LIST OF PUBLICATIONS

1. Synthesis, nucleic acid binding and cytotoxicity of polyethyleneimine-copper(II) complexes containing 1,10-phenanthroline and L-valine.

2. Studies on the interactions of polymer-anchored copper(II) complexes with tRNA.

3. Synthesis, characterization, DNA-, RNA-binding, cytotoxicity and antimicrobial activities of polyethyleneimine anchored copper(II) complexes containing 1,10-phenanthroline and L-tyrosine.
   J. Lakshmipraba, S. Arunachalam*, A. Riyasdeen, R. Dhivya, S. Vignesh , M. A. Akbarsha and R. A. James, (Manuscript Communicated)

4. DNA/RNA binding and anticancer/antimicrobial activities of polymer-copper(II) complexes.
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   J. Lakshmipraba, S. Arunachalam,* R. V. Solomon and P. Venuvanalingam (Manuscript Communicated)
Studies on the interactions of polymer-anchored copper(II) complexes with tRNA

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Sankaralingam Arunachalam

Received: 8 January 2010 / Accepted: 16 March 2010 / Published online: 1 April 2010
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Abstract The binding interactions between tRNA and polyethyleneimine-anchored copper(II) complexes with 1,10-phenanthroline and 2,2′-bipyridine ligands and varying degrees of coordination have been studied using physicochemical techniques. In both sets of polyethyleneimine-anchored complexes, the complexes with higher degree of coordination bind more strongly to tRNA than those with lower degree of coordination. Binding to tRNA appears to be mainly electrostatic in nature.

Introduction

Recently, increasing attention has been focused on the DNA-binding interactions of transition metal complexes [1, 2], which can mimic the structure of cis-platin [3]. Such transition metal complexes have long been investigated as potential agents in chemotherapy and photodynamic therapy owing to their high affinity for the double-stranded DNA helix [4]. Various spectroscopic techniques, such as NMR [5], circular dichroism (CD) [6], fluorescence [7], electron spin resonance [8], UV–Visible [6] and resonance Raman spectroscopy [9] have been applied to a multitude of different DNA sequences, and the binding modes have been distinguished. Binding of metal complexes with 1,10-phenanthroline or modified phenanthroline ligands to DNA has attracted much attention [10, 11]. These complexes can bind to DNA through a series of weak interactions, such as π-stacking associated with the intercalation of aromatic heterocyclic groups between the base pairs, hydrogen bonding, van der Waals interactions along the grooves of the DNA helix, and hydrophobic effects.

In contrast to DNA, very little attention has been paid to the binding properties of RNA with transition metal complexes. These metal complexes have been used as catalysts of RNA hydrolysis cleavage [12], shape-selective probes of RNA tertiary structure [13], agents of RNA oxidation cleavage [14] and for recognition of mismatches in RNA [15]. But only a few reports have investigated the interactions between transition metal complexes and RNA [16–18]. The future development of RNA-targeting drugs will rely on a deeper understanding of these binding processes, leading to advances in medical applications such as the inhibition of the AIDS virus and the photodynamic therapy of tumours [19]. Although all cellular RNAs have a single polynucleotide chain, they are highly versatile molecules that can fold into a multitude of secondary structures and conformations. These complex structural motifs could provide potential binding pockets for specific drug recognition sites, offering the possibility of new RNA-binding molecules that could be modulators of cellular functions [20]. Research in this area has identified different classes of RNA-binding compounds, of which the most important are the aminoglycoside antibiotics that were shown to interact with the functional sites on 16S rRNA [21]. Although aminoglycoside antibiotics can target key RNA molecules of bacteria and retroviruses, their therapeutic use is limited due to high toxicity. Therefore, there is urgent need to develop RNA-targeted small molecules for antiviral therapy. One approach to the development of such molecules has been to study the interactions of known DNA-binding compounds with fairly well-characterized interaction profiles. Wilson et al. [22] have initiated work in this direction long ago and have performed binding studies on a wide
variety of DNA intercalating and groove-binding molecules with various RNA constructs.

Drug–polymer conjugates are potential candidates for the selective delivery of anticancer agents to tumours. One of the most successful and widely studied gene delivery polymers reported to date is polyethyleneimine (PEI), an off-the-shelf polycation containing a high density of primary, secondary and tertiary amines, which has been used previously as a cationic agent as well as a flocculation agent [23]. It has been shown that PEI is one of the most potent non-viral vectors [24] for gene delivery [25], in both in vitro and in vivo studies. Recently, PEI has been investigated as a possible alternative to viral and liposomal routes for gene delivery [26]. PEI possesses a number of advantages as a polymeric complexing agent [23]. In addition to its cationic properties, PEI exhibits a high content of functional groups, good water solubility and chemical stability.

Recently, we have reported results on the interactions between some polymer–metal complexes and DNA [27, 28]. In this report, we present our studies on the interactions between tRNA and polyethyleneimine-anchored copper(II) complexes containing 1,10-phenanthroline and 2,2'-bipyridine ligands (Fig. 1). To our knowledge, this is the first report on the interactions of polymer-anchored metal complexes with RNA using spectroscopic techniques.

**Experimental**

Branched polyethyleneimine (BPEI) (MW ca. 25,000) and transfer tRNA (tRNA) from baker’s yeast were obtained from Sigma–Aldrich and used as received. Copper(II) chloride dihydrate was purchased from Merck, and 1,10-phenanthroline and 2,2'-bipyridine were obtained from Loba, India. The polymer-anchored copper(II) complexes were synthesized as reported in our previous work [27, 28]. The degree of coordination (x), defined as the number of moles of copper(II) chelate per mole of the repeating unit (amine group) of polymeric ligand, was determined by the procedure adopted earlier [28].

Electronic absorption spectra were recorded on a UV–Vis–NIR Cary 300 spectrophotometer using cuvettes of 1-cm path length, and emission spectra were recorded on a JASCO FP 770 spectrophotometer using a 5-mm exit slit. Circular dichroism spectra were recorded on a JASCO J-716 spectropolarimeter. Spectroscopic titrations were carried out in buffer solution (50 mM NaCl—5 mM Tris–HCl, pH 7.1). A solution of RNA in the buffer gave a ratio of UV absorbance at 260 and 280 nm of ~ 1.8–1.9, indicating that the RNA was sufficiently free from proteins.

RNA-binding experiments

The RNA-binding experiments were performed at 25.0 ± 0.2 °C. The tRNA concentration per nucleotide was determined by electronic absorption spectroscopy using the known molar extinction coefficient of 9,250 M⁻¹ cm⁻¹ at 260 nm [29]. Absorption titration experiments of polymer–copper(II) complexes in buffer (50 mM NaCl—5 mM Tris–HCl, pH 7.1) were performed using a fixed complex concentration to which increments of the tRNA stock solutions were added. Polymer–copper(II) complex–tRNA solutions were allowed to incubate for 10 min before the absorption spectra were recorded. For fluorescence quenching experiments, tRNA was pre-treated with ethidium bromide (EB) for 30 min. The polymer–copper(II) complexes were then added to this mixture, and their effect on the emission intensity was measured. Samples were excited at 450 nm, and emission was observed between 500 and 700 nm. Circular dichroic spectra were recorded at room temperature (ca. 25 °C).

**Results and discussion**

Electronic absorption spectroscopy is one of the most useful techniques in studying the binding of RNA to metal complexes. We have studied the binding between our polyethyleneimine-anchored copper(II) complexes and yeast tRNA by monitoring the resultant changes in absorbance and wavelength. The absorption spectra of polymer–copper(II) 1,10-phenanthroline (x = 0.326) and 2,2'-bipyridine (x = 0.501) complexes in the absence and in the presence of yeast tRNA are shown in Fig. 2. In the UV region, both the polymer–copper(II) phenanthroline and 2,2'-bipyridine complexes exhibit an intense band between 200 and 300 nm. For both the complexes, increasing the concentration of yeast tRNA results in an increase in absorbance and a slight red shift in this region. This hyperchromism indicates that the polymer–copper(II) complexes bind with tRNA electrostatically [30]. This electrostatic interaction
may be between the positively charged polymer–copper(II) units and negatively charged phosphate backbone of the tRNA. On the other hand, the yeast tRNA helix offers many hydrogen bonding sites in both the minor and major grooves, and it is likely that the amine groups of polyethyleneimine can form hydrogen bonds with RNA, which may also contribute to the observed hyperchromism. In order to compare the binding strength of the polymer–copper(II) complexes with tRNA, the intrinsic binding constant, $K_b$, has been determined using the equation [31].

$$\frac{[\text{RNA}]}{(e_a - e_i)} = \frac{[\text{RNA}]}{(e_a - e_i)} + 1/K_b(e_a - e_i)$$

where $e_a$, $e_i$ and $e_i$ are the apparent, free and fully bound extinction coefficients, at 295 and 310 nm for [Cu(phen)$_2$-BPEI][Cl$_2$-4H$_2$O] and [Cu(bpy)$_2$BPEI][Cl$_2$-4H$_2$O], respectively.

A plot of $[\text{RNA}] / (e_a - e_i)$ versus $[\text{RNA}]$ gives $K_b$ as the ratio of the slope to intercept (Inset Fig. 2). The $K_b$ values thus obtained for our polymer–copper(II) complexes with different degrees of coordination are given in Table 1. As seen from this table, the $K_b$ value for both the complexes varies with the degree of coordination ($x$); the complexes with higher degree of coordination show higher $K_b$ values. This result can be attributed to the presence of a large number of positively charged copper complexes in the polymer with the higher degree of coordination, which increases the cooperative binding ability of the polymer–copper(II) complex unit to yeast tRNA compared to samples with a lower degree of coordination. This kind of cooperative enhancement of tRNA binding by the copper(II) complex units present in the same polymer molecule is a special property of this kind of polymer–metal complexes, which is not observed in the case of ordinary metal complexes.

All our polymer–copper(II) complexes were non-emissive upon excitation of the MLCT band, either in aqueous solution or in the presence of yeast tRNA. Ethidium bromide (EB) is known to emit intense fluorescence in the presence of yeast tRNA due to the binding of EB to yeast tRNA [1]. It has been reported by Xu et al. [6] that the fluorescence of the EB–tRNA system can be reduced by the addition of a second molecule, which can compete with EB in binding to tRNA. Two mechanisms have been proposed to account for this reduction in the emission intensity, namely the replacement of molecular fluorophores, and/or electron transfer [29]. Bronich et al. [17] have reported that the addition of a copolymer (i.e., polyethylene glycol-polyethyleneimine (PEI)) to the EB-DNA complex resulting in quenching of the fluorescence due to the displacement of EB by the copolymer. There is also evidence for increase in non-intercalative fluorescence enhancement sites which are presumed to involve electrostatic binding [32]. In the present studies, the addition of the polymer–copper(II) complex samples to tRNA pre-treated with EB causes appreciable reduction in the emission intensity (Fig. 3). Again, higher reduction in emission intensity is observed in the case of polymers with higher proportion of copper(II) complex units in the

<table>
<thead>
<tr>
<th>Polymer–copper(II) complex</th>
<th>Degree of coordination ($x$)</th>
<th>$K_b$ (M$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Cu(phen)$_2$BPEI][Cl$_2$-4H$_2$O]</td>
<td>0.156</td>
<td>$7.823 \times 10^2$</td>
</tr>
<tr>
<td></td>
<td>0.307</td>
<td>$3.600 \times 10^3$</td>
</tr>
<tr>
<td>[Cu(bpy)$_2$BPEI][Cl$_2$-4H$_2$O]</td>
<td>0.326</td>
<td>$1.243 \times 10^4$</td>
</tr>
<tr>
<td></td>
<td>0.489</td>
<td>$1.013 \times 10^3$</td>
</tr>
<tr>
<td></td>
<td>0.501</td>
<td>$1.445 \times 10^3$</td>
</tr>
<tr>
<td></td>
<td>0.741</td>
<td>$3.489 \times 10^3$</td>
</tr>
</tbody>
</table>

**Table 1** The intrinsic binding constants ($K_b$) of polymer–copper(II) complexes with tRNA

**Fig. 2** Absorption spectra of [Cu(phen)$_2$BPEI][Cl$_2$-4H$_2$O] (50 µM) ($x = 0.326$) (a) and [Cu(bpy)$_2$BPEI][Cl$_2$-4H$_2$O] (50 µM) ($x = 0.501$) (b) in the absence (dotted line) and in the presence (solid lines) of increasing amounts of tRNA (4–41 µM). (Inset: Plot of [tRNA]/($e_a - e_i$) vs. [tRNA])

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polymer chain (Fig. 4), which can be ascribed to cooperative binding of copper(II) units on the same polymer chain.

The conformational changes of tRNA in the presence of our polymer–copper(II) complexes were probed using CD spectroscopy. The CD spectrum of yeast tRNA consists of a positive band around 268 nm due to ellipticity and a small negative band around at 235 nm due to right-handed helicity of yeast tRNA [18]. On incubation of the polymer–copper(II) complexes with yeast tRNA, an increase in intensity of both the positive and negative bands was observed (Fig. 5). In the case of [Cu(phen)$_2$BPEI]Cl$_2$H$_2$O, the negative band was shifted by 5 nm towards higher wavelength. However, in the case of [Cu(bpy)$_2$BPEI]Cl$_2$H$_2$O, the negative band shifts only by 1 nm. This indicates that [Cu(phen)$_2$BPEI]Cl$_2$H$_2$O binds more strongly to the helix surface. This slight increase in intensity with a red shift in negative and positive bands suggests that polymer–metal complex binds to tRNA electrostatically between the positively charged polymer–copper(II) units and negatively charged phosphate backbone of the tRNA [33]. On the other hand, the yeast tRNA helix possesses many hydrogen bonding sites which are accessible in both the minor and major grooves [34].

In most cases, viscosity measurements can be used to confirm such intercalative behaviour. However, since our systems are polymeric, we were not able to use the viscosity method, because not only changes in the structure of RNA but also the changes in the structure of the polymer–copper(II) complex would change the viscosity of the solution.
Conclusions

The binding interactions between tRNA and polyethyleneimine-anchored copper(II) complexes with 1,10-phenanthroline and 2,2′-bipyridine ligands and of various degrees of coordination have been studied using UV–Visible absorbance, fluorescence and circular dichroism spectral studies. The results suggest that the complex binds to yeast tRNA electrostatically and that these spectroscopic methods are effective for studying the interaction mode of metal complexes with RNA. For both polyethyleneimine-anchored copper(II) complexes containing 1,10-phenanthroline and 2,2′-bipyridine ligands, polymers with higher degree of complex coordination bind more strongly than samples with lower degree of coordination.

Acknowledgments

We are grateful to the University Grants Commission–Special Assistance Programme & COSIST and Department of Science and Technology and Fund for Improvement of S&T infrastructure programmes of the School of Chemistry, Bharathidasan University, and Research Schemes by Council of Scientific and Industrial Research (Grant No. 01(2075)(06/EMR-II) and University Grants Commission (Grant No. F. 32-274(2006), New Delhi, sanctioned to one of the authors, S.A. We express sincere thanks to Prof. PR. Athappan, Department of Inorganic Chemistry, Madurai Kamaraj University, Madurai, for CD studies.

References

Synthesis, nucleic acid binding and cytotoxicity of polyethyleneimine-copper(II) complexes containing 1,10-phenanthroline and L-valine

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A R T I C L E   I N F O

Article history:
Received 20 October 2010  
Received in revised form 8 April 2011  
Accepted 11 April 2011  
Available online 16 April 2011

Keywords:
BPEI, branched polyethyleneimine  
Copper(II) complex  
DNA and tRNA interaction  
Cytotoxic studies

A B S T R A C T

The polymer–copper(II) complex samples, [Cu(phen)/L-Val]·BPEI·Cl·H₂O, with varying degrees of coordination in the polymer chain, were prepared and characterized by elemental analysis and spectroscopic methods. The binding of these complex samples with both DNA and RNA has been investigated. The experimental results indicate that the polyethyleneimine–copper(II) complex samples bind with DNA and RNA mostly through surface binding; but hydrogen bonding and van der Waals interactions are also present. Evaluation of cytotoxic activity of a sample of polymer–copper(II) complex with higher degree of coordination against different cancer cell lines proved that the complex exhibited cytotoxic specificity and significant cancer cell inhibition rate.

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1. Introduction

Nucleic acids are very important genetic substances. They provide a range of binding sites and binding modes for covalent and non-covalent interactions [1–6]. Interest in the binding of metal complexes to DNA has been motivated not only by a desire to understand the basics of these interaction modes but also by the development of metal complexes into anti-inflammatory, anti-fungi, antibacterial and anticancer agents. Hence, much attention has been targeted on the design of metal-based complexes, which can bind to DNA [7–10]. It is currently admitted that RNAs play a key role in many biological processes involving living cells and are considered as the essential regulators of many steps of gene expression [11]. Furthermore, many viruses such as HIV, HCV, influenza and flaviviruses are encoded by a unique RNA molecule that serves as the main genetic material. Therefore, metal complexes that bind to RNA and disturb RNA function could be powerful tools for understanding and controlling gene expression [12–14]. Recently different ligands and metal complexes have been developed that bind to RNA structure [15–19]. Therefore, a comparative study of the interactions of transition metal complexes with RNA and DNA is needed for the future development of nucleic acids-targeting drugs. The development of reagents that can form intrastrand crosslinks with nucleic acid continues to be a subject of considerable interest in the areas of microbiology and national drug design [20]. Amino acids are the structural units of proteins that recognize a specific base sequence of DNA and they are highly water soluble. Copper is a bioessential element in all living systems. Recently, novel copper(II) complexes with amino acid ligand have been receiving considerable interest due to their biological relevance, good nucleic acid binding, antimicrobial and anticancer activities [21–23]. In addition, polynuclear copper complexes are attracting much attention currently. Incorporation of more than one copper centre in a single polymer chain produces enhanced electrostatic interactions to the anionic nucleic acid phosphate backbone [24]. Polymamine compounds are often based on their polycationic nature at physiological pH because in that environment, they are known to play an essential role for many biological processes [25]. Polyethyleneimine, (PEI), possesses quite a number of advantages as polymer chelating agent, such as good water solubility, high content of functional groups, suitable molecular weights as well as good physical and chemical stabilities [26]. There are some reports on polyethyleneimine–copper(II) complexes which are very stable and copper ions are hard to elute from the polymer domain to the bulk solution and K values are from 1 to 5 orders magnitude greater than in the polymer–chelate

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systems than in the monomeric Cu-complex systems [27]. Recently we have reported some interesting results on the various interacting nature of polymer—metal complexes with DNA and RNA [28–32].

The present work deals with synthesis and characterization of the water soluble polyethyleneimine coordinated complex samples containing ligand valine for its high water solubility and less steric hindrance, [Cu(phen)(l-Val)] BPEI·Cl·H₂O (where l-Val = l-Valine, phen = 1,10-phenanthroline and BPEI = branched polyethyleneimine) with varying degrees of coordination (x) in the polymer chain. The nucleic acid binding capability of these polymer—copper(II) complexes with ct-DNA (calf thymus DNA) and yeast tRNA (RNA) were studied by various spectroscopic and other methods. The antimitotic activities of polymer-complex as well as ordinary precursor complex on Jurkat, a T cell lymphoma cell line and Raji, a B Cell lymphoma cell line using different cell death indicator stains and MIT assay have been performed.

2. Results and discussion

2.1. Degree of coordination

The structure of the polymer—copper (II) complex is shown in Fig. 1. In this figure ‘x’ represents the degree of coordination, which is the number of moles of copper(II) chelate per mole of the repeating unit (amine group) of polymeric ligand. If the entire repeating units (amine group) in the polymer are coordinated to copper, then the value of x is 1. It can be calculated either from carbon content or copper content [33]. The degree of coordination thus obtained for the polymer—copper(II) complex samples of the present work are 0.238, 0.433 and 0.541.

2.2. Spectral characterization

For our polymer—copper(II) complexes, the bands obtained around 1466 cm⁻¹ and 1387 cm⁻¹ in IR region can be attributed to the ring stretching frequencies viz., ν(C=C) and ν(C=N) respectively of 1,10-phenanthroline. The out of plane bending values, ν(C=H) at 852 cm⁻¹ and 730 cm⁻¹ for the phenanthroline ligand are red shifted to 835 cm⁻¹ and 689 cm⁻¹ respectively in the complex. These shifts can be explained by the fact that each of the two nitrogen atoms of phenanthroline ligands donates a pair of electron to the central copper atom forming coordinate bond [29]. The band around 2925 cm⁻¹ can be assigned to C–C stretching vibration of aliphatic CH₂ of BPEI whereas the broad band observed around 3442 cm⁻¹ is assigned to the N–H stretching of BPEI [34]. Absence of band in the region of 1750–1700 cm⁻¹ indicates that COO⁻ coordinated to the complex and it is shifted to 1630 cm⁻¹. In the UV–Visible region, the intense absorption bands appear from 200 to 350 nm is attributed to intraligand transitions. The other absorption band which appears around 640 nm is assigned to d–d transition [35]. The electron magnetic resonance spectra (ESR) of Cu³ complex with a giso = 2.079 confirms the square-pyramidal geometry [36].

Examination of the polymer—copper(II) complexes using scanning electron microscopy with different magnifications are shown in Fig. 2. The image reveals that the metal complexes are coordinated on the surface of the polymer.

2.3. Nucleic acid binding studies

2.3.1. Absorption titration studies

Electronic spectroscopy is considered as an effective method to examine the binding mode of nucleic acid interaction with transition metal complexes. The binding behaviour of polymer—copper(II) complexes to nucleic acid has been followed through absorption spectral titrations. The absorption spectra of the complexes in the absence and in the presence of nucleic acid are shown in Fig. 3. As the concentration of nucleic acids increases, the absorption band of polymer—copper(II) complexes exhibits hyperchromism. In order to compare the binding strength of the polymer—copper(II) complexes with nucleic acid, the intrinsic binding constant, Kᵦ, has been determined using the equation, [NA]([cₐ – cₜ] – [NA][cₐ – cₜ] + 1)/Kᵦ(cₐ – cₜ), where [NA] is the concentration of DNA or RNA expressed in base pairs; cₜ, cₚ and cₐ are the apparent, free and fully bound copper(II) complex extinction coefficients [37]. A plot of [NA]([cₐ – cₜ]) versus [NA] gives Kᵦ as the ratio of the slope to intercept (Inset Fig. 3). The Kᵦ values thus obtained for our polymer—copper(II) complexes with different degrees of coordination are given in Table 1. As seen in Table 1 the Kᵦ value increases with degree of coordination as we have observed in similar types of systems earlier [28–32]. The polymer—copper(II) complex can bind to nucleic acid in different binding modes on the basis of their structure and charge and type of ligands. As DNA double helix possesses many hydrogen bonding sites which are accessible to both, the minor and major grooves, it is likely that the amine groups of PEI forms hydrogen bonds with DNA which contribute hyperchromism observed in the absorption spectra. In the case of tRNA the hyperchromism indicates that the polymer—copper(II) complexes bind with tRNA electrostatically. This electrostatic interaction may be between the positively charged polymer—copper(II) units and negatively charged phosphate backbone of the tRNA. On the other hand, our polymer—copper(II) complex, which possesses several methylene groups on the branched polyethylene can also bind to nucleic acid by van der Waals interactions between the methylene groups and the valine methyl groups. In our complex, the copper(II) chelate are randomly coordinated to the branched polymer, hence complete intercalation of phenanthroline ligand between a set of adjacent base pairs is sterically impossible. But this needs further clarification and large number of copper(II) chelate present in the complex which

![Fig. 1. Structure of [Cu(phen)(l-Val)] BPEI·Cl·H₂O.](image-url)
can cooperatively act to increase the overall binding ability of polymer–copper(II) complex to DNA. Interestingly, the $K_0$ values obtained for our polymer–copper(II) complex samples are very much higher than those of the other known monomer copper complexes $[\text{Cu(phen)}_2\text{Cl}_2]$ ($K_0 = 2.75 \times 10^5 \text{M}^{-1}$) and $[\text{Cu(phen)}_2\text{Cl}_4]$ ($K_0 = 4.75 \times 10^5 \text{M}^{-1}$). [38,39]. Also $K_0$ values for our complex samples are higher than known association constant of BPEI to DNA ($K_0 = 1.2 \text{ M}^{-1}$). [40]. Besides the binding constant indicates that our polymer–copper(II) complex samples can bind very strongly with DNA when compared to RNA.

2.3. Fluorescent spectral studies

All our polymer–copper(II) complexes were non-emissive upon excitation of the MLCT band, either in aqueous solution or in the presence of nucleic acid. Ethidium bromide (EB) is known to emit intense fluorescence in the presence of DNA and RNA due to the binding of EB to nucleic acid. When our polymer–copper(II) complexes are added to nucleic acid pretreated with EB, the nucleic acid-induced emission intensity of EB decreases (Fig. 4). Addition of a second nucleic acid binding molecule would quench the EB emission by either replacing the nucleic acid EB (if it binds to nucleic acid more strongly than EB and/or by accepting the excited state electrons from EB). The quenching behaviour can be analyzed through the Stern–Volmer equation [41], $I_0/I = 1 + K_{sv}r$, where, $I_0$ and $I$ are the fluorescence intensities in the absence and presence of complex, respectively, $K_{sv}$ is the linear Stern–Volmer quenching constant and $r$ is the ratio of the total concentration of complex to that of nucleic acid. A plot of $I_0/I$ versus $[\text{complex}]/[\text{NA}]$ is drawn (Inset Fig. 4), $K_{sv}$ is obtained from the ratio of slope to intercept. The $K_{sv}$ values for the polymer–copper(II) complexes thus obtained for binding with DNA are 0.043, 0.048 and 0.049 and with RNA are 0.024, 0.031 and 0.038 respectively with increasing degree of coordination. This trend is also similar to what we have observed earlier for similar type of complexes. This is also evidence for additional non-intercalative fluorescence enhanced sites which are presumed to involve electrostatic binding (surface binding).

2.3.3. Circular dichroic spectral studies

The CD spectrum of ct-DNA alone shows a positive band at 272 nm and a negative band at 245 nm due to the $\pi$–$\pi$ stacking of the base pairs and right-handed helicity of B-form DNA, respectively [42]. The changes in CD signals of DNA observed on interaction with drugs may often be assigned to the corresponding changes in DNA structure. Upon adding our polymer–copper(II) complex ($x = 0.541$) (Fig. 5a), the CD spectrum of DNA undergoes changes in both the positive and negative bands. Slight changes in intensities of both bands, and slight shifts in band position are observed. This reveals that the complex binds with DNA electrostatically (surface binding). Thus simple groove binding and electrostatic interaction of molecules show less or no perturbation on the base stacking and helicity, while intercalation enhances the intensities of both the bands stabilizing the right-handed B conformation of ct-DNA as observed for the intercalator [43]. In the case of tRNA, the CD spectrum consists of a positive band around 275 nm due to ellipticity and a small negative band around 245 nm due to right-handed helicity [44]. On adding the polymer–copper complex ($x = 0.541$) the ellipticity band intensity changes with slight blue shift and the helicity band affected which is shown in Fig. 5b, this is due to electrostatic binding of the polymer–complex with negatively charged RNA backbone. This indicates

![Fig. 1](image1.png) Absorption spectra of $[\text{Cu(phen)}(\text{l-Val})\text{BPEI}]\text{Cl}_2\text{H}_2\text{O}$ ($x = 0.541$): (a) in the absence of DNA (dotted line) and in the presence of DNA (solid line) $[\text{complex}] = 5 \times 10^{-5}$ M, $[\text{DNA}] = 0.165 \times 10^{-4}$ (b) in the absence of RNA (dotted line) and in the presence of RNA, $[\text{complex}] = 9 \times 10^{-5}$ M, $[\text{RNA}] = 0.13 \times 10^{-5}$ M. (inset: plot of $[\text{NA}]/(\lambda_a - \lambda_d)$ versus $[\text{NA}]$).
that the polymer-complex interacts with RNA also through electrosstatic mode of binding (surface binding).

2.3.4. Cyclic voltammetry studies

The application of cyclic voltammetry to the studies of transition metal complex bound to DNA provides a useful complement to the previously used methods of investigation, such as UV–visible and fluorescence spectroscopic techniques. The cyclic voltammograms of the polymer–copper(II) complex is taken tris–HCl buffer. The cationic peak potential (Epc) and the anionic peak potential (Epa) are ~ −0.70 mV and ~ +49 mV respectively and the separation of the anodic and cathodic peak potential (∆Ep) is 119 mV. The ratio between the peak current is 0.95, indicating that the complex shows a quasi-reversible redox couple of Cu(II)/Cu(I). The cyclic voltammogram of 1 mM of polymer–copper(II) complex (x = 0.541) in the absence and presence of DNA are shown in Fig. 6. The complex shows one well defined redox couple corresponding to Cu(II)/Cu(I) in the absence and presence of DNA, as expected. The measured ∆Ep value (~ 120 mV) indicates that the redox couples are quasi reversible. The i//iC falls at cal. 1.05–1.10, clearly confirming one electron transfer in the redox process. After adding DNA, the peak current of the complex increased significantly, the reduction peak potential shifted negatively. Bard et al. have pointed out that the shift direction of the electrochemical potential after reacting with DNA is related to its binding mode with DNA and a negative shift is characteristic of an electrostatic interaction [45]. In addition to changes in formal potential upon addition of DNA, the voltammetric current increases significantly as shown in Fig. 6. The dependence of cathodic currents of the polymer–copper(II) complex on different scan rate (v) is investigated and the results are shown in the inset Fig. 6. It is observed that the cathodic currents of the polymer–copper(II) complex both with and without DNA are linear to the square roots of the scan rate (v1/2), which are expected for the diffusion-controlled process [46]. So, we can interpret the changes in current upon DNA addition in terms of the diffusion of an equilibrium mixture of unbound and DNA bound complexes on the electrode surface.

2.3.5. Gel electrophoresis

The spectroscopic results reveals that the polymer–copper(II) complex with higher degree of coordination (x = 0.541) binds strongly to DNA. Binding of these complexes with DNA was also studied by gel electrophoresis using plasmid pBR322 DNA. Many polycationic organic polymer molecules interact with DNA through electrostatic interactions between phosphate groups of the DNA and oppositely charged groups of polymer [47–49]. Forrest et al. [50] and Vinogradov et al. [51] have reported that the organic polymer, polyethyleneimine (PEI), retards the DNA migration mainly through electrostatic interactions in the agarose gel electrophoresis experiments and they have suggested that the binding between DNA and participating species. In order to substantiate the binding of our polymer–copper(II) complexes to DNA, gel electrophoresis has been performed with a sample of the polymer–copper(II) complex (Fig. 7). Lane 1 is pure DNA not treated with complex. Lanes 2–6 represent the DNA treated with the polymer–copper(II) complex of various solution concentrations. As reported by previous workers on the interaction of DNA-polyethyleneimine alone, in our work also the migration of the DNA band is retarded when the concentration of polymer–copper(II) complex is 40 μM. This clearly demonstrates that the cationic segments of the branched polyethyleneimine neutralize the negative charges of DNA.

2.3.6. Cytotoxicity assay

Cytotoxic effect of [Cu(phen)(l-Val)]BEI|Cl2H2O (x = 0.541) was examined on cultured primary cell (Normal human PBMC) and established cancer cell lines of T cell and B cell origin (Jurkat and Raji respectively) by exposing the cells up to 72 h. The cytotoxicity was assessed by three different assay viz. Dye Exclusion Assay [52], Sulforhodamine B assay [53], MT assay [54].

2.3.6.1. Dye exclusion assay

For dye exclusion method the cells were assessed by bright field microscopy for their viability after 24, 48, 72 h treatment with [Cu(phen)(l-Val)]BEI|Cl2H2O (x = 0.541) of concentration ranging from 5 μM–1280 μM. The results of cytotoxic activity on human PBMC was directly based on its viable count by dye exclusion method i.e. the dead cells has the tendency of taking the dye and settle at the bottom of the well and the viable cells won’t take up the dye and hence opaque and will float in the medium, which was counted and calculated using haemocytometer.

The bar diagram shows the percentage viability of cells upon treatment with various concentration of the complex [Cu(phen)(l-Val)]BEI|Cl2H2O on human PBMC – a normal cell. At 0 h the percentage viability ranges between 95 and 98% and as the
time and concentration goes higher the viability of the cells decreases i.e. the percentage viability versus concentration has an inverse correlation. Similarly, the percentage of viable cells (viability) versus treatment time has an inverse correlation. 1280 μM of the compound after 72 h of treatment there was a huge reduction in viable cells which has gone to 9%, whereas it was 61% with 5 μM concentration after 72 h treatment (Fig. 8).

2.3.6.2. SRB assay. The graph (Fig. 9) shows the percentage inhibition of the growth of normal cell (PBMC) and two different cancer cell lines viz. Jurkat and Raji after treatment with [Cu(phen)(L-Val)BPEI]Cl·H₂O (x = 0.541). There is a direct correlation between the concentration of the complex and percentage growth inhibition. Both the cancer cell lines and the normal cells were maximum affected by [Cu(phen)(L-Val)BPEI]Cl·H₂O, at 1280 μM and the IC₅₀ of which varies from 10 μM, 24 μM, 18 μM for PBMC, Raji cell line and Jurkat cell line respectively. When the precursor ordinary complex namely, [Cu(phen)(L-Val)H₂O]Cl·H₂O was treated with cell lines showed that the IC₅₀ value was 5100 μM for PBMC, 900 μM for Jurkat and 1300 μM for Raji.

2.3.6.3. MTT assay. The plot of concentration of polymer-complex, [Cu(phen)(L-Val)BPEI]Cl·H₂O, versus percentage cytotoxicity in terms of mitochondrial enzyme dehydrogenase was shown in this graph (Fig. 10). This also supports the results of dye exclusion method and SRB assay, i.e. as the concentration of complex increases, the percentage cytotoxicity also increases. Maximum number of cells was affected at 1280 μM after 72 h. The IC₅₀ value of the polymer-complex is 7.5 μM for PBMC, 20 μM for Jurkat and 15 μM for Raji. When the precursor ordinary complex, [Cu(phen)(L-Val)H₂O]Cl·H₂O was treated with cell lines the IC₅₀ values obtained were 81 μM, 185 μM, 145 μM for PBMC, Raji cell line and Jurkat cell line, respectively. Out of these two methods the MTT assay requires cellular metabolic activity to convert the colourless tetrazolium to the purple-coloured formazan dye; therefore, it detects only viable cells, whereas the SRB method does not distinguish between viable and dead cells. This difference, however, does not compromise the ability of the SRB assay to detect cytotoxic effects of a drug. Studies undertaken by several groups showed that results from the SRB assay correlated well with those of the MTT assay, although the IC₅₀ values of compounds tested using the SRB method were higher [55–57].

These results prove that the polymer-complex is more cytotoxic to normal and cancer cell lines compared to that of ordinary precursor complex.

Bright field microscopic analysis of treated cells (Fig. 11) revealed that our polymer-complex is toxic to both normal and cancer cells. When these cells were analyzed after 24, 48 and 72 h treatment we could see the toxic effect of the compound by way of their morphology. The dead cells settled at the bottom of the well. The percentage cytotoxicity increases as the concentration goes on higher and the time duration increases. That is [Cu(phen)(L-Val)BPEI]Cl·H₂O inhibited the growth of normal human PBMC, T and B lymphoma cell lines significantly in a dose and duration dependent manner. The overall impression is that [Cu(phen)(L-Val)BPEI]Cl·H₂O inhibited the growth of both primary and established cell lines and the effect is based on dose and duration.
3. Conclusion

We have synthesized water soluble polyethyleneimine—copper (II) complexes containing 1,10-phenanthroline and l-Valine with three different degree of coordination and characterized them by spectrochemical and elemental analysis. The binding of these complex samples with nucleic acids (DNA and RNA) was studied using UV–Visible absorption, emission spectroscopies, circular dichromism spectroscopy, cyclic voltammetry and gel electrophoresis. The results indicate that the complexes bind with nucleic acids; for DNA this binding is through electrostatic mode (surface binding), and other binding mode such as van der Waals, hydrogen bonding, without changing the helical nature of DNA and in the case of tRNA it is electrostatic binding and other non-covalent interactions with affecting the right-handed helicity of tRNA. The cytotoxic effect of the complex (x = 0.541) was studied using different cancer cells. The compound is toxic to cancer cells as well as normal cells. The concentration and IC50 values of the compound vary among different types of cancer cells. Both SRB and MTT assay methods indicated that the polymer-complex, [Cu(phen)l-l-Val] (BPEI)Cl H2O, is more cytotoxic to normal and cancer cell lines compared to that of ordinary precursor complex, [Cu(phen) (l-Val)[H2O]]Cl H2O.

4. Experimental section

4.1. Materials

Calf thymus DNA, yeast tRNA (tRNA) and branched polyethyleneimine (BPEI) (Mw ca. 25,000) were obtained from Sigma–Aldrich, Germany, and were used as such. Copper(II) chloride dihydrate, 1,10-phenanthroline were purchased from Merck, India and l-Valine was obtained from Loba Chemie, India. The spectroscopic titrations were carried out in the buffer (50 mM NaCl—5 mM Tris—HCl, pH 7.1) at room temperature. A solution of calf thymus DNA in the buffer gave a ratio of UV absorbance at 260 and 280 nm of ~1.8—1.9:1, indicating that the DNA was sufficiently free of protein [58]. Plasmid pBR322 DNA was purchased from Genei, India. All the experiments involving the interaction of the polymer—copper(II) complex with nucleic acids were carried out with twice distilled water in buffer containing 5 mM Tris—HCl/50 mM NaCl at pH 7.0. The complex, [Cu(phen)l-l-Val] H2OCl H2O, was prepared as reported earlier [35]. Jurkat, a T cell lymphoma cell line and Raji, a B Cell lymphoma cell lines were obtained from National Centre for Cell Sciences (NCCS) Pune, India.

4.2. Physical measurements

The carbon, hydrogen and nitrogen contents of samples were determined at SAIF, Lucknow, India. Absorption spectra were recorded on a UV–Vis–NIR Cary300 Spectrophotometer using cuvettes of 1 cm path length, and emission spectra were recorded on a JASCO FP 770 spectrophotofluorimeter. Scanning electron microscopic images were taken at SASTRA University, India. FT-IR spectra were recorded on an FT-IR JASCOO 460 PLUS spectrophotometer with samples prepared as KBr pellets. EPR spectra were recorded on JEOL-FA200 EPR spectrometer. Electrochemical measurements were made on an Princeton EG and G-Parc model potentiostat. A three-electrode system comprising a glassy carbon working electrode, a platinum wire auxiliary electrode and a saturated calomel reference (SCE) electrode was used for voltammetry studies. The buffer (5 mM Tris—HCl/50 mM NaCl, pH 7.2) solution was used as supporting electrolyte.
4.3. Synthesis and stability of polymer–copper(II) complexes

To a solution of BPEI (0.15 g) dissolved in ethanol (15 mL), [Cu(phen)(l-Val)(H2O)]Cl·H2O (1.7 g) in water was added slowly with stirring. The mixture was heated between 50 and 60 °C for 15 h in a water bath. After being warmed enough, the dark blue solution was dialyzed approximately at 15 °C against distilled water for 4–5 days. Afterwards the solvent was evaporated by a rotary evaporator under reduced pressure at room temperature. A dark blush filmy substance was obtained. It was pulverized and dried. Yield, 0.27 g (Anal. Calc.: C 46.90; H 5.57; N 14.55; O 5.83. Found: C 46.90; H 5.48; N 15.15; O 5.67% and x = 0.541).

The polymer–copper(II) complex samples with various amounts of copper(II) complex units bound to the polymer chain were synthesized by varying the amount of [Cu(phen)(l-Val)(H2O)]Cl·H2O in the reaction solution was varied between 0.6 and 1.0 g along with changing time between 6 and 10 h and keeping the range of temperature same. These complexes are very stable in solution when we occasionally kept the solutions of our polymer–copper(II) complexes (which we are used in our experiments) in dialysis bag we never observed the presence of any free quantity of copper complex ion or copper ion in the solution outside the dialysis bag (which we verified through the spectrophotometric method) which indicated that our polymer-complexes are very stable during the handling of our experiments.

4.4. DNA and tRNA binding experiments

The nucleic acid binding experiments were performed at 25.0 ± 0.2 °C. The DNA concentration per nucleotide was determined by electronic absorption spectroscopy using the known molar extinction coefficient value of 6600 M−1 cm−1 and 9250 M−1 cm−1 for RNA at 260 nm [59,60].

Absorption titration experiments of polymer–copper(II) complexes in buffer (50 mM NaCl–5 mM Tris–HCl, pH 7.1) were performed by using a fixed complex concentration to which increments of the nucleic acid stock solutions were added. Polymer–copper(II) complex–nucleic acid solutions were allowed to incubate for 10 min before the absorption spectra were recorded. Equal solution of nucleic acid was added to both complex solution and reference solution to eliminate the absorbance of nucleic acid itself.

For fluorescence quenching experiments nucleic acids were pretreated with ethidium bromide (EB) for 30 min. The polymer–copper(II) complexes were then added to this mixture and their effect on the emission intensity was measured. Samples were excited at 450 nm and emission was observed between 500 and 700 nm.

Circular dichroic spectra were recorded at room temperature using the same tris buffer.

For the cyclic voltammetry experiment, the electrode surfaces were freshly polished with alumina powder and then sonicated in ethanol and distilled water for 1 min prior to each experiment. The electrode rinsed with distilled water thoroughly. Cyclic voltammetric experiments were performed at 25.0 ± 0.2 °C in a single compartment cell with a three-electrode configuration, classy carbon working electrode, platinum wire auxiliary electrode and saturated calomel as reference electrode using tris buffer. The solution was deoxygenated with nitrogen gas for 20 min prior to experiments.

For the gel electrophoresis experiments, supercoiled pBR322 DNA (0.1 μg) was treated with the polymer–copper(II) complex in 50 mM Tris–HCl, 18 mM NaCl buffer, pH 7.2. The samples were electrophoresed for 3 h at 50 V on a 0.8% agarose gel in Tris–acetate acid–EDTA buffer. The gel was stained with 0.5 μg/mL of ethidium bromide and photographed under UV light.

4.5. Cell culture

4.5.1. Control cell

Normal Human Peripheral Blood Mononuclear Cells (PBMC) were used as control cells and were isolated from the peripheral blood of apparently healthy normal human beings after getting informed consent. The cells were separated by density gradient centrifugation [61]. The separated cells were cultured in RPMI 1640 Media (HiMedia, Mumbai, India) supplemented with 10% Foetal Calf Serum (FCS) (HiMedia, Mumbai, India), 100 IU/ml of Penicillin (HiMedia, Mumbai, India) and 100 μg/ml of Streptomycin (HiMedia, Mumbai, India).

4.5.2. Cancer cell lines

The cancer cell lines were cultured in RPMI 1640 Medium (HiMedia, Mumbai, India) supplemented with 10% Foetal Calf Serum (FCS) (HiMedia, Mumbai, India). 100 IU/ml of Penicillin (HiMedia, Mumbai, India) and 100 μg/ml of Streptomycin (HiMedia, Mumbai, India) were also added to the media as antibiotics to control the growth of contaminating microorganisms. The cells were cultured in 96 well tissue culture plates (Greiner, USA), and kept at 37 °C in a humidified atmosphere of Sperecentage CO2 in a CO2 incubator (TC2323, Shel Lab, U.S.A). All the experiments were performed using cancer cell lines (Jurkat, Raji) of 10–15 passage and freshly isolated normal cell (PBMC).
4.6. Cytotoxicity assay

4.6.1. Preparation of the complex solution

A known concentration of the complex, \([\text{Cu(phen)}]([\text{Val}][\text{BPEJ}])\)

\(\text{Cl}_2\text{H}_2\text{O}\) was dissolved in known volume of PBS pH-7, to get stock solution of the complex. The stock solutions were membrane filtered to remove microbes if any. The stock solutions were then diluted to get various concentrations of the complexes. The solution of the precursor ordinary complex, \([\text{Cu(phen)}][\text{[Val]}][\text{H}_2\text{O}])\)

\(\text{Cl}_2\text{H}_2\text{O}\) was also made in the same way.

4.6.2. Dye exclusion assay [52]

100 µl of different concentrations of the compounds were added to a series of wells in a 96 well tissue culture plate containing (Greiner, USA) 1 \(\times 10^5\) cells per well. PBS was added as the solvent control. PHA (proliferator) and LPS (cytotoxin) were added as known controls. Immediately after the addition of cells as well as the compound and just before incubation in the CO2 incubator, the cell morphology was monitored under inverted phase contrast microscope (Nikon TM24). By doing this, the gross morphological changes were observed and photographed. Then the cells were incubated for 24, 48 and 72 h and after the incubation period the viable cell counts were made using trypan blue dye and haemocytometer. The percentage viability was calculated by plotting the number of viable cells versus concentration of the complexes.

4.6.3. Sulforhodamine B assay [53]

100 µl of different concentrations of the compounds were added to a series of wells in a 96 well tissue culture plate (Greiner, USA) containing 1 \(\times 10^5\) cells per well. PBS was added as the solvent control. PHA (proliferator) and LPS (cytotoxin) were added as known controls. Then the cells were incubated at 37 °C in a humidified atmosphere of 5% CO2 in a CO2 incubator (TC2323, Shel lab, U.S.A) for 72 h. After 72 h the cells were fixed in the substratum by adding 50 µl of cold (4 °C) TCA (50%). The final TCA concentration was kept at 10%. After adding TCA, the plates were left for 30 min at 4 °C and were washed five times with deionized water. After washing, the plates were air dried at room temperature. The fixed cells were stained with 0.4% sulforhodamine B (SRB) (Sigma, USA) dissolved in 1% acetic acid. The staining was done for 30 min. SRB stains only the cellular proteins. The excess stain was washed with 1% acetic acid. The stained proteins were solubilized using 10 mM trizma base in PBS. The colour developed was detected at 490 nm (measurement) and 630 nm (reference) using a micro plate reader (Biorad, USA). The intensity of the colour developed is directly proportional to the protein concentration of the cells. The assay was performed in triplicates and the respective mean values were calculated and used for further calculation. The percentage inhibition was calculated from this data using the formulae given below:

\[
\text{Percentage of control cell growth} = \frac{\text{Mean OD of treated cell sample} - \text{Mean OD of the cell at day zero}}{\text{Mean OD of untreated cells (control)}} \times 100
\]

\[
\text{percentage growth inhibition} = 100 - \text{percentage of control cell growth}
\]

\[
\text{Percentage inhibition} = \frac{\text{Mean OD of untreated cell(control)} - \text{Mean OD of treated cell}}{\text{Mean OD of untreated cells(control)}} \times 100
\]

4.6.4. MTT assay [54]

100 µl of different concentrations of the compounds were added to a series of wells in a 96 well tissue culture plate (Greiner, USA) containing 1 \(\times 10^5\) cells per well. PBS was added as the solvent control. PHA (proliferator) and LPS (cytotoxin) were added as known controls. Then the cells were incubated at 37 °C in a humidified atmosphere of 5% CO2 in a CO2 incubator (TC2323, Shel lab, U.S.A) for 72 h. After the incubation period 20 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; (MTT) (Sigma, USA) (5 mg/ml in Phosphate Buffered Saline (PBS)) was added to each well and the plates were wrapped with aluminium foil and incubated at 37 °C for 4 h. By this treatment a purple formazone product was formed due to the reduction of MTT by mitochondrial enzyme dehydrogenase of the cells. This formazone was dissolved by the addition of 100 µl of acetic isopropanol (0.04 N HCl in proponal) to each well. Then the absorbance was monitored at 570 nm (measurement) and 630 nm using a micro plate reader (Biorad, USA). The assay was performed in triplicates and the respective mean values were calculated and used for further calculation. The percentage inhibition was calculated from this data using the formula:

\[
\text{Percentage inhibition} = \frac{\text{Mean OD of untreated cell(control)} - \text{Mean OD of treated cell}}{\text{Mean OD of untreated cells(control)}} \times 100
\]

Acknowledgement

We are grateful to the UGC-SAP and COSIST and DST-FIST programmes of the Department of Chemistry, Bharathidasan University. One of the authors, SA, thank the Department of Science and technology, Government of India for sanction of a research scheme (Grant No. SR/S1/IC-13/2009). We thank Prof. M. B. Viswanathan, Department of Plant science, Bharathidasan University, for providing the gel documentation facility. We express sincere thanks to Prof. Pr. Athappan and Prof. A. Ramu, Department of Inorganic Chemistry, Madurai Kamaraj University, Madurai, for CD studies.

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