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ARTICLE

Ferrocenyl methylene units and copper(II) phenanthroline complex units anchored on branched poly(ethyleneimine) – DNA binding, antimicrobial and anticancer activity†

Cite this: DOI: 10.1039/x0xx00000x

Received 14th April 2014,
Accepted 00th April 2014

DOI: 10.1039/x0xx00000x

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Ilayaperumal Pradeep,[a] Sengan Megarajan,[a] Sankaralingam Arunachalam,*[a] Rajakumar Dhivya,[b] Annadurai Vinothkanna,[c] Mohammad Abdulkadher Akbarsha,[b] and Soundarapandian Sekar[c]

A polymer conjugate containing copper(II) phenanthroline complex units and ferrocenyl methylene units bound to the same polymer backbone was synthesized and characterized by spectroscopic and analytical techniques. We could observe both EPR spectrum for the paramagnetic copper(II) units and proton NMR spectrum due to the diamagnetic ferrocenyl methylene units. Binding interaction of this complex with calf thymus (CT-DNA) has been investigated by absorption, emission, cyclic voltammetry, and circular dichroism. The complex displays significant binding properties to the CT-DNA. In fluorimeteric studies, the binding mode of the complex with CT-DNA was investigated using Ethidium bromide(EB) as a fluorescence probe. The binding of copper(II) complex units with DNA has been facilitated by the presence of ferrocenyl methylene units in the same polymer molecule. This polymer conjugate shows good anticancer activity against HepG2 cells and antimicrobial studies have shown better activity.

Introduction

Polymer-drug conjugates

Polymer-drug conjugates are becoming increasingly used as multi-agent therapeutics as opposed to single agent, for diseases such as cancer.[1-2] These polymer therapeutics exhibit a number of advantageous properties including high stability, allowing for extended time in the circulatory system and a high drug-loading capacity. The mechanism of action of these combination polymers at cellular level has highlighted that conjugate conformation in solution and the drug release rates are key parameters for the activity.[3-4] These factors make them suitable carriers for anticancer drugs.[5] These properties overcome many of the short comings that are inherent with metal based drugs by reducing side-effects and enhancing the therapeutic efficacy.[6]

[a,*]*School of Chemistry, Bharathidasan University, Tiruchirappalli – 620024, INDIA. Fax:+91-431-2407043/45; Tel: +91-431-2407053; E-mail: arunasurf@yahoo.com*
[b]*Mahatma Gandhi -Doerenkamp Center, Bharathidasan university, Tiruchirappalli – 620024, INDIA.*
[c]*Department of Industrial Biotechnology, Bharathidasan University, Tiruchirappalli– 620024, INDIA*

†Electronic Supplementary Information (ESI) available: [Characterisation, DNA Binding studies data, *in vitro* studies images].

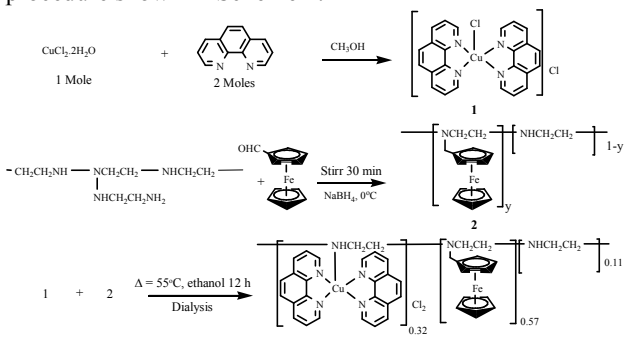
Cyclodextrin based polymers, poly(ethyleneglycol) – camptothecin, carboxymethyldextran–exatecan and poly(ethyleneglycol)– poly(aspartic acid)–doxorubicin micelles are a few other polymer-based drugs that have entered the clinical trials at various levels since their inception as a viable therapeutic strategy.[7] Polyethyleneimine has a high density of amines in its structure that allows one to easily modify the polymer. Different redox moieties can be easily attached to this polymer

Polymer-metal complexes

Polymer-metal complexes have been the focus of considerable research because of their potential use in biosensing applications, to construct novel biosensors and can be applied to environmentally benign electro-functional systems, such as electrically switchable drug delivery systems.[8-11] Pendent type polymer metal complexes are a new class of materials in which the metal complexes are attached to polymer through coordinate-covalent bond.[12]

Our group has already developed a few polyethyleneimine-based metal complex drugs.[13-16] Copper is a biologically relevant element and a large number of copper(II) complexes with ferrocene moiety have been synthesized and explored for their biological activities.[17-19] Among these copper complexes, attention has been mainly focused on the copper(II) complexes with 1,10- phenanthroline ligand, due to their high nucleolytic efficiency, anti-tumor, antimicrobial[20] and photo induced cleavage.[17-18]

In this work we have reported the synthesis, DNA binding and antimicrobial as well as anticancer activity of a new polymer conjugate in which copper(II) phenanthroline complex units as well as ferrocenyl methylene units are attached to a branched polyethyleneimine molecule by a systematic synthetic procedure shown in Scheme 1.



Scheme 1 Synthesis procedure for polymer conjugates.

Experimental Section

Chemicals and Solutions

REAGENTS

Ferrocene carboxaldehyde, branched polyethyleneimine (BPEI) 25 KDa, calf thymus DNA, were obtained from Sigma Aldrich, $CuCl_2.2H_2O$, all salts and acids were purchased from Merk and Loba chemie (India). Tris-HCl was purchased from Loba Chemie, and sodium chloride (NaCl) was purchased from SISCO Research laboratories, India.

5mM TRIS –HCl BUFFER PREPARATION

A solution of CT- DNA in the buffer gave a ratio of UV absorbance at 260 nm to 280 nm of ~1.8–1.9: 1, indicating that the DNA was sufficiently free of protein. All the experiments involving the interaction of the polymer conjugate with CT DNA were carried out with twice distilled water in buffer containing 5 mM Tris-HCl/ 50 mM NaCl at pH 7.1.

Antimicrobial studies

Antimicrobial activity was evaluated for a range of microorganisms by assessing minimum inhibitory concentration (MIC) by broth microdilution method using 96-well microtiter plates. Disk diffusion assay using Mueller-Hinton agar plates for bacteria and Sabouraud dextrose agar for yeast. Gram positive bacteria (*Staphylococcus aureus* MTCC 2940 and *Bacillus subtilis* NCIM 2063), Gram negative bacteria (*Escherichia coli* MTCC 1610, *Vibrio cholerae* MTCC 3904 and *Salmonella typhi* MTCC 3917) and yeast (*Candida albicans* MTCC 227) were the reference strains used for both the tests (MTCC = Microbial Type Culture Collection, Chandigarh, India and NCIM = National Collection of Industrial Microorganisms, Pune, India).

Cytotoxicity studies

MTT ASSAY

A miniaturized viability assay using 3-(4,5-di-methyithiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was carried out with HepG2 cells according to the method described in Mosmann, 1983.[21] The gross morphological changes in the treated cells were observed in a phase contrast microscope (Carl Zeiss, India, Germany) and photographed. The cells were then assayed by addition of 20 µL of 5 mg/µL in phosphate-buffered saline (PBS). The plates were wrapped with aluminium foil and incubated for 4 h at 37° C. the purple formazan product was dissolved by addition of 100 µL of 1% 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 duplicates and used to calculate the respective means. AAS results have shown that 2000 µg/ml of our polymer conjugate (x=0.32) sample contains 1.76×10^{-7} mol/dm^3 of copper complex units. Therefore an IC_{50} value of the 24(± 0.5) µg/ml for the polymer conjugates corresponds to $2.1(\pm 0.5) \times 10^{-9}$ mol/dm^3 of copper complex units.

AO / EB STAINING

The HepG2 cells were seeded in 6-well plates and allowed to reach 80% confluence. The cells were then treated with the IC_{50} concentration of compounds Fc-BPEI, $Cu(phen)_2Cl_2$, $[Cu(phen)_2(BPEI)Cl]Cl$ & Polymer conjugates and incubated for 24 h. The cells were trypsinized and pelleted and then suspended in PBS. A drop of cell suspension was placed on a glass slide and stained with AO(acridine orange) and EB(ethidium bromide) (1% in PBS, separately; mixed in 1:1 ratio) (Sigma Chemical Co., St. Louis, MO, USA), and a cover slip was laid over it to reduce light diffraction. At random 100 cells were observed in a fluorescent microscope (Carl Zeiss, Jena, Germany) fitted with a 377–355 nm filter and examined at ×400 magnification.

Instrumentation

^{1}H NMR spectroscopy was performed on a Brucker 400 operating at 400 MHz in $CDCl_3$. FT-IR spectra were recorded on a FT-IR JASCO 460 PLUS spectrophotometer with samples prepared as KBr pellets. The UV–Visible absorption spectra were recorded at room temperature on a UV-1800 Shimadzu UV spectrophotometer.

Fluorescence experiments were carried out on a JASCO FP650 Spectrofluorometer (Japan), and the emission was measured

from 500 nm to 700 nm with an excitation wavelength of 450 nm. The excitation and emission slits were fixed at 3 nm, 5 nm and scanning speed to 500 nm/min.

The CHN contents of samples were determined by Perkin-Elmer 2400 Series CHNS/O Analyzer, Department of chemistry, IIT Madras, India. EPR spectra were recorded on JEOL-FA200 EPR spectrometer.

Electrochemical measurements were made on a 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 7.1) solution was used as supporting electrolyte. Prior to use, all electrodes were polished successively on alumina and washed thoroughly with doubly distilled water after each polishing step. Room temperature 25(±1°C) was maintained during the experiments.

Circular dichroism experiments were carried out using a quartz cell of 0.5 cm path length. Each CD spectrum was collected after averaging over at least 4 accumulations using a scan speed of 100 nm and 1 s response time.

AAS recorded on sensAA (Dual), GBC Scientific Equipment, Department of Department of Marine Science, Bharathidasan University, India. SEM with EDAX recorded on JEOL JSM-6390, Karunya University, India.

Polymer conjugates synthesis

SYNTHESIS OF $[Cu(phen)_2Cl]Cl$

$[Cu(phen)_2Cl]Cl$ was synthesised as per literature method.[22-24] Briefly, one mmole of $CuCl_2.2H_2O$ was dissolved in 30 ml of distilled water in a 100 ml beaker, to this 2 mmoles of 1,10-phenanthroline in 5 ml of ethanol, was added in warm condition solution above, and then whole mixture was heated to 80°C. The Green shining precipitate formed as the residue was filtered off. The precipitate was re-crystallized in ethanol and water mixture (1:4 v/v), Green shining crystals were obtained. [Yield = 0.515g].

SYNTHESIS OF FERROCENYL METHYLENE BRANCHED POLYETHYLENEIMINE (FC-BPEI)

The Fc-BPEI was synthesised by a reported procedure[9] (Scheme 1). Briefly In a round-bottom flask, 0.4319 g of BPEI (avg. MW ca.25 KD) was dissolved in 20 mL of methanol, and a solution of 0.428 g (2 mM) of ferrocenecarboxaldehyde dissolved in 5 mL of methanol was added to it dropwise under constant agitation. The resulting dark red solution was stirred for 2 h and cooled in an ice bath. Sodium borohydride 0.0757 g (2 mM) was added, upon which the solution lightened in colour. After 1 h, the methanol was removed under vacuum, and the residue was extracted overnight with diethyl ether to remove any nonreacted aldehyde and ferrocenylmethanol. The ether was then decanted, and the residue was washed with diethyl ether before being dried under vacuum. The residue was extracted with benzene, and insoluble salts were removed by filtration. Finally, the solvent was removed from the filtrate under vacuum to give 0.7887 g of Fc-BPEI. ^{1}H-NMR (400MHz, $CDCl_3$, δ): 4.1-4.3 (br, Fc ring H), 3.6 (s, Fc-CH_2-NH), 2.5-2.8 (br, -CH_2N-) (See Fig. 1A.).

SYNTHESIS OF THE POLYMER CONJUGATES

In a round bottom flask $[Cu(phen)_2Cl]Cl$ (1.25g) was taken in 30 ml ethanol, to this Fc-BPEI (0.75 g) was added. The reaction was carried out at 55-60 ^oC about 12h. After that the reaction mixture dialysed for a week against water. Finally our polymer conjugate was obtained like a filmy substance [yield: 1.75g ≈ 70%].

The spectral analysis data: 1H-NMR(400 MHz, $CDCl_3$, δ): 4.8-4.1 (m, cyclopentadiene protons), 2.6 (s, -CH_2-, 2H), 1.8-2.0, (b, –NH-CH_2-CH_2-NH- (ethylene diamine protons), see Fig. 1B.; IR(KBr): ν = 3427 (N-H str), 2920, 2847, 1649 (C=N), 1461 (C=C) the characteristic peaks of ferrocene was observed at 820 and 1414 cm^{-1} (see ESI Fig. S1.); UV-vis (*ethanol*): λ_{max} = 270, 440, and 661 nm (see Fig. 2.); The solid state EPR spectra of the compound was recorded in X-band frequencies at room temperature as well as in frozen solution (77 K). The polymer conjugate exhibits well defined single isotropic features near g = 2.0399 at room temperature. Such isotropic lines are usually the result of intermolecular spin exchange, which broadens the lines. This intermolecular spin exchange is caused by the strong spin coupling which occurs during a coupling of two paramagnetic species. Five coordinated copper(II) complexes may possess two geometries (trigonal bipyramidal and square pyramidal), which are characterized by ground states $d_x^2{}_{-y}^2$ or d_z^2. In frozen solution, The EPR spectra of the polymer–copper(II) complex showed axial spectra with well-defined g_{II} (2.1444) and $g_\perp$ (1.9316) features together with hyperfine lines, It shows $g_{II} > g_\perp$ value, which suggests a square pyramidal structure (see Fig. 3). From the Atomic absorption spectroscopy we have calculated the Cu and Fe weight percentages. From the weight percentage we have calculated the degree of coordination. The cross-section SEM morphology of the polymer conjugates is shown in Fig. 4 we can see metals coated sheet like layer. CHN analysis (atomic %): C 44.28, H 6.60, N 15.26; Fe and Cu concentrations (ppm) have been determined by AAS and SEM with EDAX (see Fig. 4 and Fig. S2).

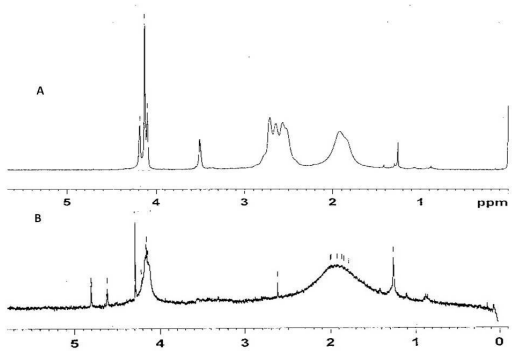


Fig. 1. ^{1}H-NMR of compounds (A) Fc-BPEI and (B) polymer conjugates in $CDCl_3$.

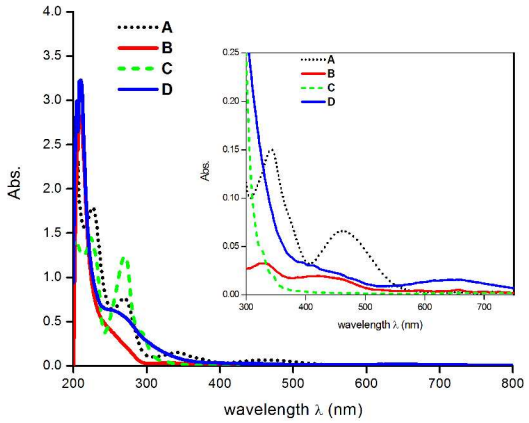


Fig. 2. UV-Vis spectrum of (Black short dotted line) - $Cu(phen)_2Cl_2$, (Red line) - $[Cu(phen)_2(BPEI)Cl]$, (Green short dashed line) - FC-BPEI and (Blue line) - polymer conjugate in ethanol (insert figure: Scale: wave length = 300-750nm; absorbance = 0-0.25)

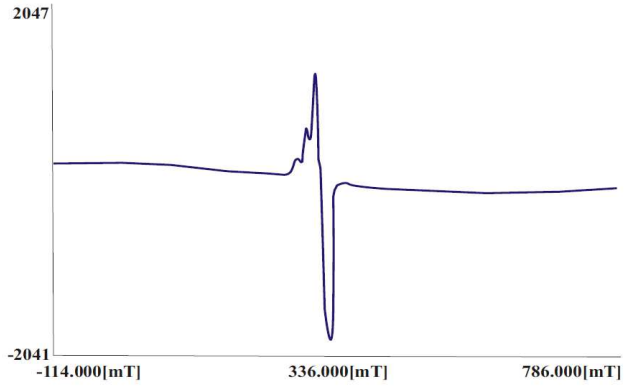


Fig. 3. EPR spectrum of the Cu(II) polymer conjugates at LNT.

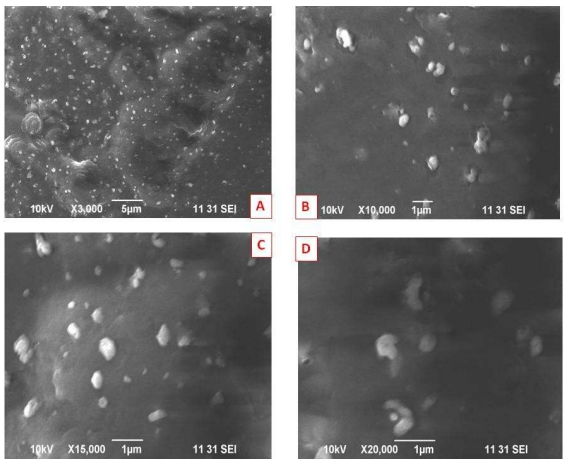


Fig. 4. SEM image A-D different magnifications of the polymer conjugate.

Results and Discussion

Synthesis and characterization of polymer conjugate

The synthesized polymer conjugates characterized by ^{1}H NMR, IR, UV, EPR, AAS, CHN, and SEM with Edax.

Binding Studies

The binding efficiency of the copper complex units of polymer conjugates with CT-DNA was obtained through UV-Vis absorption and competitive emission studies. The π-π* transition of phenanthroline ligand in the Cu(II) complex units has shown both slight blue shift and hyperchromism at 271 nm in presence of CT-DNA (see Fig. 5). This indicates that the mode of binding of copper complex units with DNA may be mostly through electrostatic interaction with partial intercalation.[25]

The intrinsic binding constant was calculated according to the following equation

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

where, [DNA] is the concentration of DNA. ε_a, ε_f, and ε_b correspond to the apparent extinction coefficient, the extinction coefficient for the free compound and its entirely DNA-bound combination, respectively. In the plots of [DNA]/ (ε_a - ε_f) versus [DNA], K_b was given by the ratio of the slope to intercept.

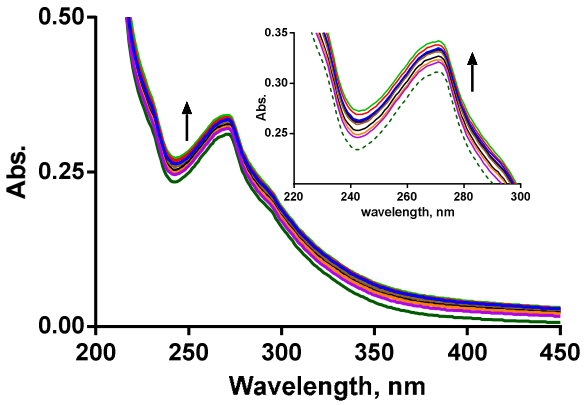


Fig. 5. Absorption spectra of the polymer conjugate, Dotted line Complex (= 20μM), lines with increasing order absence and presence of DNA by the addition of 10 μl respectively. [DNA] / [Complex] = 2.4.

This binding constant (K_b) is found to be 1.49 (±0.02)×10^6 M^{-1} and it is 500 times compared with that of the similar copper complexes $[Cu(phen)_2Cl_2]$[26] (2.7 × 10^3 M^{-1}), $[Cu_2(Phen)_2Cl_4]$[23] (4.75 × 10^4 M^{-1}), $[Cu(phen)_2BPEI]Cl_2.4H_2O$[25] (7.96 × 10^5 M^{-1}) and Ferrocene[27](3.45 × 10^2 M^{-1}). Also compared to the neat polymer copper complex, $[Cu(phen)_2(BPEI)Cl]Cl$ reported by us previously[25] the binding constant of polymer conjugates is higher which clearly shows that the presence of ferrocenyl

methylene moiety along with the copper complex units in the same polymer chain has facilitated this binding.

Competitive binding studies

As the polymer conjugates is not fluorescent we have performed competitive emission experiments with ethidium bromide as fluorescent material. The quenching of emission from EB bound CT-DNA with the successive addition of our polymer conjugate has also lead to the conclusion that this compound was able to replace EB from inside the CT-DNA. The emission intensity of EB-DNA is quenched on adding the polymer conjugates. The emission spectra of DNA-EB system in the absence and the presence of complex are demonstrated in Fig. 6. This clearly exposes decrease in the fluorescence intensity of the probe molecule (EB) by adding the complex. The decrease of the fluorescence intensity is because of releasing free EB molecules from DNA–EB complex. Fluorescence quenching is explained by the Stern–Volmer equation:

$$F_0/F = 1 + K_{sv}[Q] \quad (2)$$

where F_0 and F demonstrate the fluorescence intensities in the absence and in the presence of quencher, respectively. K_{sv} is quenching constant and [Q] is the concentration of quencher[28]. The binding constant (K_f) for the complex with DNA was assayed using the equation.

$$\text{Log}(F_0 - F/F) = \text{Log}\, K_f + n\, \text{Log}[Q] \quad (3)$$

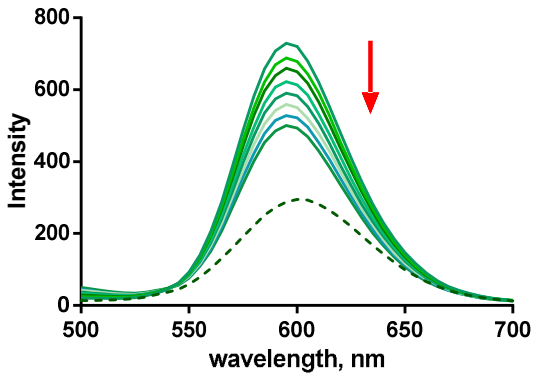


Fig. 6. Caption The Emission spectra of the compound. Dashed line EB alone, Dashed with dotted line EB(= 5μM) + [DNA] (= 5μM) and [Compound] = 35 mM each addition 10 μl (decreased intensity by the addition of 10 -100 μl respectively).

In this equation, F_0 and F are the fluorescence intensities of the fluorophore in the absence and presence of different concentrations of CT-DNA, respectively, n is the number of equivalent binding sites, which can be determined by the slope based on Equation 3. The values of K_f and n were found to be $K_f = 7.66(\pm 0.02) \times 10^5\ M^{-1}$, n = 0.9213; respectively.[29]

Cyclic voltammetry

Electrochemical investigations on the polymer conjugates-nucleic acid interaction provides a useful complement to the spectroscopic methods. It is known that the electrochemical potential of a copper complex will shift positively when it intercalates into nucleic acid, and if it is bound to nucleic acid by electrostatic interaction, the potential would shift in a negative direction.[30-31]

The cyclic voltammograms (CV) of the polymer conjugates in the absence and presence of CT DNA in tris-HCl buffer solution are shown in Fig. 7. No new peaks appeared after the addition of CT DNA. The cyclic voltammogram of polymer conjugates showed quasi reversible cyclic voltammetric responses at 0.432 V and 0.542 V for Fc^+-Fc couple along with the Cu(II) - Cu(I) couple at 0.176 V and 0.35 V versus SCE in Tris-HCl buffer medium respectively. After the each 100μl addition of CT DNA the voltammetric response was noted, the values of cathodic peak current I_{pc} showed 0.176 V to 0.118V negative shift and $\Delta E_{(Cu(II)-Cu(I))}$ values are 0.174 V to 0.242V. This shows that copper complex units in the polymer conjugates only involved in electrostatic interaction with DNA and ferrocene moiety was not involved but the Ferrocene moiety facilitates the electrostatic groove binding[32] because of the voltammetric response $\Delta E_{(Fc^+-Fc)}$ 0.11V and I_{pc} values are -1.201μA to -0.756 μA and I_{pa} values are -11.24 μA to -10.27 μA.

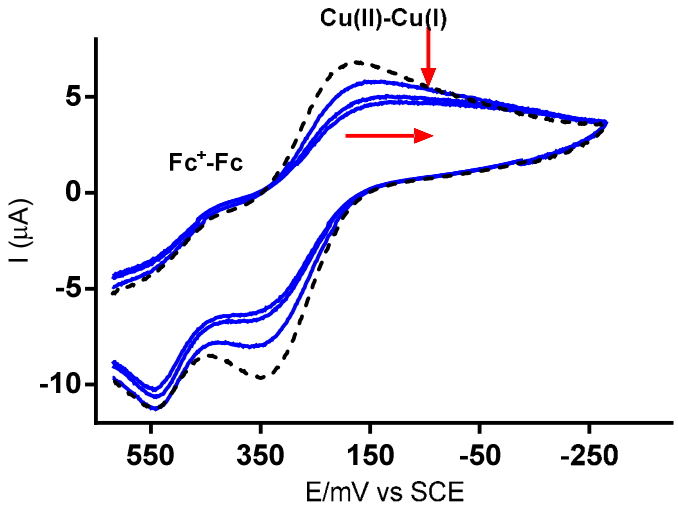


Fig. 7. Cyclic voltammetry measurements scan rate 50 mV/s. dashed-dot line polymer conjugates (20 μM) alone 50, 100, 150 μl, [CT-DNA] (= 35 mM) each addition.

These observations indicate the non-coordinating intercalative binding of copper complexes through 1,10 -phenanthroline ligand moiety between the DNA base pairs[33-34]. Hence it can be concluded that the copper(II) complex binds to DNA via intercalation, by insertion of the copper(II) complex between the base pairs of the DNA duplex strand. The binding constant,

K, of the interaction of the complex with DNA was assigned according to the following equation[34]:

$$\log (1/[DNA]) = \log(K) + \mathrm{Log}(I_{Free} / I_{Free} - I_{Bond}) \qquad (4)$$

Here K is the apparent binding constant. I_{Free} and I_{Bond} are the peak current of the free guest and adduct, respectively. The plot of log(1/[DNA]) versus $\log(I_{Free}/(I_{Free}-I_{bond}))$ according to Equation (3) is fairly linear as shown in Fig. 7. Binding constant of copper(II) complex and DNA was calculated to be $0.64(\pm 0.001) \times 10^4$ M^{-1} using DPV data.

Circular Dichroism

Circular dichroic spectral techniques give us useful information on how the conformation of the CT DNA chain is influenced by the bound complex, i) The CD spectrum of CT DNA consists of a positive band at about 260–280 nm that could be due to base stacking, ii) A negative band at 243 nm that could be due to helicity and it is also characteristic of DNA in a right-handed B form.

The changes in CD signals of CT DNA observed on its interaction with drugs may often be assigned to the corresponding changes in CT DNA structure. i) Simple groove binding and the electrostatic interaction of small molecules show less or no perturbation on the basestacking and helicity bands, ii) Intercalation enhances the intensities of both the bands stabilizing the right-handed B conformation of CT DNA, as observed for the classical intercalator methylene blue.[35]

Here we have performed circular dichroism studies of CT-DNA in presence and absence of polymer conjugates of the present study (see Fig. 8). in the Tris buffer solution. The CD technique revealed that polymer conjugates were able to change the ellipsity of DNA molecule considerably by the electrostatic and groove binding.

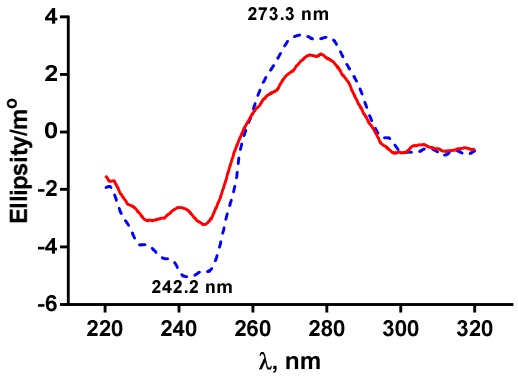


Fig. 8. Circular dichroism spectra in the absence (dashed line) and in the presence of [Complex] 30μM and [DNA] 90μM in the Tris buffer solution.

Antimicrobial studies

Antimicrobial studies have shown that minimum inhibitory concentration (MIC) of the polymer conjugates differs with the nature of the pathogens in the range of 250-350 μg/ml (see Table 1). Zone of inhibition of the compound by disk diffusion assay performed better for bacteria than yeast (Table 1). In both cases, the antimicrobial activity was less for the synthesized compound than most of the reference antibiotics (Table 1).[36-37]

Table 1. MIC values and Zone of inhibition obtained for the synthesized compound and reference antibiotics against the pathogenic microbes

	Escherichia coli MTCC 1610	*Staphylococcus aureus* MTCC 2940	*Salmonella typhi* MTCC 3917	*Vibrio Cholerae* MTCC 3904	*Bacillus Subtilis* NCIM 2063	*Candida albicans* MTCC 227
MIC value of the compound (μg/ml)	250	250	250	300	300	350
Diameter of zone of inhibition of compound (mm) at MIC level/disk	12	12	12	11	11	8
Diameter of zone of inhibition of compound (mm) at 500μg /disk	15	14	14	15	12	8
Diameter of zone of inhibition (mm) of reference antibiotics (μg/disk in parenthesis)						
Amphicilin (10)	16	17	10	22	15	26
Ciprofloxacin (5)	48	34	31	46	32	30
Co Trimoxazole (25)	42	36	11	25	11	16
Amoxyclav (30)	22	20	12	18	12	28
Nitrofurantoin (300)	26	17	14	22	0	26
Norfloxacin (10)	44	34	26	46	31	36
Fosfomycin (200)	46	40	10	19	32	28
Amikacin (30)	40	36	29	40	30	40
Gentamycin (10)	40	40	29	44	26	34
Ceftriaxone (30)	36	36	23	28	30	36

Zone of inhibition increased with increase in the concentration of the compound when tested at MIC level (250-350 μg/disk) and at a higher concentration (500μg/disk). The trends in MIC and disk diffusion assay indicate that the compound can find applications as bacteriostatic agent against Gram positives and Gram negatives.

Cytotoxicity studies

Anticancer activity of the polymer conjugates was found out with the HepG2 cells which are suitable in vitro model systems for the study of polarized human hepatocytes. The polymer conjugates was dissolved in 1% DMSO, diluted in culture medium and used to treat the HepG2 cells over a complex concentration of 10 to 100 μg/ml for a period of 24 h. 1% DMSO was used as the solvent control. A miniaturized viability assay using 3-(4,5-di-methyithiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was carried out according to a literature method.[21]

The percentage inhibition was calculated, from this data, using the formula:

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

The IC_{50} value was determined as the concentration of the compounds that is required to reduce the absorbance to half that of the control. This IC_{50} value in terms of copper complex units for the polymer conjugates with HepG2 cells is found to be $2.1(\pm 0.5) \times 10^{-9}$ mol/dm^3 indicating that compared to BPEI[38]

and other mimic systems, the activity of the polymer conjugates is very high. (See Table 2.)

Table 2. The IC_{50} values for *in vitro* cytotoxic studies.

Compounds	IC_{50}, for 24 hrs.
$[Cu(phen)_2Cl]Cl$	10 ± 0.5 µg/ml ($20.2 \pm 0.5 \times 10^{-6}$ mol/dm^3)
$[Cu(phen)_2BPEI]Cl_2 4H2O$	10 ± 0.5 µg/ml ($87.9 \pm 0.5 \times 10^{-6}$ mol/dm^3)
$[Cu(phen)_2$ (Fc-BPEI)] Cl_2	24 ± 0.5 µg/ml ($2.1 \pm 0.5 \times 10^{-9}$ mol/dm^3)

Values in parentheses indicate corresponding concentration of copper complex units.
Fc-BPEI 69 ± 0.5 µg/ml ([Fc] = $8.1 \pm 0.5 \times 10^{-7}$ mol/dm^3).

Evaluation of Apoptosis

To detect apoptosis, at a basic level, we adopted AO/EB staining, to visualize and quantify the number of viable and apoptic cells. Viable cells exhibit a large green nuclei, whereas apoptic cells shows signs of nuclear condensation or nuclear bead formation. These morphological changes are due to the activation of caspase cascades[39] which cleaves the specific substrates responsible for DNA repair activation which would reveal the changes in the gross cytology of the cell[40], and with special reference to cytoplasm and nucleus. After treatment of HepG2 cells with the complexes at their respective IC_{50} concentrations for 24 h, the cells were observed for the gross cytological changes. The treated cells revealed all the above cytological changes: (i) viable cells show light green fluorescing nuclei with highly organized structure; (ii) early apoptotic cells show bright green fluorescing nuclei with chromatin condensation and nuclear fragments; (iii) late apoptotic cells show orange to red fluorescence with condensed or fragmented chromatin; and (iv) necrotic cells show red fluorescing without chromatin fragmentation (see Fig. 9). These cytological changes indicated that the cells were committed to cell death, mostly through apoptosis and to a certain extent necrosis. It shows that the polymer conjugates acts as a good anticancer agent.

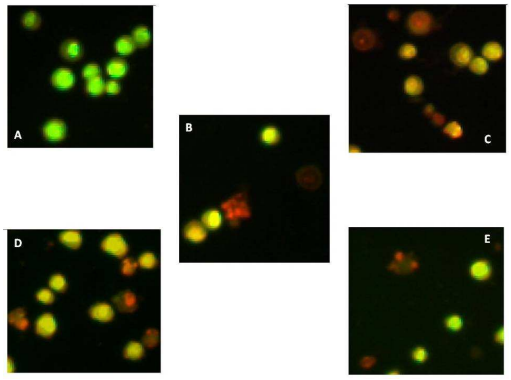


Fig. 9. AO / EB staining assay of the A- Control , B- $[Cu(phen)_2Cl]Cl$, C- Fc-BPEI , D- polymer conjugates, E- $[Cu(phen)_2(BPEI)Cl]Cl$, polymer conjugates in 24 hrs.

Conclusions

In summary, a new polymer conjugate containing both paramagnetic copper(II) phenanthroline complex units and diamagnetic ferrocenyl methylene units in a single polymer chain has been developed. The copper (II) phenanthroline complex units bind with DNA more strongly than both known the simple copper(II) complexes and polymer attached copper(II) complexes. In vitro cytotoxicity study demonstrates good anticancer activity of the polymer conjugates have good retention power and Ferrocenyl moiety plays good role. In addition antimicrobial studies show that the polymer conjugates are bacteriostatic agents.

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