and the structural change is cooperative and produce identical melting profiles of native chromatin and chromatin with 50µM and 100 µM copper. The CD melting profile in the presence and absence of copper at 275 nm (data not shown) is evidence for the above results. Both CD and UV melting results showed that there was not much effect of temperature on DNA in chromatin, this may be because the DNA bases in chromatin are protected by the association of the DNA with the chromatin proteins, so that the
bases of DNA depends on the accessibility of DNA in chromatin to Cu (II) (Zimmer et al, 1974). It is concluded that the basic conformation of DNA in native chromatin is determined largely by histones, and nonhistone proteins. It is also seen that DNA is greatly stabilized against thermal melting in the DNA-histone complex.

**Fluorescence Studies:** Ethidium bromide replacement experiment was carried out to verify the local structural information due to the interaction of copper with chromatin. The fluorescence spectra of chromatin-EB complex excited at 530 nm and emission spectra are scanned from 550 to 650 nm (Fig 3). Upon addition of copper chloride to chromatin, the fluorescence intensity of chromatin-EB complex decreased with increasing concentration of copper. The decrease of chromatin-EB emission with the addition of copper indicates that binding of Cu$^{2+}$ ions with chromatin-EB complex forms a new non-fluorescent complex of Cu-chromatin–EB, which causes the fluorescence quenching of chromatin–EB complex (Lawrence et al, 1976). This data show that Cu$^{2+}$ ions bind to DNA in chromatin, resulting in chemical DNA denaturation and that the binding of copper is mainly concentration dependent.

**Discussion**

The DNA was organized in the form of compact condensed structure in the nucleus. The higher order of organization of genomes in chromatin is responsible for the regulation of gene expression (Joffe et al, 2010; Postberg et
Chromatin organization is a dynamic process occurring in the living cells by continuously opening and reorganizing according to cellular needs (Rippe, 2007; Mazloom et al, 2010; Jasencakova et al, 2010). Defects in the chromatin organization are responsible for the physiological and pathological process. Chromatin conformational changes may even lead to the manifestation of diseases (Misteli, 2010; Crutchley et al, 2010). Chromatin organization at a particular time depends on the state of DNA, composition of DNA binding histone proteins, metals and their modification (Fukuda et al, 2006; Chen et al, 2010). Many studies reported that copper induced alterations in chromatin structures are emerging as key players in maintenance of genome stability, aging and in the development of AD (Byung-Eun Kim et al, 2008; Turski and Thiele, 2009).

Copper is physiologically important metal required for a number of biological functions. However, excessive levels of copper in brain can lead to intracellular toxicity. The redox properties shows significant changes in the structural conformation of DNA implicating DNA instability, which result in neuronal dysfunction and critical failure of biological functions and ultimately cell death contributing to neurological disease. Recent reports (Rao, 1993; 2009) show, that copper plays an important role several neurodegenerative disorders. Perturbation in the copper homeostasis can lead to an increase of copper in the brain and liver. Copper has a high capacity to generate free radicals, even at low concentrations. In the present study, the ability of copper to participate in an array of conformational and structural stability in DNA in chromatin was analyzed. CD spectra of chromatin showed a characteristic B-DNA conformation, having a
positive peak at 275 nm is shown in Fig. 2a. CD spectra of chromatin between 250-300 nm are dominated by DNA, as proteins contribute very little to CD spectra of this region (Adler et al., 1973). Upon gradual addition of copper to chromatin, a decrease in the magnitude of both the positive and negative bands was noticed. Structural changes in DNA elicited by copper are a function of the nature and binding affinity for target sites on DNA. Copper first binds to anionic phosphate (Zimmer et al., 1971) groups of the backbone and subsequently to the bases, specifically to $N_7$ of Guanine (Bryan and Frieden, 1967) and $N_3$ cytosine (Fritzsche and Zimmer, 1968) of DNA by competing with hydrogen bands. This disrupts the interactions between the bases of DNA and thereby weakening base stacking (Dehkordi, 2012) by tilting the bases leads to change in the winding angle. As the winding angle increases, the magnitude of the band decreases with a greater degree of twisting of the chain. Hydrogen bonding forces play an essential role in the binding (Forster et al., 1979; Gupta et al., 1980), which may affect sugar puckering and change in the conformation of guanine from Anti- to Syn (Courtois et al. 1968). The negative super helical tension and rearrangement in coordination caused by copper may drive local transitions to alternate conformational change in the DNA structures in chromatin. Binding of $Cu^{2+}$ with adenine is similar in interaction with the $N_7$ position and the phosphate site would represent a third copper-base binding site. However copper does not bind to Thymine. Both the bases of GC pair are known involve the copper complexes. Results of UV absorbance and fluorescence studies are also show the ability of copper ions to cleave the phosphodiester bonds in nucleotides. Therefore
suggesting that copper binds both to the phosphate and bases and induces the conformational changes in DNA. Copper exists in the cell nucleus at relatively high concentration and closely associates with chromosomes and play an important role in higher levels of chromatin organization (Bryan et al, 1981).

Thermal denaturation studies of UV show a small variation in $T_m$. This suggests that copper binds to anionic phosphate backbone and to the bases depending upon the accessibility of DNA in chromatin. The results of CD melting studies show that protein bound to DNA in chromatin stabilizes DNA. Higher stabilization is due to the compact binding of the histones. Studies of both CD and UV show that there is not much effect of temperature on DNA in chromatin. However, our previous reports show the binding of copper to DNA, decrease the $T_m$ by destabilization of the double helix. Higher thermal stability of the chromatin may be because the DNA bases in chromatin are protected by associating with the histone and nonhistone proteins. The basic conformation of DNA in native chromatin is therefore determined largely by histones, nonhistone proteins and the varying degrees of accessibility of the DNA in chromatin to Cu (II).

DNA conformation is an important aspect for the gene expression, any changes in B-DNA conformation will affects the integrity of DNA that altered the expression of genes and is associated with multiple intracellular signal transduction pathways. The altered integrity of DNA may affect the normal process of DNA replication, transcription and inhibit RNA polymerase activity in vitro. Copper induced conformational change and stability of DNA in chromatin
are recognized as crucial intermediaries that may cause neurotoxicity and have an important role in the pathogenesis of Alzheimer’s disease (AD) and other neurological disorders. Hence, our findings suggest that copper induced DNA conformation in chromatin may have biological relevance.

**Conclusions**

Copper binds to both phosphate and the bases of DNA cause conformational and functional changes in DNA. Copper induced DNA damage and conformational changes in chromatin is viewed as one of the important factors in the pathogenesis of Alzheimer’s disease (AD) and other neurological disorders. Copper is redox-active metal that are constantly involved in generation of ROS, which may be an important process in the pathogenesis of AD and PD. It is also concluded that histone and non histone proteins present in chromatin protect DNA from oxidative DNA damage. Further studies are needed to examine the role of proteins in protecting the oxidative DNA damage.
Fig 1a. Absorbance spectra of Chromatin with varied concentrations of copper
Fig 1b. Absorbancespectra of chromatin with varied temperature
Fig 1c. Absorbance spectra of chromatin-copper (50µM) with varied temperature
Fig 1d. Melting profile of chromatin and copper. a, Chromatin, b. Chromatin with 100 μM copper
Fig 2a. Circular dichroism studies showing the conformational change of chromatin-copper interactions.
Fig 2b. Circular dichroism studies of chromatin with varied temperature
Fig 2c. Circular dichroism studies of chromatin-copper (50 µM) with varied temperature
Fig 2d. Circular dichroism studies of chromatin-Copper (100µM) with varied temperature Chromatin – Cu™
Fig 3. Fluorescence emission spectrum of chromatin-copper


