Available online at www.sciencedirect.com
ScienceDirect
European Journal of Medicinal Chemistry 43 (2008) 2082–2091
EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY
http://www.elsevier.com/locate/ejmech

Original article

DNA binding and biological studies of some novel water-soluble polymer–copper(II)–phenanthroline complexes

Rajendran Senthil Kumar [a], Sankaralingam Arunachalam [a,*], Vaiyapuri Subbarayan Periasamy [b], Christo Paul Preethy [b], Anvarbatcha Riyasdeen [b], Mohammad Abdulkader Akbarsha [b]

[a] *Department of Chemistry, Bharathidasan University, Palkalaiperur, Tiruchirappalli 620 024, Tamil Nadu, India*
[b] *Department of Animal Science, Bharathidasan University, Tiruchirappalli 620 024, Tamil Nadu, India*

Received 14 July 2007; received in revised form 14 September 2007; accepted 17 September 2007
Available online 29 September 2007

Abstract

Some novel water-soluble polymer–copper(II)–phenanthroline complex samples, $[Cu(phen)_2(BPEI)]Cl_2 \cdot 4H_2O$ (phen = 1,10-phenanthroline, BPEI = branched polyethyleneimine), with different degrees of copper complex content in the polymer chain have been prepared by ligand substitution method in water–ethanol medium and characterized by infrared, UV–visible, EPR spectral and elemental analysis methods. The binding of these complex samples with DNA has been investigated by electronic absorption spectroscopy, emission spectroscopy and gel retardation assay. Electrostatic interactions between DNA molecule and polymer–copper(II) complex molecule containing many high positive charges have been observed. Besides these ionic interactions, van der Waals interactions, hydrogen bonding and other partial intercalation binding modes may also exist in this system. The polymer–copper(II) complex with higher degree of copper complex content was screened for its antimicrobial activity and antitumor activity.

Keywords: Polymer–copper(II) complexes; Antitumor activity; Antimicrobial activity; DNA binding; Polyethyleneimine

1. Introduction

Deoxyribonucleic acid (DNA) is the primary target molecule for most anticancer and antiviral therapies according to cell biology [1]. Investigations of the interactions of DNA with transition metal complexes are basis to design new types of the pharmaceutical molecules to elucidate the mechanism involved in the site specific recognition of DNA and to determine the principles governing the recognition [2,3]. Various types of interaction of these metal complexes with DNA have been reported. An electrostatic interaction that extends the negatively charged phosphates outside the DNA double helix, interaction with grooves of DNA, and intercalation model in which the base pairs of DNA unwind to accommodate the intercalating agent are some important binding modes.

Copper is a physiologically important metal element that plays an important role in the endogenous oxidative DNA damage associated with aging and cancer [4]. Among the copper complexes explored so far, attention has been mainly focused on the copper(II) complexes of 1,10-phenanthroline ligand due to their high nucleolytic efficiency [5] and numerous biological activities such as antitumor [6], anti-candida [7], and antimicrobial [8,9] activities, etc. These complexes have also been widely utilized as foot printing agents of both proteins and DNA [10], probes of the dimensions of the minor groove of duplex structures [11], and identifiers of transcription start sites [12]. Recently, it has been reported that a binuclear copper(II) complex containing 1,10-phenanthroline and a trinuclear copper(II) complex containing di-(2-picolyl)amine bind strongly with DNA and cleave more effectively than their

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

0223-5234/$ - see front matter © 2007 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.ejmech.2007.09.017

corresponding monomeric complexes [13–16]. The bis-(phen) copper complex is proposed to bind to DNA by partial intercalation of one phenanthroline ring, while the other makes favorable contacts in the minor groove [17]. The reports by Burrows et al. [18] advocate design studies on metal complexes with nitrogen-donor ligands that can participate in both hydrogen bonding and π–π interactions for molecular recognition and crystal engineering applications. We envisioned that cooperative effect arising from such non-covalent interactions would be a valuable principle in the development of new metal based probes which recognize biomolecular targets with high specificity.

It has been noticed that drug–polymer conjugates are potential candidates for the selective delivery of anticancer agents to tumor tissue [19] and only a very few reports are available for the use of drug–polymers as anticancer drugs [20]. Polyethyleneimine (PEI) is a cationic polymer exhibiting the highest positive charge density potential [21] and, recently, PEI has appeared as a possible alternative to viral and liposomal routes of gene delivery [22]. 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. [23].

We are interested in the synthesis of polymer–copper(II)–phenanthroline complex composed of polymeric ligand and copper complex units in which the copper complex units are linked to the polymeric ligand by a coordinate bond. The linker might provide additional favorable interactions, such as hydrogen bonding and van der Waals forces, within the groove to further enhance DNA binding and specificity. Recently, we have reported interesting results on the various interaction nature of some polymer–cobalt(III) and copper(II) complexes containing amino acids [24,25]. In this paper we report the synthesis and DNA binding studies of some polymer–copper complex (containing 1,10-phenanthroline) samples with varying amounts of copper complex units in the polymer chain. Also, we report the antitumor and antimicrobial activities of a sample of this complex.

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. 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 was calculated either from the carbon content [26,27] or copper content [28]. The degree of coordination (*x*) thus obtained for the polymer–copper(II) complex samples synthesized are 0.094, 0.103 and 0.143. The *x* value of the polymer–copper(II) complex was found to reach a constant level of about 0.143. 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 0.143. This may be ascribed to the steric hindrance between metal complex units in the polymer chain which prevents further coordination [26].

2.2. Spectral characterization

The IR bands, ν (C–H) 853 cm^{-1} and 737 cm^{-1}, observed for phenanthroline are redshifted to 848 cm^{-1} and 729 cm^{-1} in the complex. 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 [29]. Besides, it is also confirmed by the shift of ν (C–N) of phenanthroline from about 1670 cm^{-1} in the free ligand to 1621 cm^{-1} after coordination [30]. The bands around 2925 cm^{-1} and 2853 cm^{-1} can be assigned to C–H asymmetric and symmetric stretching vibration of aliphatic CH_2 of BPEI. The broad band observed around 3433 cm^{-1} is assigned to the N–H stretching of BPEI [23].

In the UV–visible region, the intense absorption bands appeared from 220 to 270 nm are attributed to ligand based transitions. Another band which appeared around 654 nm is assigned to ligand field transitions.

The EPR spectrum of the polymer–copper(II) complex ($x = 0.143$) was recorded in X-band frequencies in frozen solution (77 K) and is shown in Fig. 2. The complex exhibits well-defined single isotropic feature near $g = 2.0914$. Such isotropic lines are usually a result 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 coupling of two paramagnetic species.

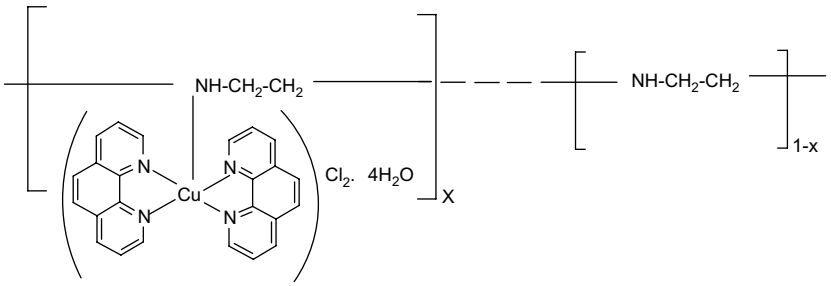

Fig. 1. Structure of $[Cu(phen)_2(BPEI)]Cl_2 \cdot 4H_2O$.

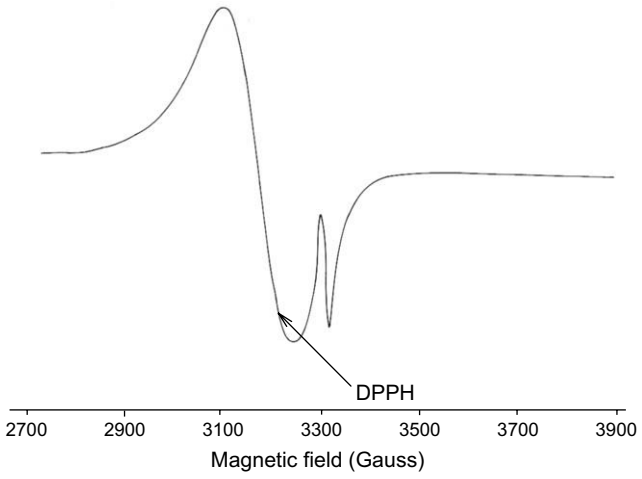

Fig. 2. EPR spectrum of $[Cu(phen)_2(BPEI)]Cl_2 \cdot 4H_2O$ ($x = 0.143$) in DMSO at liquid nitrogen temperature.

2.3. DNA-binding studies

2.3.1. Absorption spectral studies

The application of electronic absorption spectroscopy in DNA binding studies is one of the most useful techniques. The absorption spectra of polymer–copper(II) complex ($x = 0.143$) in the absence and presence of calf thymus DNA are shown in Fig. 3. In the UV region, the complex shows two bands at 225 and 266 nm, which can be attributed to the $\pi-\pi^*$ transition of the coordinated phenanthroline ligand. Upon the addition of incremental amounts of DNA, the absorption band of the complex exhibits hyperchromism and blue shift. A similar hyperchromism has been observed for the Soret bands of certain porphyrins when interacted with DNA but has not yet been clearly explained [31].

The polymer–copper(II)–phenanthroline complex can bind to the double stranded DNA in different binding modes on the basis of their structure and charge and type of ligands.

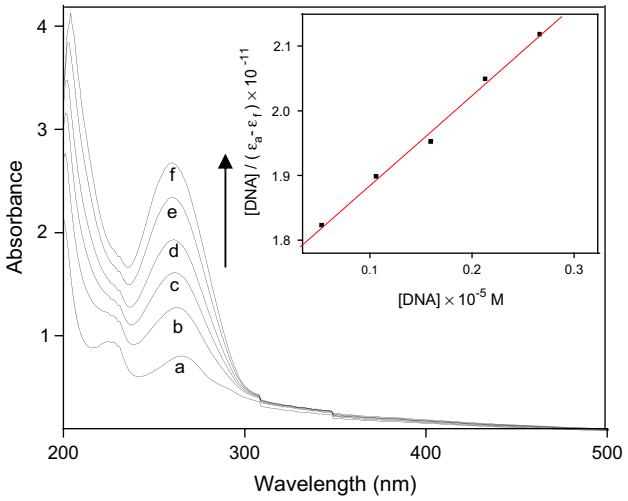

Fig. 3. Absorption spectra of $[Cu(phen)_2(BPEI)]Cl_2 \cdot 4H_2O$ ($x = 0.143$) in the absence (a) and in the presence of increasing amounts of DNA (b–f), [Complex] = 15 μM, [DNA] = 0.5–2.6 μM. Arrow shows the absorbance changes upon increasing DNA concentrations. Inset: plot of $[DNA]/(\varepsilon_a - \varepsilon_f)$ *vs* [DNA].

Our polymer–copper(II) complexes containing several methylene groups in the branched polyethylene (BPEI) can bind to DNA by van der Waals interactions between the methylene groups and the thymine methyl groups [32]. On the other hand, since DNA possesses several hydrogen bonding sites which are accessible both in minor and major grooves, it is likely that the amine groups of BPEI form hydrogen bonds with N-3 of adenine or O-2 of thymine in the DNA, which may contribute to the hyperchormism observed in the absorption spectra. The hyperchromic effect may also be due to the electrostatic interaction between positively charged polymer (BPEI) and the negatively charged phosphate backbone at the periphery of the double helix CT DNA [33].

Structurally, intercalation with DNA may be one of the binding patterns, since our polymer–copper(II) complexes contain phenanthroline ligands which should provide aromatic moiety extending from the metal centre through which overlapping occurs with base pairs of DNA by intercalation. It has been reported that simple bis(1,10-phenanthroline) copper(II) complexes apparently bind to DNA by intercalation [17,34]. Recently, Zhang et al. [13] have indicated that a binuclear copper(II) complex containing 1,10-phenanthroline ligand intercalatively binds into the base pairs of DNA. In our complexes the copper(II)–phenanthroline complex moieties 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. Also, we could not do circular dichroism measurements due to the same problem of precipitation at the necessary concentrations of complex and DNA.

The intrinsic binding constant, K_b, for the interaction of polymer–copper(II) complex samples with DNA was obtained from absorption titration data at 266 nm 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]:

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

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]/($\varepsilon_a - \varepsilon_f$) *vs* [DNA] gives K_b as the ratio of the slope to the intercept.

The K_b values of polymer–copper(II) complex with different degrees of coordination of copper(II)-chelate content are shown in Table 1 which indicates that complex with highest degree of copper complex content has highest 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 highest degree of copper content shows strongest binding with DNA. This may be due to the high positive charges and large number of phenanthroline moieties present in the complex which cooperatively act to increase the overall binding ability of the polymer–copper(II) complex molecule to DNA. The K_b values obtained for our polymer–copper(II) complex samples are very much higher than any other known mononuclear and binuclear copper(II) complexes containing 1,10-phenanthroline ($Cu(phen)_2Cl_2$ (K_b, $2.75 \times 10^3\ M^{-1}$) [16], $Cu_2(phen)_2Cl_4$ (K_b, $4.75 \times 10^4\ M^{-1}$) [13]) and higher than known association constant of PEI to DNA (K_a, $1.2 \times 10^5\ M^{-1}$) [37].

2.3.2. Fluorescent spectral studies

No fluorescence was observed for the polymer–copper(II) complexes at room temperature in aqueous solution or in the presence of calf thymus DNA. So the binding of polymer–copper(II) complexes and DNA cannot be directly presented in the emission spectra. Hence, competitive ethidium bromide (EB) binding studies were undertaken to gain support for the extent of binding of polymer–copper(II) complex with DNA. The molecular fluorophore, EB, emits intense fluorescence light in the presence of CT DNA due to its strong intercalation between the adjacent DNA base pairs [38]. It has been previously reported that the fluorescent light can be reduced by the addition of a second molecule [39], indicating the competition of second molecule with EB in binding to DNA. Two mechanisms have been proposed to account for this reduction of EB emission, the replacement of the molecular fluorophore and/or electron transfer. The non-replacement based quenching has been correlated with DNA-mediated electron transfer from the excited EB to an acceptor (i.e., metal ion). Recently, Ni et al. [14] found that fluorescence reduction of fluorophore NR (neutral red dye) in the presence of DNA by $Cu(phen)_2^{2+}$ may be due to the replacement of the DNA intercalator, i.e., NR. This means that the complex interacts with the same site as NR does. Zhang et al. [13] have also reported that binuclear copper(II) complex of 1,10-phenanthroline ligand can competitively bind to the EB–DNA system by intercalation. On the other hand, Bronich et al. [40] have reported that the addition of a copolymer (i.e. polyethyleneglycol–polyethyleneimine (PEI)) to EB–DNA complex can result in quenching of the fluorescence due to the displacement of EB by the copolymer. Xiao and Zhan [33] have studied the effect of polymeric Schiff base–nickel complexes on the fluorescence of EB–DNA system and they have suggested that a portion of the metal complex possessing a planar aromatic moiety can intercalate with adjacent base pairs, which in turn can inhibit EB binding to calf thymus DNA competitively. In our studies the addition of the polymer–copper(II) complex 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 decrease in the emission intensity. The polymer–copper(II) complex with higher degree of coordination has led to a larger reduction in emission intensity in the presence of DNA. This may be due to the larger number of copper(II) chelates (containing phenanthroline ligands) present in the polymer chain which hinders intercalation of EB into DNA and thus decreasing the binding ability between EB and calf thymus DNA.

Table 1
The intrinsic binding constants (K_b) of $[Cu(phen)_2(BPEI)]Cl_2 \cdot 4H_2O$ with calf thymus DNA

Complex	x	λ_{max}, nm (ε, $M^{-1}\,cm^{-1}$)	K_b (M^{-1})
$[Cu(phen)_2(BPEI)]Cl_2 \cdot 4H_2O$	0.094	264 (50,853)	5.95×10^5
	0.103	265 (51,993)	6.86×10^5
	0.143	265 (53,685)	7.96×10^5

2.3.3. Gel electrophoresis

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

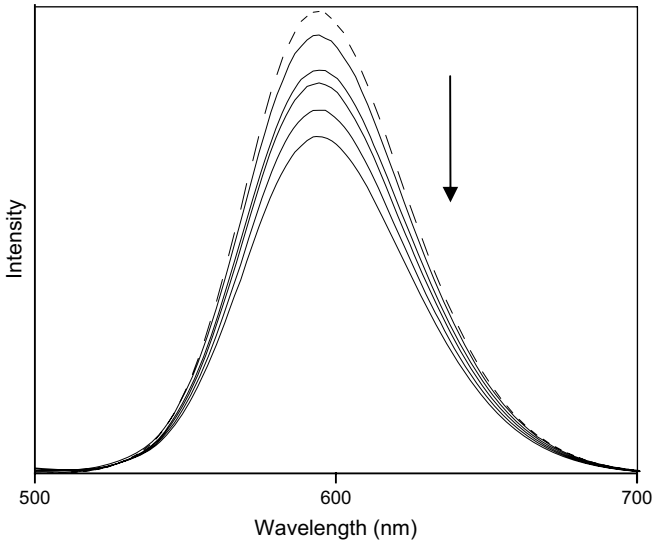

Fig. 4. Emission spectra of EB bound to DNA in the absence (---) and in the presence of $[Cu(phen)_2(BPEI)]Cl_2 \cdot 4H_2O$ (—) ($x = 0.143$), [EB] = 4 μM, [DNA] = 4 μM, [Complex] = 0–4 μM.

groups of the DNA and oppositely charged groups of polymer [41,42]. Forrest et al. [43] and Vinogradov et al. [44] 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. [45] observed that 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 has also been observed due to the intercalation by ruthenium complexes [46]. In order to substantiate the ionic binding of our polymer–copper(II) complex to DNA, gel retardation assay was performed on pBR322 DNA (Fig. 5). Lane 1 is pure DNA not treated with the complex. Lanes 2–8 represent the DNA treated with increasing amounts of the polymer–copper(II) complex. As seen from the figure (lanes 2 and 3), the DNA band migrates completely with increasing the complex concentration (10 and 20 μM), but at higher complex concentration (30–50 μM), the migration of DNA band diminishes (lanes 4–6) and in lanes 8 and 9, due to very high concentration (60 and 70 μM), and complete retardation of the DNA band has been observed. This clearly demonstrates that the cationic segments of the polyethyleneimine neutralize the negative charges of DNA which could have facilitated further due to the increased intercalation of the planar phen groups of copper(II) complex moieties. The same behaviour in the electrophoresis has been observed earlier by us in the case of some polyethyleneimine–copper(II)/cobalt(III) complexes [24,25].

2.3.4. Antibacterial and antifungal screening

The polymer–copper(II) complex ($x = 0.143$) was screened *in vitro* for its anti 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 sulfoxide (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 exhibited considerable activity against Gram positive *Staphylococcus aureus* and Gram negative *Escherichia coli*, but inactive against

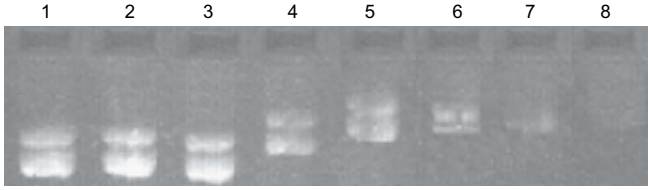

Fig. 5. Gel retardation assay of pBR322 DNA by $[Cu(phen)_2(BPEI)]Cl_2 \cdot 4H_2O$ ($x = 0.143$). Lane 1: DNA alone, lanes 2–8: DNA + polymer–copper(II) complex in the concentration of 10, 20, 30, 40, 50, 60, 70 μM.

Table 2
Antimicrobial activities of $[Cu(phen)_2(BPEI)]Cl_2 \cdot 4H_2O$ ($x = 0.143$)

Test organisms	Diameter of inhibition zone (mm)		
	BPEI [25]	Standard	$[Cu(phen)_2(BPEI)]Cl_2 \cdot 4H_2O$
Staphylococcus aureus	7	16	25
Bacillus subtilis	9	17	23
Escherichia coli	8	16	20
Pseudomonas aeruginosa	7	17	14
Candida albicans	6	12	25

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

Gram negative *Pseudomonas aeruginosa*. In our biological experiments, using polymer–copper(II) complex ($x = 0.143$), we have observed high antimicrobial activity against Gram positive bacteria *S. aureus* and *Bacillus subtilis* and Gram negative bacteria *E. coli* and *P. aeruginosa*. 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 the yeast *C. albicans*. In all the cases except Gram negative *P. aeruginosa*, the polymer–copper(II) complex has shown very high activity than the standard antibacterial and antifungal agents, ciprofloxacin and clotrimazole, respectively (Fig. 6). In our early study we have reported the antimicrobial activity of the polymer (BPEI) alone [25]. It showed some activity against all microorganisms but the effect was very small compared to polymer–copper(II)–phenanthroline 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.

2.3.5. Cytotoxicity assay

We examined the effects of the polymer–copper(II) complex ($x = 0.143$) on cultured NCI-H460 human lung cancer cells by exposing cells for 24 and 48 h to the medium containing the complex at 10–80 μg/mL concentration. Bright field microscopic analysis with control (Fig. 7a) and treated cells (Fig. 7b) revealed that the complex produced dose- as well as duration-dependent cytotoxic effect in the cells. The results of MTT assay (Fig. 8) indicated that the complex inhibited the growth of the lung cancer cells significantly in a dose- and

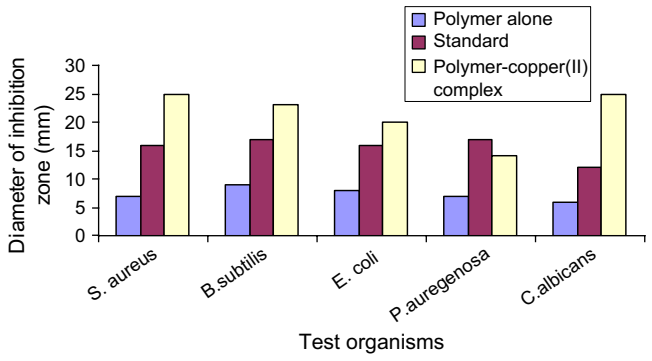

Fig. 6. Graphs showing diameter inhibition zone *vs* test organisms (standard – ciprofloxacin for bacteria and clotrimazole for fungus).

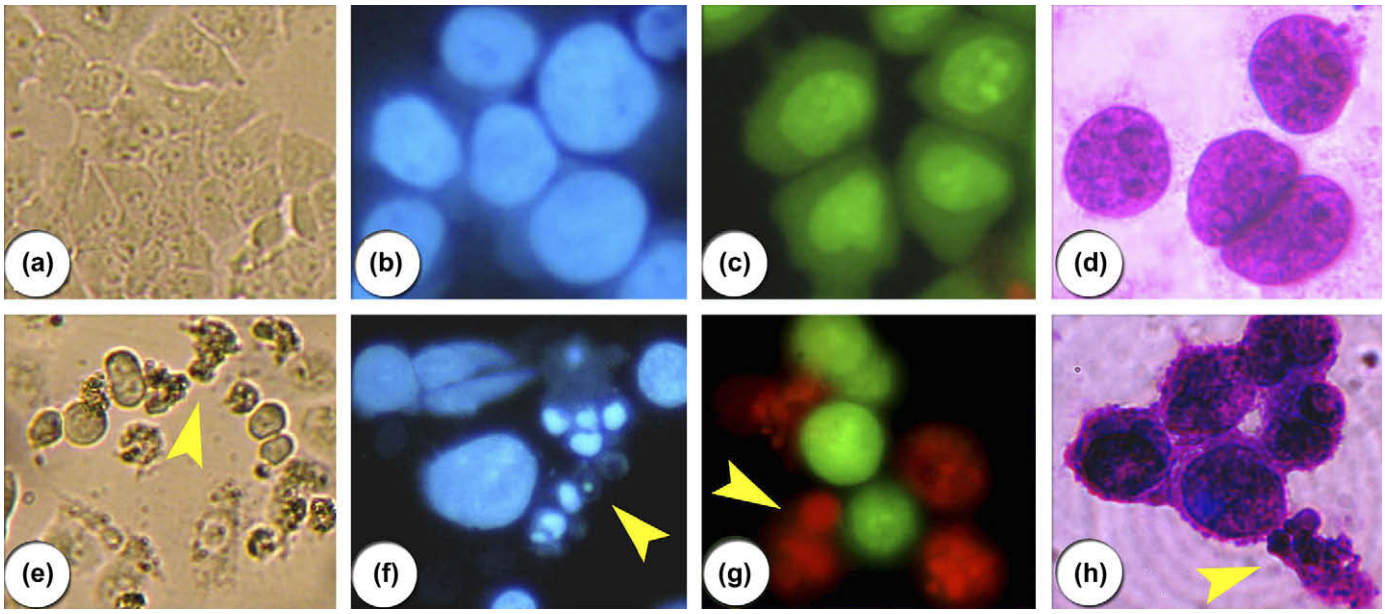

Fig. 7. Detection of nuclear changes by triple comparative staining preparation of NCI-H460 cells treated for 24 h with 10 μM CPY. Bright field microscopy of untreated cells (a); treated cells (b). Fluorescent microscopy of Hoechst 33258 stained untreated cells (c); treated cells (d). Fluorescent microscopy of acridine orange/ethidium bromide stained untreated cells (e); treated cells (f). Light microscopy of Leukostat stained untreated cells (g); treated cells (h). Arrow heads indicate dot-like chromatin (apoptotic cells).

duration-dependent manner. For 24 h treatment period, higher concentrations of the complex were required to kill the cells whereas for 48 h treatment the cell killing occurred at lower concentrations. The IC_{50} value of the complex was slightly higher for the 24 h treatment groups, i.e., in the range of 17.5–19.5 μg/mL, whereas for the 48 h treatment groups the IC_{50} value fell in the range of 11.5–13.2 μg/mL (Fig. 8).

2.3.6. Hoechst 33258 staining

After the cells were treated with IC_{50} concentrations of polymer–copper(II) complex ($x = 0.143$) (10–40 μg/mL) for 24 and 48 h the cells were observed for cytological changes adopting Hoechst 33258 staining. The observations revealed that the complex brought about cytological changes such as chromatin fragmentation, binucleation, cytoplasmic vacuolation, nuclear swelling, cytoplasmic blebbing and late apoptosis indication of dot-like chromatin and condensation (Fig. 7d) whereas untreated cells did not show such changes (Fig. 7c). Data collected from the manual counting of cells with normal and abnormal nuclear features are shown in Fig. 9. Both apoptotic and necrotic cells increased in dose-dependent manner.

2.3.7. Special staining for cell death assessment

To quantify cell death, we adopted Leukostat as well as AO/EB staining. Treatment of cells with polymer–copper(II) complex ($x = 0.143$) for 24 and 48 h induced apoptosis. Using Leukostat triple staining, apoptosis was inferred based on the changes in the overall profile of the cell, and with special reference to chromatin condensation and apoptotic body formation. Normal cells were uniformly stained, both in respect of nuclei and cytoplasm (Fig. 7g) whereas cells in early apoptosis had normal profile of the cell membrane but the chromatin was condensed and the nuclei were irregularly shaped, cells in late apoptosis had intensely stained cell membrane with blebs and darkly stained highly condensed nuclei, and cells undergoing necrosis had lost the nuclear basophilia and, in their final stage, usually appeared as ‘‘ghosts’’ due to cell lysis (Fig. 7h).

AO/EB staining adopting fluorescence microscopy also revealed apoptosis from the perspective of fluorescence. After H460 cells were exposed to various concentrations of polymer–copper(II) complex ($x = 0.143$) for 24 and 48 h, the cells were classified into four types according to the fluorescence emission and the morphological feature of chromatin condensation in the stained nuclei. (1) Viable cells had uniformly green fluorescing nuclei with a highly organized structure (Fig. 7e). (2) Early apoptotic cells (which still had intact

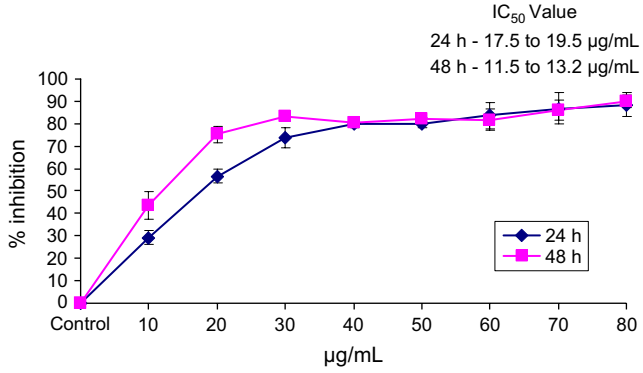

Fig. 8. Graphs showing % inhibition *vs* different concentrations for NCI-H460 cancer cells exposed to the complex $Cu(phen)_2(BPEI)Cl]Cl_2 \cdot 4H_2O$. The treatment with the complex affects viability of cells in dose- as well as duration-dependent manner. The IC_{50} range of $[Cu(phen)_2(BPEI)]Cl_2 \cdot 4H_2O$ for NCI-H460 cells is shown on top right. The values are indicated in range.

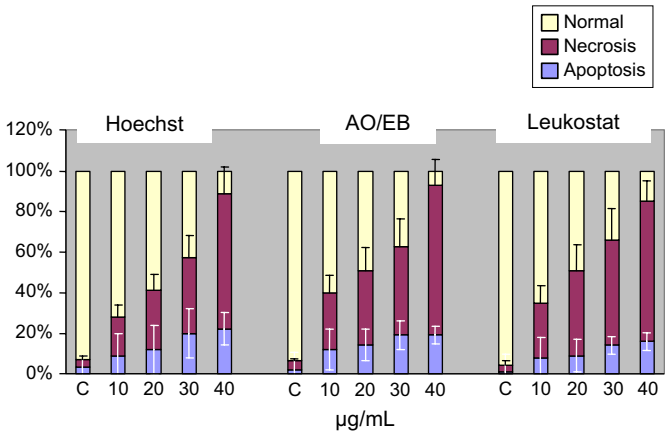

Fig. 9. Stacked column of 100% for comparison of percent cells in normal, necrosis and apoptosis as revealed by AO/EB, Leukostat and Hoechst 33258 staining followed by manual counting. All staining techniques reveal similar data, indicating that all are efficient, though morphological and cytological manifestations differ.

membranes but had started undergoing DNA fragmentation) had green fluorescing nuclei, but perinuclear chromatin condensation was visible as bright green patches or fragments. (3) Late apoptotic cells had orange to red fluorescing nuclei with condensed or fragmented chromatin. (4) Necrotic cells had uniformly orange to red fluorescing nuclei with no indication of chromatin fragmentation and the cells were swollen to large size (Fig. 7f). The results suggest that polymer–copper(II) complex ($x = 0.143$) treatment caused more cells to take to death in NCI-H460 cells.

Data from the three staining methods were compared. Data on cells indicating cell death morphologies, induced on treatment with various concentrations of polymer–copper(II) complex ($x = 0.143$) for 24 and 48 h, followed by staining with Hoechst 33258, Leukostat and AO/EB, collected from manual counting of cells, are presented in Fig. 9, which indicates that all the three staining methods are efficient in revealing the incidence of both apoptosis and necrosis.

2.3.8. Efficiency of the complex in killing cancer cells

Thus, polymer–copper(II) complex synthesized by us was tested in NCI-H460 cancer cell line adopting antiproliferation and cell morphological assays to find the efficiency in killing the cells and the underlying specific mechanism. Our polymer–copper(II) complex has a dose- and duration-dependent relationship in dealing with the cancer cell. Exposing NCI-H460 cells to polymer–copper(II) complex ($x = 0.143$) led to severe disruption of cellular morphologies such as nuclear abnormalities, membrane 'blebbing' and chromatin fragmentation, which are characteristic of cells dying through specific cell death mechanism. These morphological features were classified based on nuclear as well cytoplasmic features, which reveal that a higher percentage of cell death is produced in 48 h with a lesser concentration than 24 h and also response to dosage. At 40 µg/mL is higher percentage of necrosis than other lower doses.

3. Conclusion

Some new water-soluble polymer–copper(II) complexes with different degrees of copper(II)–phenanthroline complex moieties on the polymer chain have been synthesized and characterized by infrared, UV–visible, EPR spectral and elemental analysis methods. The DNA binding studies of these polymer–copper(II) complexes with different percentage of copper(II)–phenanthroline complex moieties have been examined by absorption spectroscopy, fluorescence spectroscopy and gel retardation assay. We conclude that the presence of multiple copper(II)–phenanthroline complex moieties and free NH groups, in a single big size polymer molecule, can enhance the various binding abilities of such systems with DNA. The electrostatic interaction between DNA molecule and polymer–copper(II) complex molecule containing many high positive charges has been observed. Besides this ionic interaction, van der Waals interaction, hydrogen bonding and other partial intercalation binding modes may also exist in this system. The polymer–copper(II) complex showed good antimicrobial activity against Gram positive and Gram negative bacteria and fungi than the standard antibiotic and antifungal drugs, ciprofloxacin and clotrimazole, respectively. The antiproliferative and morphological effects of polymer–copper(II) complex sample with high degree of coordination appear to be specific on killing of cells. In future, this complex might prove to be of application in target-based cancer therapy since the mechanism of cell death appears to be essentially apoptosis but necrosis also is one of the desired endpoints in cancer therapy. From these perspectives, it would be pertinent to confirm the mechanisms of cell killing in the present context by adopting appropriate molecular techniques including expression of pro- and anti-apoptotic genes and caspases in the case of apoptosis, and oxidative damage in the case of necrosis.

4. Experimental

Copper chloride hexahydrate and 1,10-phenanthroline were purchased from Merck, India. Calf thymus DNA and branched polyethyleneimine (BPEI) (M_w ca. 25,000) obtained from Sigma–Aldrich, Germany, were used as such. Plasmid pBR322 DNA was purchased from Genei, India. The complex $[Cu(phen)_2Cl]Cl$ was prepared as reported earlier [47]. The carbon, hydrogen and nitrogen contents of samples were determined at SAIF, Lucknow, India. Absorption spectra were recorded on an 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 an FT-IR Perkin–Elmer spectrophotometer with samples prepared as KBr pellets. EPR spectra were recorded on Varian E-112 EPR spectrometer 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. Ciprofloxacin and clotrimazole discs were purchased from HiMedia Laboratories Pvt. Ltd., India.

4.1. 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), $[Cu(phen)_2Cl]Cl$ (1.5 g, 2.82 mmol) 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 (the scanning electron micrograph of the complex is shown in Fig. 10). Yield, 0.2 g (Calcd C 46.15, H 7.47, N 17.83, found: C 46.15, H 7.51, N 17.07 and $x = 0.143$).

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 $[Cu(phen)_2Cl]Cl$ in the reaction solution was varied between 0.6 and 1.0 g. Also the reaction period has been kept between 6 and 10 h.

4.2. Copper analysis

Copper content in the polymer chain of the polymer–copper(II) complex was estimated by a slight modification of the reported procedure [28]. A known weight of the complex (0.02 g) was dissolved in 2 mL of concentrated nitric acid. The solution was gently warmed. After being cooled, the pH of the solution was adjusted to >5 with 6 M NaOH. The solution was transferred into 10 mL volumetric flask and made upto the mark with aqueous NH_3. The absorbance of this solution was measured against a reagent blank. From the absorbance, the concentration of copper was calculated. Knowing the concentration of copper present in a given weight of the polymer–copper(II) complex, the ratio of the number of moles of amine groups coordinated to the copper chelate to the total number of moles of amine groups in the polymer chain was calculated.

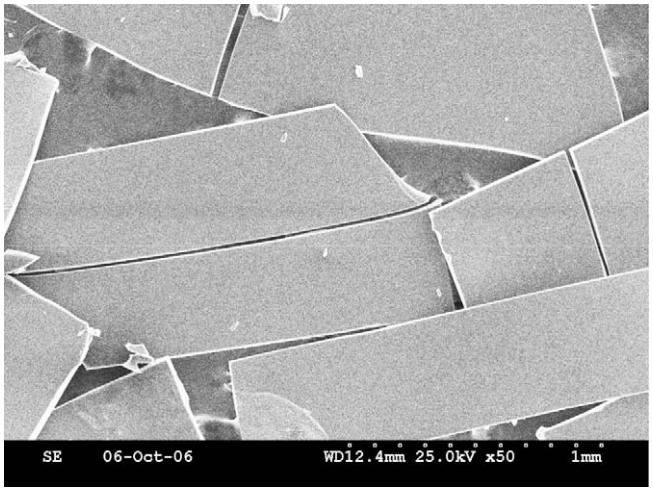

Fig. 10. Scanning electron micrograph of $[Cu(phen)_2(BPEI)]Cl_2 \cdot 4H_2O$ ($x = 0.143$).

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 [48]. 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 [49]. 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) was performed by using a fixed complex concentration (15 μM) with increasing amounts of DNA over a range of 0.5–2.6 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 ($x = 0.143$) was tested for their effect on certain human pathogenic bacteria and a fungus by disc diffusion method [50]. The complex was stored dry at room temperature and dissolved in DMSO (1%). Both the Gram positive (*S. aureus* and *B. subtilis*) and Gram negative (*E. coli* and *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 in Sabouraud dextrose agar medium, incubated at 27 °C for 72 h followed by periodic subculturing to fresh medium and was 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) were 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.

4.5. Cell culture

NCI-H460 human lung cancer cells were obtained from National Centre for Cell Science (NCCS), Pune, India. The cells were cultured in RPMI 1640 medium (Sigma–Aldrich, St. Louis, MO, USA), supplemented with 10% fetal bovine serum (Sigma, USA) and 100 U/mL of penicillin and 100 μg/mL of streptomycin as antibiotics (HiMedia, Mumbai, India), in 96-well culture plates, at 37 °C, in a humidified atmosphere of 5% CO_2, in a CO_2 incubator (Heraeus, Hanau, Germany). All the experiments were performed using cells from passage 15 or less.

4.6. Cytotoxicity assay [51]

The $[Cu(phen)_2(BPEI)]Cl_2 \cdot 4H_2O$ complex was first dissolved quantitatively in dimethyl sulfoxide (DMSO, Sigma, USA) to make the stock solution. This solution was diluted to get various concentrations of the complex in the range of 10–80 μg/mL. Two hundred microliters of this solution was added to a series of wells containing 5×10^3 NCI-H460 cells per well. DMSO solution was used as the solvent control. After 24 and 48 h of treatment, the gross morphological changes in NCI-H460 cells were observed and photographed using an inverted microscope (Carl Zeiss, Jena, Germany). Immediately thereafter, 20 μL of MTT solution (5 mg/mL in phosphate-buffered saline (PBS)) was added to each well and the plates were wrapped with aluminum foil and incubated for 4 h at 37 °C. The purple formazan product was dissolved by the addition of 100 μL of 100% DMSO to each well. The absorbance was monitored at 570 nm (measurement) and 630 nm (reference) using a 96-well plate reader (Bio-Rad, Hercules, CA, USA). Data were collected for four replicates each and used to calculate the respective means. The percentage inhibition was calculated, from this data, using the formula:

$$\frac{\text{Mean absorbance of untreated cells (control)} - \text{Mean absorbance of treated cells}}{\text{Mean absorbance of untreated cells (control)}} \times 100.$$

4.7. Hoechst 33258 staining [52]

The cell pathology was detected by staining the nuclear chromatin of trypsinized cells (5.0×10^4/mL) with 1 μL of Hoechst 33258 (1 mg/mL, aqueous) for 10 min at 37 °C. A drop of cell suspension was placed on a glass slide and a coverslip was laid over to reduce light diffraction. At random 300 cells were observed in a fluorescent microscope (Carl Zeiss, Germany) fitted with a 377–355 nm filter and observed at 400× magnification, and the percentage of cells reflecting pathological changes was calculated. Data were collected for four replicates and used to calculate the mean and the standard deviation.

4.8. Acridine orange (AO) and ethidium bromide (EB) staining [52]

Acridine orange and ethidium bromide staining was performed as described by Spector et al. [52]. Twenty-five microliters of cell suspension of each sample (both attached, released by trypsinization, and floating), containing 5×10^5 cells, was treated with AO and EB solution (one part of 100 μg/mL AO and one part of 100 μg/mL EO in PBS) and examined under a fluorescent microscope (Carl Zeiss, Germany) using an UV filter (450–490 nm). Three hundred cells per sample were counted in tetraplicates for each dose point. Cells were scored as viable, apoptotic or necrotic as judged by the staining, nuclear morphology and membrane integrity, and percentages of apoptotic and necrotic cells were then calculated. Morphological changes were also observed and photographed.

4.9. Leukostat staining [52]

Leukostat staining was performed as described by Spector et al. [52]. Smears of cells (attached, released by trypsinization, and floating were pooled) were fixed by dipping the slide for 10 s in Leukostat fixative solution (2 mg/mL malachite green in 100% methanol). The cytoplasm was stained by dipping for 10 s in Leukostat staining solution 1 (0.1% eosin Y, 0.1% formaldehyde, 0.4% sodium phosphate dibasic, and 0.5% potassium phosphate monobasic) and counterstained by dipping for 10 s in Leukostat staining solution 2 (0.04% azure, 0.04% methylene blue, 0.4% sodium phosphate dibasic, and 0.5% potassium phosphate monobasic). The preparations were examined at 400× magnification in a light microscope (Carl Zeiss, Germany). The cells were scored as normal, apoptotic or necrotic as judged by staining and nuclear/cell morphology. Percentages of apoptotic cells and necrotic cells were then calculated. The morphological changes were also observed and photographed.

Acknowledgements

We are grateful to the UGC-SAP & COSIST and DST-FIST programmes of the Department of Chemistry and Department of Animal Science, Bharathidasan University. We thank Dr. L. Uma and Dr. D. Prabhakaran, National Facility for Marine Cyanobacteria, for providing the Gel documentation facility.

Council of Scientific and Industrial Research (CSIR), New Delhi, is gratefully acknowledged for financial support (Grant No. 01(2075)/06/EMR-II) to A.S. and a Senior Research Fellowship to R.S.K.

References

[1] L.R. Kelland, Eur. J. Cancer 41 (2005) 971–979.
[2] E.V. Hackl, V.L. Galkin, Y.P. Blagoi, Int. J. Biol. Macromol. 34 (2004) 303–308.
[3] K. Jiao, Q.X. Wang, W. Sun, F.F. Jian, J. Inorg. Biochem. 99 (2005) 1369–1375.
[4] B.N. Ames, M.K. Shigenaga, T.M. Hagen, Proc. Natl. Acad. Sci. USA 90 (1993) 7915–7922.
[5] D.S. Sigman, Biochemistry 29 (1990) 9097–9105.
[6] J.D. Ranford, P.J. Sadler, Dalton Trans. (1993) 3393–3399.
[7] G. Majella, S. Vivienne, M. Malachy, D. Michael, M. Vickie, Polyhedron 18 (1999) 2931–2939.
[8] J.D. Saha, U. Sandbhor, K. Shirisha, S. Padhye, D. Deobagkar, C.E. Ansond, A.K. Powelld, Bioorg. Med. Chem. Lett. 14 (2004) 3027–3032.
[9] M.A. Zoroddu, S. Zanetti, R. Pogni, R. Basosi, J. Inorg. Biochem. 63 (1996) 291–300.
[10] M.D. Kuwabara, C. Yoon, T.E. Goyne, T. Thederahn, D.S. Sigman, Biochemistry 25 (1986) 7401–7408.
[11] J.A. Cowan, Curr. Opin. Chem. Biol. 5 (2001) 634–642.
[12] T.B. Thederahn, A. Spassky, M.D. Kuwabara, D.S. Sigman, Biochem. Biophys. Res. Commun. 168 (1990) 756–762.
[13] Q.G. Zhang, F. Zhang, W.G. Wang, X.L. Wang, J. Inorg. Biochem. 100 (2006) 1344–1352.
[14] Y. Ni, D. Lin, S. Kokot, Anal. Biochem. 352 (2006) 231–242.
[15] M. Komiyama, S. Kina, K. Matsumura, J. Sumaoka, S. Tobey, V.M. Lynch, E. Anslyn, J. Am. Chem. Soc. 124 (2002) 13731–13736.
[16] T. Gupta, S. Dhar, M. Nethaji, A.R. Chakravarty, Dalton Trans. (2004) 1896–1900.
[17] O. Zelenko, J. Gallagher, D.S. Sigman, Angew. Chem. Int. Ed. Engl. 36 (1997) 2776–2778.
[18] A.D. Burrows, C.W. Chan, M.M. Chowdhry, J.E. McGrady, D.M.P. Mingos, Chem. Soc. Rev. 24 (1995) 329–339.
[19] B.A. Howell, E.W. Walls, R. Rashidianfar, Makromol. Chem., Macromol. Symp. 19 (1988) 329–339.
[20] B. Schechter, R. Arnon, M. Wilchek, React. Polym. 25 (1995) 167–175.
[21] A.V. Harpe, H. Petersen, Y. Li, T. Kissel, J. Controlled Release 69 (2000) 309–322.
[22] W.T. Godbey, K.K. Wu, A.G. Mikos, J. Controlled Release 60 (1999) 149–160.
[23] K. Geckeler, G. Lange, H. Eberhardt, E. Bayer, Pure Appl. Chem. 52 (1980) 1883–1905.
[24] R.S. Kumar, S. Arunachalam, Polyhedron 25 (2006) 3113–3117.
[25] R.S. Kumar, S. Arunachalam, Polyhedron 26 (2007) 3255–3262.
[26] E. Tsuchida, H. Nishide, T. Ohkawa, Bull. Sci. Res. Lab. 69 (1975) 31–34.
[27] Y. Kurimura, E. Tsuchida, M. Kaneko, J. Polym. Sci. Part A-1 9 (1971) 3511–3519.
[28] J.W. Kolis, D.E. Hamilton, N.K. Kildahl, Inorg. Chem. 18 (1979) 1826–1831.
[29] L. Jin, P. Yang, Polyhedron 16 (1997) 3395–3398.
[30] T.I.A. Gerber, J. Coord. Chem. 56 (2003) 1397–1407.
[31] R.F. Pasternack, E.J. Gibbs, J.J. Villafranca, Biochemistry 22 (1983) 2406–2414.
[32] C.L. Liu, J.Y. Zhou, Q.X. Li, L.J. Wang, Z.R. Liao, H.B. Xu, J. Inorg. Biochem. 75 (1999) 233–240.
[33] Y.N. Xiao, C.X. Zhan, J. Appl. Polym. Sci. 84 (2002) 887–893.
[34] J.M. Veal, R.L. Rill, Biochemistry 30 (1991) 1132–1140.
[35] S. Satyanarayana, J.C. Cabrowiak, J.B. Chaires, Biochemistry 32 (1993) 2573–2584.
[36] A.M. Pyle, J.P. Rehmann, R. Meshoyrer, C.V. Kumar, N.J. Turro, J.K. Barton, J. Am. Chem. Soc. 111 (1989) 3051–3058.
[37] Y.L. Zhou, Y.Z. Li, Spectrochim. Acta A 60 (2004) 377–384.
[38] J. Olmsted, D.R. Kearns, Biochemistry 16 (1977) 3647–3654.
[39] B.C. Baguley, M. Lebret, Biochemistry 23 (1984) 937–943.
[40] T.K. Bronich, H.K. Nguyen, A. Eisenberg, A.V. Kabanov, J. Am. Chem. Soc. 122 (2000) 8339–8343.
[41] T.K. Bronich, A.V. Kabanov, L.A. Marky, J. Phys. Chem. B 105 (2001) 6042–6050.
[42] M.X. Tang, F.C. Szoka, Gene Ther. 4 (1997) 823–832.
[43] M.L. Forrest, J.T. Koerber, D.W. Pack, Bioconjugate Chem. 14 (2003) 934–940.
[44] S.V. Vinogradov, T.K. Bronich, A.V. Kabonov, Bioconjugate Chem. 9 (1998) 805–812.
[45] J. Annaraj, S. Srinivasan, K.M. Ponvel, P.R. Athappan, J. Inorg. Biochem. 99 (2005) 669–676.
[46] E.C. Long, J.K. Barton, Acc. Chem. Res. 23 (1990) 271–273.
[47] C.M. Harris, T.N. Lockyer, H. Waterman, Nature 192 (1961) 424–425.
[48] J. Marmur, J. Mol. Biol. 3 (1961) 208–218.
[49] M.F. Reichmann, S.A. Rice, C.A. Thomas, P. Doty, J. Am. Chem. Soc. 76 (1954) 3047–3053.
[50] A.W. Bauer, N.M. Kirby, J.C. Sherris, M. Turck, Am. J. Clin. Pathol. 45 (1966) 493–496.
[51] T. Mosmann, J. Immunol. Methods 65 (1983) 55–63.
[52] D.L. Spector, R.D. Goldman, L.A. Leinwand, Culture and biochemical analysis of cells, , In: Cell: A Laboratory Manual, vol. 1, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1998, pp. 15.1–15.24.