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Original article

DNA binding and antimicrobial studies of polymer–copper(II) complexes containing 1,10-phenanthroline and L-phenylalanine ligands

Rajendran Senthil Kumar, Sankaralingam Arunachalam*

School of Chemistry, Bharathidasan University, Tiruchirappalli 620 024, Tamil Nadu, India

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ABSTRACT

Some water-soluble polymer–copper(II) complexes, $[Cu(phen)(L\text{-phe})(BPEI)]ClO_4 \cdot 4H_2O$ (phen = 1,10-phenanthroline, L-phe = L-phenylalanine, and BPEI = branched polyethyleneimine), with various amounts of copper(II) chelates in the polymer chain, were prepared by ligand substitution method in water–ethanol medium and characterized by infra-red, UV–visible, EPR spectral and elemental analysis methods. Binding of these complexes with calf thymus DNA (CT DNA) has been investigated by absorption spectroscopy, emission spectroscopy and gel electrophoresis techniques. The experimental results indicate that the amount of copper(II) chelate content in the polymer backbone have marked effect on the binding affinity to CT DNA. Interactions like electrostatic interaction, van der Waals interaction, hydrogen bonding and/or partial intercalation binding modes exist in this system. A sample of polymer–copper(II) complex was tested for its antibacterial and antifungal activity against certain human pathogenic organisms and it was found to have good antibacterial and antifungal activities.

1. Introduction

DNA plays an important role in the life process since it contains all the genetic information for cellular function. However, DNA molecules are prone to be damaged under various conditions like interactions with some molecules. This damage may lead to various pathological changes in living organisms. The binding interaction of transition metal complexes with DNA is of interest for both therapeutic and scientific reasons [1–3]. These transition metal complexes are known to bind to DNA via both covalent and non-covalent interactions. In covalent binding the labile ligand of the complexes is replaced by a nitrogen base of DNA such as guanine N7. On the other hand, the non-covalent DNA interactions include intercalative, electrostatic and groove (surface) binding of cationic metal complexes along outside of DNA helix, along major or minor groove.

Copper is a biologically relevant element and many enzymes that depend on copper for their activity have been identified. Because of its biological relevance, a large number of copper(II) complexes have been synthesized and explored for their biological activities [4–6]. Among these copper complexes, attention has been mainly focused on the copper(II) complexes of 1,10-phenanthroline ligand due to their high nucleolytic efficiency, anti-tumor, anti-candida, and antimicrobial [7–9] activities etc.

Amino acids are the basic structural units of proteins that recognize a specific base sequence of DNA. An amino acid with a side chain aromatic ring, eg. phenylalanine, contributes mainly to the stabilization of proteins through hydrophobic interactions and the formation of hydrophilic environments [10]. Copper complexes containing amino acids have been studied as models for the behaviour of copper enzymes [11] and some copper complexes with amino acid ligands were reported to exhibit potent anti-tumor and artificial nuclease activities [12–14].

A polymer–metal complex is composed of a polymeric ligand and metal ion in which the metal ion is attached to the polymer ligand by a coordination bond. It has been noticed that drug–polymer conjugates are potential candidates for the selective delivery of anti-cancer agents to tumor tissue [15]. Polyethyleneimine (PEI) is a cationic polymer exhibiting the highest positive charge density potential [16] and, recently, PEI has appeared as a possible alternative to viral and liposomal routes of gene delivery [17]. PEI possesses quite a number of advantages as polymer chelating agent, such as good water solubility, high content of functional groups, good physical and chemical stability, etc. [18]. Concerning biochemistry and medicine, further research involving PEI–metal complexes could throw light upon certain biological processes and bring interesting results for the treatment of diseases. Recently, we have reported our interesting results on the interaction of some polymer–cobalt(III)/copper(II) complexes with DNA [19–21].

In this paper we report the synthesis and DNA binding studies of some novel water-soluble polymer (PEI)–copper(II) complex

* Corresponding author. Tel.: +91 431 2407053.
E-mail address: arunasurf@yahoo.com (S. Arunachalam).

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(containing 1,10-phenanthroline and L-phenylalanine ligands) with varying amounts of copper(II) complex moieties in the polymer chain. Also, we report the antimicrobial activities of a sample of this complex against certain human pathogenic organisms.

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 degree of coordination. The degree of coordination (*x*), means the number of moles of copper(II) chelate per mole of the repeating unit (amine group) of polymeric ligand. If all the repeating units (amine groups) in the polymer are coordinated to copper, then the value of *x* is 1. The degree of coordination (*x*) of the copper chelate in the polymer–copper(II) complex could be calculated from the carbon content [22,23] or copper content [24]. The degree of coordination (*x*) thus obtained for the polymer–copper(II) complex samples synthesized were 0.113, 0.128 and 0.162. The *x* value of the polymer–copper(II) complex was found to reach a constant level of about 0.162. Even if the copper chelate was added in large excess or the reaction mixture was warmed for more than 12 h, we could not obtain polymer–copper(II) complex samples with degree of coordination more than 0.162. This may be ascribed to the steric hindrance between metal complex units in the polymer chain which prevents further coordination [22].

2.2. Spectral characterization

The IR values, δ(C–H) 853 cm^{-1}, 737 cm^{-1} observed for phenanthroline are redshifted to 843 and 734 cm^{-1}. This shift can be explained by the fact that the two nitrogen atoms of phenanthroline ligands donate a pair of electrons each to the central copper metal forming a coordinate covalent bond [25]. The bands around 2924 cm^{-1} can be assigned to C–H stretching vibration of aliphatic CH_2 of BPEI. The broad band observed around 3420 cm^{-1} is assigned to the N–H stretching of BPEI [18] and the band around 1086 cm^{-1} has been assigned to ν(Cl–O) of perchlorate anions [15].

The electronic spectra of polymer–copper(II) exhibit two bands in the 220–275 nm regions which are assigned to ligand based transitions. Another band which appeared around 676 nm is assigned to ligand field transitions.

The room temperature EPR spectrum of the polymer–copper(II) complex shows a single broad signal (inset of Fig. 2). The complex exhibits single isotropic feature near $g = 2.033$. Such single isotropic lines are usually results of intermolecular spin exchange, which broadens the lines. This intermolecular type of spin exchange is caused by the strong spin coupling which occurs during a collision of two paramagnetic species. The liquid nitrogen temperature spectrum of the complex shown in Fig. 2. The values of $g_{\|}$ and $g_{\perp}$ are 2.252 and 2.078, respectively. The existence of $g_{\|} > g_{\perp} > 2.0023$ suggest that $d_{x}^{2}-d_{y}^{2}$ is the ground state with the d^9 (Cu^{2+}) configuration [26,27].

2.3. DNA binding studies

2.3.1. Absorption spectral studies

The binding of polymer–copper(II) complexes to DNA helix has been characterized through absorption spectral titrations, by following changes in absorbance and shift in wavelength. The experiments were performed by maintaining a constant concentration of the complex while varying the DNA concentration. Fig. 3 shows the absorption spectra of polymer–copper(II) complex ($x = 0.162$) in the absence and in the presence of calf thymus DNA. In the UV region, the absorption bands around 272 nm, observed in the case of polymer–copper(II) complex, can be attributed to the π–π^* transition of the coordinated ligands. Addition of increasing amounts of calf thymus DNA results in hyperchromism and bule shift. A similar hyperchromism has been observed also for a Cu(II) complex bearing NH and methyl groups [15,28,29] and for the soret bands of certain porphyrins when interacted with DNA but this features has not been yet well explained [30].

The polymer–copper(II) complex can bind to the double-stranded DNA in different binding modes on the basis of their structure, charge and type of ligands. As DNA double helix possesses many hydrogen bonding sites which are accessible both in the minor and major grooves, it is likely that the amine groups of polyethyleneimine forms hydrogen bonds with DNA, which may contribute to the hyperchromism observed in the absorption spectra. On the other hand, our polymer–copper(II) complexes containing several methylene groups of the branched polyethyelene(BPEI), can bind to DNA by van der Waals interactions [31,32] between the methylene groups and the thymine methyl groups [29]. The hyperchromic effect may also be due to the electrostatic interaction [33] between positively charged polymer (BPEI) and the negatively charged phosphate backbone at the periphery of the double helix CT DNA [34]. In our polymer–copper(II) complex, the copper(II) chelates are randomly coordinated to the branched polymer, hence complete intercalation of the phenanthroline ligand between a set of adjacent base pairs is sterically impossible, but some type of partial intercalation can be envisioned [35].

Viscosity and circular dichroism measurements could have been helpful to us to confirm the intercalative behaviour. But as our systems are polymeric and DNA being another polymer we were not able to use viscosity method, because not only changes in the structure of DNA but also the changes in the structure of the polymer–copper(II) complex would change the viscosity of the solution. So it will not be possible to resolve the changes in the viscosity behaviour if at all there is a change in the viscosity behaviour. The solubility of polymer–copper(II) complex samples are very low, so we could not do circular dichroism measurements also.

The intrinsic binding constant (K_b) for the interaction of polymer–copper(II) complex samples with DNA was obtained from

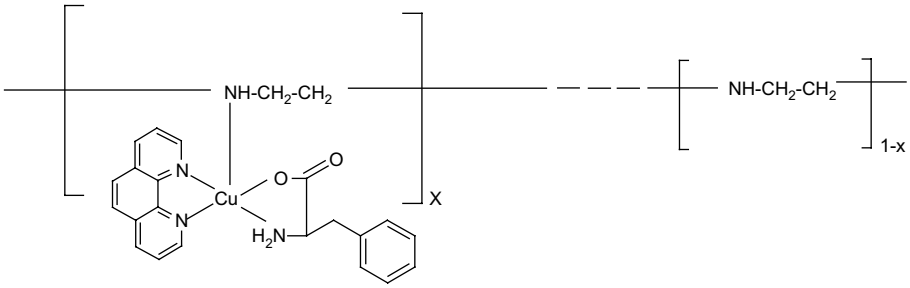

Fig. 1. Structure of [Cu(phen)(L-phe)(BPEI)]$ClO_4 \cdot 4H_2O$ ($x = 0.162$, where *x* is the degree of coordination).

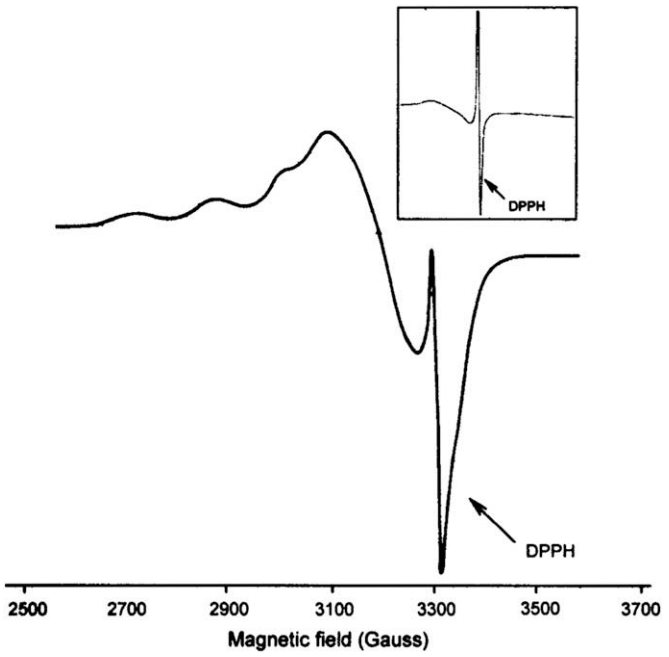

Fig. 2. EPR spectrum of [Cu(phen)(L-phe)(BPEI)]$ClO_4 \cdot 4H_2O$ ($x = 0.162$) in DMSO at liquid nitrogen temperature (inset: solid state EPR spectrum at room temperature).

absorption titration data (at 272 nm ($\varepsilon = 32{,}438\ M^{-1}\ cm^{-1}$)) where only polymer–copper(II) complex absorbs in the concentration range used. No absorption could be observed for DNA. Our attempts to determine the binding constant for the polymer–copper(II) complex samples using the visible region of the spectrum were unsuccessful due to the necessity of using very high concentration of the polymer–copper(II) complex at which the complex–DNA adduct precipitates out of the solution. The intrinsic binding constant, K_b, has been determined using the equation [36],

$$[DNA]/(\varepsilon_a - \varepsilon_f) = [DNA]/(\varepsilon_b - \varepsilon_f) + 1/K_b(\varepsilon_b - \varepsilon_f),$$

where [DNA] is the concentration of DNA in base pairs, ε_a, ε_f and ε_b correspond to A_{obsd}/[total copper complex units], the extinction coefficient of the complex in its free form and the extinction coefficient of the complex in the fully bound form, respectively. A plot of [DNA]/($\varepsilon_a - \varepsilon_f$) versus [DNA], gives K_b as the ratio of the slope to the intercept.

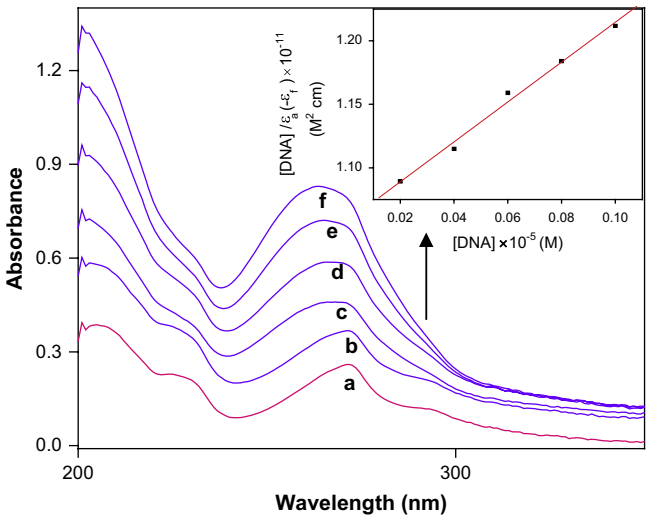

Fig. 3. Absorption spectra of [Cu(phen)(L-phe)(BPEI)]$ClO_4 \cdot 4H_2O$ ($x = 0.162$) in the absence (a) and in the presence of increasing amounts of DNA (b–f), [Complex] = 8 μM, [DNA] = 0.2–1 μM. Arrow shows the absorbance changes upon increasing DNA concentrations. Inset: plot of [DNA]/($\varepsilon_a - \varepsilon_f$) versus [DNA].

The K_b values of polymer–copper(II) complex with different degree of coordination of copper(II) chelate content are shown in Table 1 which indicate that complex with higher degree of copper complex content has higher K_b value. The significant difference in DNA binding affinity of the three polymer–copper(II) complex samples can be understood as a result of the fact that the complex with higher degree of copper content shows stronger binding with DNA. This may be due to the high positive charges and large number of copper(II) complex moieties present in the complex which cooperatively act to increase the overall binding ability of the polymer–copper(II) complex molecule to DNA. Interestingly, the K_b values obtained for our polymer–copper(II) complex samples are very much higher than those for the other known mononuclear and binuclear copper(II) complexes containing 1,10-phenanthroline ($Cu(phen)_2Cl_2$ (K_b, $2.75 \times 10^3\ M^{-1}$)) [37], $Cu_2(phen)_2Cl_4$ (K_b, $4.75 \times 10^4\ M^{-1}$) [38]. Also K_b values for our complex samples are higher than known association constant of PEI to DNA (K_b, $1.2 \times M^{-1}$) [39]. This shows that comparatively our polymer–copper(II) complex samples can bind very strongly with DNA.

2.3.2. Fluorescent spectral studies

The polymer–copper(II) complex samples, however, are non-fluorescent on excitation in the visible region. Hence, competitive binding studies using ethidium bromide (EB) bound to DNA were carried out for polymer–copper(II) complex samples. Ethidium bromide (EB) is a well known cationic dye widely used as probe for native DNA [40]. The fluorescence intensity of EB is very weak, but it is greatly increased when EB is specifically intercalated into the base pairs of double-stranded DNA. It was previously reported that the fluorescent light could be quenched by the addition of a second molecule [41]. Recently Ni et al. [42] found that fluorescence reduction of fluorophore NR (neutral red dye) in the presence of DNA by $Cu(phen)^{2+}$ may be due to the replacement of the DNA intercalator, NR. This means that the complex interacts with the same site as NR does. Zhang et al. [38] also indicate that a binuclear copper(II) complex of 1,10-phenanthroline ligand competitively binds to the EB–DNA system by intercalation. On the other hand, Bronich et al. [43] have reported that the addition of a copolymer (i.e. polyethyleneglycol–polyetheyleneimine(PEI)) to EB–DNA complex results in quenching of the fluorescence due to the displacement of EB by the copolymer. Xiao and Zhan [34] have studied the effect of polymeric Schiff base–nickel complexes on the fluorescence of EB–DNA system and they suggested that a portion of the metal complex, possessing a planar aromatic moiety, intercalates to adjacent base pairs, which inhibits EB binding to calf thymus DNA competitively. In our studies the addition of the polymer–copper(II) complexes to DNA pretreated with EB causes appreciable reduction in the emission intensity (Fig. 4), indicating that the replacement of the EB fluorophore by the polymer–copper(II) complex, results in a decrease in the emission intensity. This behaviour can be analysed through Stern–Volmer equation [44],

$$I_o/I = 1 + K_{sv}r,$$

Table 1
The intrinsic binding constants (K_b) and Stern–Volmer constants (K_{sv}) of [Cu(phen)(L-phe)(BPEI)]$ClO_4 \cdot 4H_2O$ with calf thymus DNA.

Complex	Degree of coordination (x)	K_b (M^{-1})	K_{sv}
[Cu(phen)(L-phe)(BPEI)]$ClO_4 \cdot 4H_2O$	0.113	4.34×10^5	0.145
	0.128	4.82×10^5	0.165
	0.162	6.72×10^5	0.193

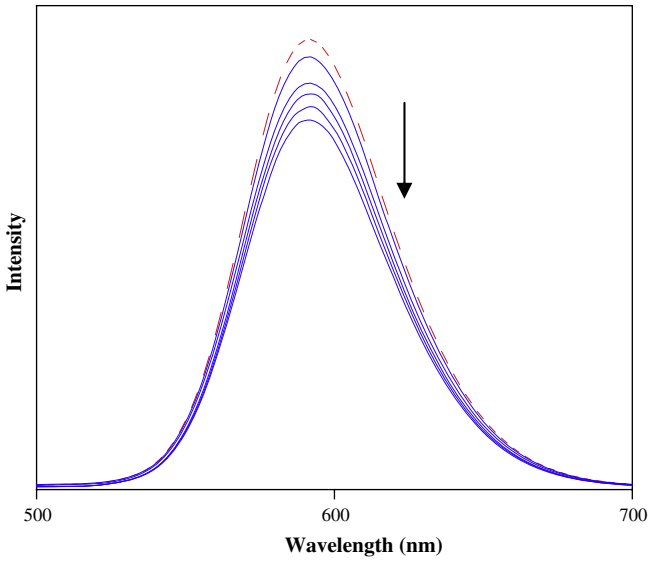

Fig. 4. Emission spectra of EB bound to DNA in the absence (—) and in the presence of [Cu(phen)(L-phe)(BPEI)]$ClO_4 \cdot 4H_2O$ (—) ($x = 0.162$), [EB] = 4 μM, [DNA] = 4 μM, [polymer-copper(II) complex] = 0–4 μM. Arrow shows the intensity changes upon increasing complex concentrations.

where I_o and I are the fluorescence intensities in the absence and the presence of complex, respectively. K_{sv} is a linear Stern–Volmer quenching constant, r is the ratio of the total concentration of complex to that of DNA. The quenching plot (Fig. 5) illustrates that the quenching of EB bound to DNA by the polymer–copper(II) complexes are in good agreement with the linear Stern–Volmer equation, which also indicates that the polymer–copper(II) complexes bind to DNA. In the plot of I_o/I versus [Complex]/[DNA], K_{sv} is given by the ratio of the slope to intercept. The K_{sv} values for our polymer–copper(II) complexes thus obtained are given in Table 1. The data suggest that the interaction of polymer–copper(II) complex (higher degree of coordination sample) with DNA is stronger than other two polymer–copper(II) complex samples, which is consistent with the above absorption spectral results.

2.3.3. Gel electrophoresis studies

Preliminary results from the absorption and fluorescence spectral studies show that the polymer–copper(II) complex with high copper(II) chelate content binds strongly to DNA. Binding of this complex with DNA was also studied by gel electrophoresis using plasmid pBR322 DNA. This DNA moves on agarose gel under the influence of electrical field. This movement is retarded when they are bound to other molecules.

Many polycationic polymer molecules interact with DNA through electrostatic interactions between phosphate groups of the DNA and oppositely charged groups of polymer [16,45,46] Forrest et al. [47] and Vinogradov et al. [48] have reported that the polymer, polyethyleneimine (PEI), retards the DNA migration in agarose gel electrophoresis and they suggest that the binding between DNA and polyethyleneimine (PEI) is thought to occur mainly through electrostatic interactions among the participating species. Recently Annaraj et al. [49] observed that the phenanthroline containing mixed ligand copper(II) complexes retard the mobility of DNA due to the intercalation of the planar phenanthroline groups. Similar retardation in the DNA mobility is also observed due to the intercalation by ruthenium complexes [50]. 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 ($x = 0.162$) (Fig. 6). Lane 1 represents gel moving pattern of DNA alone. Lanes 2–7 represents gel retardation behaviour of polymer–copper(II) complex (with various concentrations) treated with DNA. As reported by previous workers on the interaction of DNA–polyethyleneimine, in our work also the migration of the DNA band is retarded as the concentration of polymer–copper(II) complex in the solution is increased. This clearly demonstrates that the cationic segments of the branched polyethyleneimine are neutralizing the negative charges of DNA, which could have facilitate further due to the increased increased intercalation of the planar phen groups of copper(II) complex moieties present in the polymer chain. The same behaviour in the electrophoresis has been observed earlier by us in the case of some polyethyleneimine–copper(II)/cobalt(III) complexes [19–21].

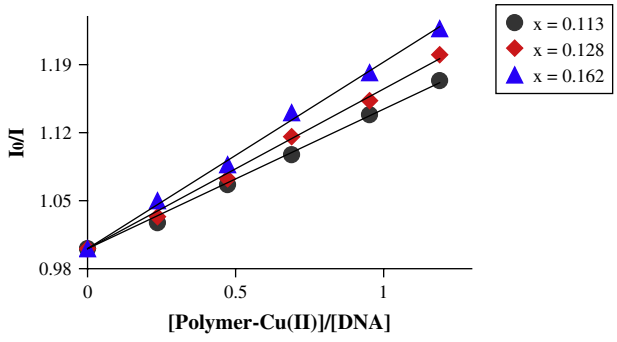

Fig. 5. Fluorescence quenching curves of EB bound to DNA by polymer–copper(II) complexes with different degree of coordination.

2.3.4. Antibacterial and antifungal screening

The polymer–copper(II) complex ($x = 0.162$) was screened *in vitro* for its microbial activity against certain pathogenic bacterial and fungal species using disc diffusion method. The complex was found to exhibit considerable activity against Gram positive and Gram negative bacteria and the pathogenic yeast *Candida albicans*. The test solutions were prepared in dimethyl sulphoxide (1%) and the results of the antimicrobial activities are summarized in Table 2.

Zoroddu et al. [9] have reported that neither phenanthroline ligand alone nor copper(II) chloride itself showed any significant activity against the Gram negative and Gram positive bacteria but the copper(II)–phenanthroline complex has exhibited considerable activity against Gram positive *Staphylococcus aureus* and Gram negative *Escherichia coli*, but inactive against Gram negative *Pseudomonas aeruginosa*. In our biological experiments, using polmer–copper(II) complex ($x = 0.162$), we have observed high antimicrobial activity against Gram positive bacteria (*S.aureus*, *Bacillus subtilis*), Gram negative bacteria (*E. coli*, *P. aeruginosa*) and the yeast *C. albicans*. The polymer–copper(II) complex has shown high activity against Gram positive than Gram negative bacteria. The polymer–copper(II) complex is also very active against fungus. Also we carried out the tests with polymer (BPEI) [19] and ordinary copper complex [Cu(phen)(L-Phe)(H_2O)]ClO_4 separately. Both showed some activity against all microorganisms but the effect is

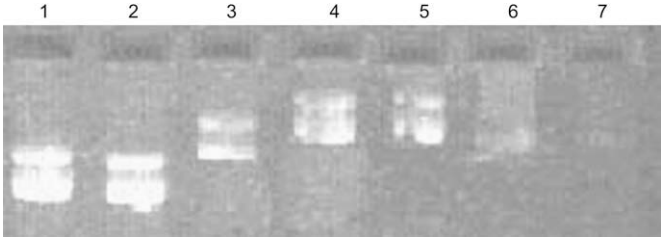

Fig. 6. Gel retardation behaviour of pBR322 DNA by [Cu(phen)(L-phe)(BPEI)]-$ClO_4 \cdot 4H_2O$ ($x = 0.162$). Lane 1: DNA alone, Lane 2–7: DNA + polymer–copper(II) complex in the concentration of 10, 20, 30, 40, 50, 60 μM.

Table 2
Antimicrobial activities of [Cu(phen)(L-phe)(BPEI)]$ClO_4 \cdot 4H_2O$ ($x = 0.162$).

Test organisms	Diameter of zone inhibition (mm)				
	[Cu(phen)(L-phe)(H_2O)]ClO_4	BPEI [19]	Standard [19]	[Cu(phen)(L-Phe)(BPEI)]$ClO_4 \cdot 4H_2O$	Standard
Staphylococcus aureus	6	7	15	18	16
Bacillus subtilis	3	9	16	17	17
Escherichia coli	4	8	17	15	16
Pseudomonas auregenosa	5	7	17	13	17
Candida albicans	4	6	10	17	12

Standard – ciprofloxacin for bacteria and clotrimazole for fungus.
Solvent – DMSO (showed nil effect against the microorganisms under test).

very small compared to polymer–copper(II) complex. It may be concluded that our polymer–copper(II) complex inhibits the growth of bacteria and fungi to a greater extent compared to the standard drugs.

3. Conclusion

Some water-soluble polymer–copper(II) complexes with varying amounts of copper(II) chelate content in the polymer chain have been synthesized and characterized by infra-red, UV–visible, EPR spectral and elemental analysis methods. The DNA binding studies of these polymer–copper(II) complexes have been examined by absorption spectroscopy, fluorescence spectroscopy and gel retardation assay. We conclude that the presence of multiple copper(II) complex moieties and free NH groups, in a single big size polymer molecule, can enhance the various binding ability of such systems with DNA and interactions like electrostatic interaction, van der Waals interaction, hydrogen bonding and/or partial intercalation binding modes exist in this system. The polymer–copper(II) complex showed good antimicrobial activity against Gram positive and Grame negative bacteria and fungi.

4. Experimental

4.1. Materials and methods

Calf thymus DNA and branched polyethyleneimine (BPEI) (M_w 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-phenylalanine was obtained from Loba, India. Plasmid pBR322 DNA was purchased from Genei, India. The complex, [Cu(phen)(L-phe)(H_2O)]ClO_4, was prepared as reported earlier [51].

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 spectrofluorimeter. FT-IR spectra were recorded on a FT-IR Perkin Elmer spectrophotometer with samples prepared as KBr pellets. EPR spectra were recorded on Varian E-112 EPR spectrometer at room temperature and at LNT (77 K), the field being calibrated with diphenylpicryl hydrazyl (DPPH, $g = 2.0037$) at SAIF, I.I.T., Chennai, India. The antimicrobial screening studies were carried out at Periyar College of Pharmaceutical Sciences, Tiruchirappalli, India and the bacteria and fungus species were obtained from National Chemical Laboratory (NCL), Pune, India.

4.2. Synthesis of polymer–copper(II) complex samples

To a solution of BPEI (0.15 g, 3.40 mmol of monomer unit) dissolved in ethanol (15 mL), [Cu(phen)(L-phe)(H_2O)]ClO_4 (0.8 g) in water was added slowly with stirring. The mixture was heated between 50 and 60 °C for 12 h in a water bath. After being warmed enough, the dark blue solution was dialysed 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 bluish filmy substance was obtained. It was pulverized and dried. Yield, 0.18 g. Calcd. (%) C 37.72, H 6.98, N 15.92, found (%): C 37.72, H 7.14, N 16.16 and $x = 0.162$.

The polymer–copper(II) complex samples with various amounts of copper(II) complex units bound to the polymer chain were synthesized by some modifications in the above experimental procedure. That is the amount of [Cu(phen)(L-phe)(H_2O)]ClO_4 in the reaction solution was varied between 0.2 and 0.6 g. Also the reaction period has been kept between 6 to 10 h.

4.3. DNA binding experiments

A solution of calf thymus DNA in the aqueous buffer solution (50 mM NaCl–5 mM Tris–HCl, pH 7.1) 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 [52] Milli-Q water was used to prepare the solutions. The DNA binding experiments were performed at 30.0 ± 0.2 °C. The DNA concentration per nucleotide was determined by electronic absorption spectroscopy using the known molar extinction coefficient value of 6600 $M^{-1}\,cm^{-1}$ at 260 nm [53,54]. Electronic absorption titration of the polymer–copper(II) complex samples in the aqueous buffer solution (50 mM NaCl–5 mM Tris–HCl, pH 7.1) were performed using a fixed complex concentration (8 μM) with increasing amounts of DNA over a range of 0.2–1 μM.

For fluorescence experiments, DNA was 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. The samples were excited at 450 nm and emission was observed between 500 and 700 nm. These experiments were carried out in 50 mM NaCl–5 mM Tris–HCl at pH 7.1 in aqueous media.

For the gel electrophoresis experiments, super-coiled 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–acetic acid–EDTA buffer. The gel was stained with 0.5 μg/mL of ethidium bromide and photographed in UV light.

4.4. Microbial assay

The *in vitro* antimicrobial screening of the polymer–copper(II) complex ($x = 0.162$) was tested for their effect on certain human pathogenic bacteria and fungus by disc diffusion method. The complex was stored dry at room temperature and dissolved in DMSO (1%). Both the Gram positive (*S. aureus*, *B. subtilis*) and Gram negative (*E. coli*, *P. aeruginosa*) bacteria were grown in nutrient agar medium and incubated at 37 °C for 48 h followed by frequent subculture to fresh medium and were used as test bacteria. The yeast *C. albicans* grown into sabouraud dextrose agar medium, incubated at 27 °C for 72 h followed by periodic subculturing to fresh medium and were used as test fungus. Then the Petri dishes were inoculated with a loop full of bacterial or fungal culture and spread throughout the Petri dishes uniformly with a sterile glass spreader. To each disc the test samples (10 μg/mL) and reference ciprofloxacin (1 μg/disc for bacteria) or clotrimazole (10 μg/disc for fungus) was added with a sterile micropipette. The plates were then incubated at 35 ± 2 °C for 24–48 h and 27 ± 1 °C for bacteria and fungus, respectively. Plates with disc containing respective solvents served as control. Inhibition was recorded by measuring the diameter of the inhibitory zone after the period of incubation.

All the experiments were repeated thrice and the average values are presented.

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