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DNA interaction of [Cu(dmp)(phen-dion)] (dmp = 4,7 and 2,9 dimethyl phenanthroline, phen-dion = 1,10-phenanthroline-5,6-dion) complexes and DNA-based electrochemical biosensor using chitosan–carbon nanotubes composite film

Soheila Kashanian [a,*], Mohammad Mehdi Khodaei [a,*], Hamideh Roshanfekr [b], Hossein Peyman [b]

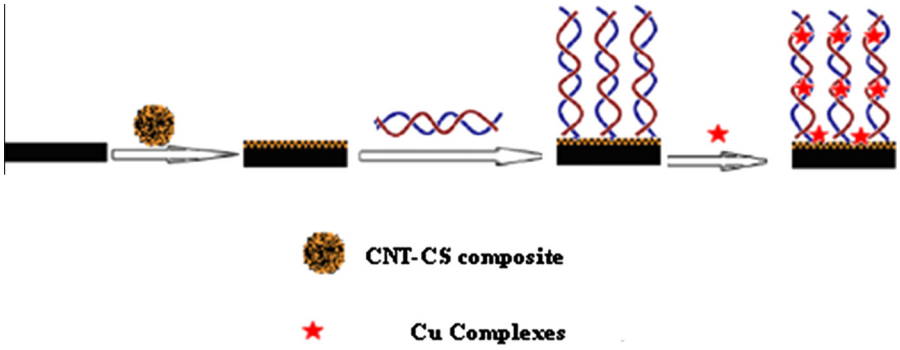
[a] Faulty of Chemistry, Sensor and Biosensor Research Center (SBRC) & Nanoscience and Nanotechnology Research Center (NNRC), Razi University, P.O. Box: 67149, Kermanshah, Iran
[b] Islamic Azad University, Ilam Branch, Department of Chemistry, Ilam, Iran

HIGHLIGHTS

- This study is a good suggestion to design improved drugs that target the cellular DNA.
- It was studied the relationship between DNA-binding affinities and ligands shape.
- This study suggested some good candidates for electroactive hybridization probes.

GRAPHICAL ABSTRACT

Binding interaction of two water soluble copper(II) complexes [Cu(4,7-dmp)(phen-dione)Cl]Cl (1) and [Cu(2,9-dmp)(phen-dione)Cl]Cl (2), with calf thymus DNA (CT-DNA) has been investigated by instrumental methods. Moreover, it was investigated the interaction of these complexes with immobilized DNA on a chitosan–carbon nanotube support.



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ABSTRACT

The interaction of two new water-soluble [Cu(4,7-dmp)(phen-dione)Cl]Cl (1) and [Cu(2,9-dmp)(phen-dione)Cl]Cl (2) which dmp is dimethyl-1,10-phenanthroline and phen-dion represents 1,10-phenanthroline-5,6-dion, with DNA in solution and immobilized DNA on a chitosan–carbon nanotubes composite modified glassy carbon electrode were investigated by cyclic voltammetry and UV–Vis spectroscopy techniques. In solution interactions, spectroscopic and electrochemical evidences indicate outside binding of these complexes. To clarify the binding mode of complexes, it was done competition studies with Hoechst and Neutral red as groove binder and intercalative probes, respectively. All these results indicating that, these two complexes (1) and (2) interact with DNA via groove binding and partially intercalative modes, respectively. The electrochemical characterization experiments showed that the nanocomposite film of chitosan–carbon nanotubes could effectively immobilize DNA and greatly improve the electron-transfer reactions of the electroactive molecules that latter finding is the result of strong interactions between captured DNA and Cu complexes. This result indicates that these complexes could be noble candidates as hybridization indicators in further studies. At the end, these new complexes showed excellent antitumor activity against K562 (human chronic myeloid leukemia) cell lines.

* Corresponding authors. Fax: +98 831 4274559.
E-mail addresses: kashanian_s@yahoo.com (S. Kashanian), mm_khodaei@yahoo.com (M.M. Khodaei).

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Introduction

DNA and ligand interactions, which can form large complexes, play a major role in biological processes such as gene transcription or DNA replication. A large number of natural and synthetic ligands have been discovered, which bind to DNA with intercalative or non-intercalative mechanisms, and some of them with cytotoxic activity are used in cancer chemotherapy [1]. There is substantial and continuing interest in redox and spectroscopically active metal complexes that bind and interact with DNA [2–5]. Such complexes are also studied with the purpose of developing novel hybridization indicators in DNA biosensors.

Copper is a biologically relevant element and its complexes have demonstrated a wide range of pharmacological activity such as antiviral [6], anticancer [7] and anti-inflammatory activity [8]. Copper-based reactions of DNA are particularly interesting since they have the potential for the in vivo applications. Nature of the reactive species responsible for interaction, and the type of forces, which provide the non-covalent interaction between the complex and DNA target, are the main point of views which are usually addressed. The intrinsic redox chemistry due to the redox-active metal ion as well as the extrinsic stereoelectronic and steric properties of diimine ligand complexes are considered to identify the overall reactivity and DNA binding properties [9]. So, the application of mixed ligand complexes permits variation in the geometry, size and hydrophobicity by systematically adjusting complex ligands and their substituents, and provides the opportunity to determine how these factors contribute to the affinity in DNA binding. The mechanism of interaction can be investigated in three different ways, i.e., interaction in solution, DNA modified electrode and compound-modified electrode, which we studied the first and the second means in this work.

Electrochemical DNA biosensors encompass a nucleic acid recognition layer that is immobilized over an electrochemical transducer. The role of the nucleic acid recognition layer is either to detect the changes which occur in the DNA structure during interaction with DNA-binding molecules or to detect selectively a specific sequence of DNA. Generally speaking, electrochemical DNA biosensors can be used to study DNA interaction with different complexes over a wide potential range, at any ionic strength, and over a wide pH range.

Recently, nanomaterials have received increasing attention to design DNA biosensors due to their distinctive chemical and physical properties caused by quantum confinement, small size, and macro quantum tunnel effects [10,11]. As a more common material, carbon Nanotubes[1] (CNTs) have been continually used as an ideal material to fabricate electrochemical biosensors since they were discovered in 1991 by Iijima [12]. However, lack of stability and uniformity of CNTs affect their direct immobilization onto the electrode surface. Therefore chitosan has also been applied to improve DNA immobilization process [13–15]. Moreover, low conductivity of these films, leading to delay in electron transfer to some extent. In order to overcome mentioned deficiency, functionalized conductive polymers were electropolymerized on the surface of electrode [16–18]. Nevertheless, the synthesis of the functionalized monomers has always been complicated, which consequently has limited its application. Hence in order to improve conductivity, films were doped with CNTs. Chitosan–CNT film can be used to increase the electrochemical signal of the DNA indicator and to improve sensitivity of biosensor.

In this report, we explore the interaction of mixed ligand complexes of the type [Cu(dmp)(phen-dione)Cl]Cl (Fig. 1) with B DNA using a variety of biophysical and spectroscopic methods. A precise understanding of DNA binding properties is essential for their potential applications as chemical probes of nucleic acid conformation and drug design.

[1] *Abbreviations*: CNTs, carbon nanotubes; CS, Chitosan; NR, neutral red; HO, hoechst; TB, trypan blue; ds-DNA, double stranded DNA; ss-DNA, single stranded DNA.

Materials and methods

Chemistry

The highly polymerized of CT-DNA and Chitosan were purchased from Sigma Corp and used as received. [Cu(dmp)(phen-dione)Cl]Cl complexes (Fig. 1) were prepared according to literature methods [19]. HEPES, Tris-base, NaOH, Neutral Red (NR), NaCl, Hoechst (HO), potassium ferro-/ferricyanide were purchased from Merck. Multiwall carbon nanotubes (MWCNTs) with purity of 95% (10–20 nm diameters) and 1 μm length were obtained from Nanolab (Brighton, MA). K562 (human chronic myeloid leukemia) cell line was kindly provided from Medical Biology Research Center, Kermanshah University of Medical Sciences. RPMI 1640, fetal bovine serum (FBS) from Gibco, New York, USA and L-glutamine, $NaHCO_3$, sodium pyruvate, penicillin, and streptomycin were purchased from Sigma–Aldrich.

Synthesis of [Cu(dmphen)(phen-dione)Cl]Cl

[Cu(dmp)(phen-dione)Cl]Cl complexes (Fig. 1) were prepared according to literature methods [19]. For complex (1), a solution of $CuCl_2.2H_2O$ (170 mg, 1 mmol) in 10 mL EtOH was added to a solution of the 4,7-dimethyl-1,10-phenanthroline (208 mg, 1 mmol) in 10 mL EtOH and the mixture was stirred at room temperature for 1 h. To the solution was then added 1,10-phenanthroline-5,6-dione (210 mg, 1 mmol) and the resulting solution was stirred at room temperature for 24 h. The solution was slowly evaporated and the resulting green fine crystals were collected by filtration. Yield: 463 mg, 83%. Anal. Calc. for $C_{26}H_{18}CuN_4O_2Cl_2$: C, 56.49; H, 3.25; N, 10.13. Found: C, 56.61; H, 3.30; N, 10.35%. IR (KBr): ν (cm^{-1}) 3060, 2985, 1701, 1626, 1574, 1428, 1299, 1067, 838, 723, 557, 425.

And complex (2) was synthesized in an identical manner which was described for complex 1 with 2,9-dimethyl-1,10-phenanthroline (L2) in the position of L1. Yield: 451 mg, 81%. Anal. Calc. for $C_{26}H_{18}CuN_4O_2Cl_2$: C, 56.49; H, 3.25; N, 10.13. Found: C, 56.31; H, 3.29; N, 10.41%. IR (KBr): ν (cm^{-1}) 3036, 2956, 2920, 1700, 1618, 1577, 1420, 1297, 1029, 861, 728, 550, 420.

DNA binding measurements and instrumentation

Disodium salt of CT DNA was stored at 4 °C. The stock solution of DNA was prepared according to our previous works [20,21]. Solutions of DNA in the HEPES buffer (10 mM, pH 7.2) gave the ratio of UV absorbance at 260 and 280 nm, A_{260}/A_{280}; of 1.9 indicating that the DNA was sufficiently free of protein. Concentrated stock solutions of DNA were prepared in buffer. The concentration of DNA was measured using its extinction coefficient at 260 nm (6600 M^{-1} cm^{-1}) after dilutions. Stock solutions were stored at 4 °C and were used no more than 4 days.

Concentrated stock solutions of the copper complexes were prepared by dissolving the complexes in H_2O and diluting suitably with the corresponding buffer to the required concentrations for all of the experiments.

The absorption spectra were recorded on an Agilent 8453 UV–Vis spectrophotometer equipped with a thermostated bath (Huber polysat cc1) using cuvettes of 1 cm path length. In a typical experiment, 2.5 mL solutions of complexes (1×10^{-4} M for complex 1 and 2) were transferred into a cuvette. Absorbance titration was conducted by adding concentrated stock solutions of CT-DNA

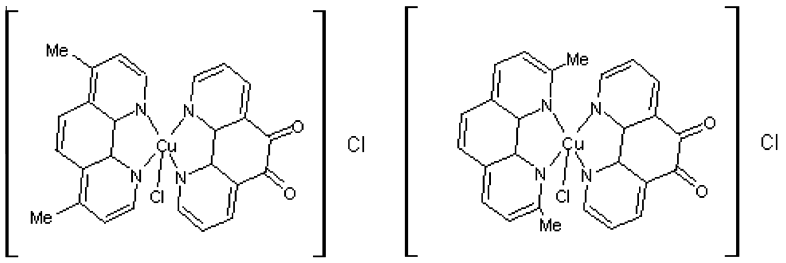

Fig. 1. Schematic structure of [Cu(4,7-dimethyl-1,10-phenanthroline)(phen-dione)Cl]Cl (1) and [Cu(2,9-dimethyl-1,10-phenanthroline)(phen-dione)Cl]Cl (2).

(1.0×10^{-3} M) directly to the cuvette. UV–Vis spectra were recorded in the range of 200–350 nm about 3 min after each addition (10 μL) of DNA solution.

For competition studies with NR, 2.5 mL solution of NR (1×10^{-5} M) were transferred into a cuvette. Absorbance titration was conducted by adding concentrated stock solutions of CT-DNA (1.0×10^{-3} M) directly to the cuvette. This solution was allowed to stand for 5 min, and then spectrophotometric data were collected from solutions of different DNA concentrations. Then, different amounts of Cu complexes were added to a series of solution containing NR and DNA (NR/DNA = 3.0).

For NaCl effect studies a series of assay solutions containing various amounts of NaCl and a fixed amount of DNA–Cu complexes ([Cu complexes] = 5×10^{-5} M; [DNA] = 2.5×10^{-4} M) were prepared to measure the fluorescence intensity [21]. These measurements were carried out with a JASCO spectrofluorimeter (FP 6200).

The cyclic voltammetry measurements were performed using an AUTOLAB model (PG STAT C), with a three-electrode system: a 0.10 cm-diameter Glassy Carbon (GC) disc as working electrode, an Ag/AgCl electrode as reference electrode, and a Pt wire as counter electrode. Electrochemical experiments were carried out in a 25 mL voltammetric cell at room temperature. All potentials are referred to the Ag/AgCl reference. Their surfaces were freshly polished with 0.05 mm alumina prior to each experiment and were rinsed using double distilled water between each polishing step. The supporting electrolyte was 10 mM of HEPES buffer solution (pH 7.2) which was prepared with double distilled water. CV measurements were recorded by keeping the concentration of Cu(II) complex constant (5×10^{-4} M) while varying the DNA concentration from 0 to 5×10^{-5} M [22,23]. In the competition experiments cyclic voltammograms (CV) were recorded by progressive addition of Cu complexes from 0–15×10^{-5} M to DNA-Hoechst solutions at a constant concentration ([DNA] = 5×10^{-5} M, [HO] = 1.00×10^{-5} M).

Preparation of CS-CNTs nanocomposite modified electrode

CS-CNTs nanocomposite was prepared according to literature methods [24]. Briefly, Chitosan solution was prepared in 2% acetic acid. MWCNTs were refluxed in concentrated HNO_3–H_2SO_4 (V/V, 1:1) for 2 h, were filtered and washed with double distilled water until the filtrate was neutral, and finally were dried under vacuum. Then, 2 mg of CNTs were dissolved in 2% acetic acid containing 2 mg chitosan and were ultrasonicated for 2 h to obtain a uniform mixture. CS–CNT solution (5 μL) was spread uniformly to the freshly polished electrode surface that is noted as CS–CNTs/GCE. In this way, a robust chitosan film doped with CNTs was formed.

DNA probe immobilization

To obtain the DNA-modified CS–CNTs/GCE, 5μL of 0.5 gL^{-1} dsDNA and ssDNA were pipetted on the surface of the CS–CNT/GCE. After air drying, the electrode was rinsed thoroughly with distilled water to eliminate the nonadsorbed DNA. Thus, the DNA-modified electrode (ssDNA/CS–CNTs/GCE or dsDNA/CS–CNTs) was designed.

Cell cultures and treatments

The human chronic myeloid leukemia K562 cell line was provided to treat with the complexes. K562 cell line was cultured in RPMI 1640, supplemented with 10% fetal bovine serum (FBS), 10 U/mL penicillin, 100 μg/mL streptomycin, 0.2 mg/mL L-glutamine, 0.002 g/mL $NaHCO_3$ and 0.0011 g/mL sodium pyruvate in an atmosphere of 96% air/5% CO_2, with 96% humidity, at 37 °C. Cells were seeded at a density of 4×10^5 cells/mL, and were incubated at 37 °C. After 24 h they were treated with Cu complexes, while in exponential growth phase, for the indicated time periods. The stock solutions of both complexes were prepared in H_2O immediately before use. The complexes were added to the cell media where they remained constantly for the appropriate time periods [21].

Cytotoxicity assay by trypan blue staining

A trypan blue (TB) exclusion assay [21] was done to detect the cytotoxicity induced by Cu compounds. 100 μL aliquots of the exponentially growing were incubated with various volumes of the stock compounds that were diluted in cell medium to reach various final concentrations ranging from 0.00, 1.28, 3.20, 8.00, 20.00, 50.00 μM. After 24 and 48 h, 20 μL of samples was added to 20 μL TB (0.4%) in a test tube. The total viable cell number was determined with a hemocytometer chamber. The cell exposure time to trypan blue did not exceed 5 min, because extensive exposure is possible to cause an increase in the dead cell population (trypan blue positive) due to the trypan blue toxicity. The total number of cells and the number of blue-stained cells were counted on a haemocytometer by observing under a microscope [25].

To calculate the concentration of each complex that produces a 50% inhibition of cell growth (IC50), the concentration of viable cells was calculated and results were finally expressed as the% cell viability versus compounds concentration.

Cell proliferation

The study of the proliferative capacity of the leukemia cells includes determination of the number of cells that were divided in the culture in the presence/absence of the complexes under study, as well as creation of the growth curves. To achieve this purpose, in the same manner as previously described exponentially growing cells were seeded at a density of 4×10^5 cells/mL, in duplicate, and 24 h later they were treated with Cu complexes, at 37 °C in a humidified 5% CO_2 atmosphere. After 24 and 48 h, each sample was mixed with TB (0.4%) thoroughly and total cell number was

counted on a haemocytometer by observing under a microscope [20].

Results and discussion

Interaction between Cu complexes and DNA in solution

The following experiments were performed to characterize and distinguish the mode of binding of Cu complexes to CT-DNA.

Absorption titration

Electronic absorption spectroscopy is universally employed to determine the binding characteristics of metal complex with DNA [26–28].

It is well known that red shift and hypochromism in the absorption spectral bands accompany the intercalative binding of molecules to DNA [29]. In addition, the spectra of intercalators are more perturbed than those of groove binders and the extent of hypochromism generally parallels the strength of intercalative binding [30,31]. Also, it is convenient to investigate the interaction through detecting UV–Vis spectra due to the correlation with metal to ligand charge transfer (MLCT) or ligand to metal charge transfer (LMCT) in copper complexes [20,21]. The UV absorption spectra of [Cu(4,7-dmp)(phen-dione)Cl]Cl (1) and [Cu(2,9-dmp)(phen-dione)Cl] (2) were recorded in the absence and presence of DNA. In the ultraviolet region from 200 to 350 nm, the two mentioned metal complexes exhibited intense ligand-based $\pi \rightarrow \pi^*$ bands (~227 nm) and a broad metal-to-ligand ($\pi_M \rightarrow \pi_L{}^*$) charge transfer (MLCT) band (270–274 nm). It can be predictable that, the UV curves of two complexes are almost the same.

As it was shown in Fig. 2, titration of DNA with the two complexes gave rise to some spectral changes (data was shown for complex 2). With increasing DNA concentrations, for both complexes, it was shown hypochromicity in UV spectra. Also, the λ_{max} for complex 1 increased nearly 2 nm. Such a small change in λ_{max} is more representative of groove binding nature, leading to small perturbations. Such small increases in the λ_{max} and the hypochromicity have been observed in the case of some porphyrin and copper complexes on their interaction with DNA [32–34].

The copper (II) complexes can bind to the double stranded DNA in different binding modes based on 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 carbonyl groups of phen-dione ligand in the copper (II) complexes form hydrogen bonds with DNA. On the other hand, our copper (II) complexes, which possess dmp-phen groups, can bind to DNA by van der Waals interactions between the mentioned groups and the DNA bases. In our complexes, complete intercalation of the dmp-phen ligands between a set of adjacent base pairs is sterically impossible, but perhaps type of partial intercalation or groove binding may be observed, but this needs further clarification of the DNA binding mode of the complex by other measurements.

In order to compare quantitatively the binding strength of the two complexes, their intrinsic binding constants for binding with CT-DNA were obtained by monitoring the changes in the absorbance for both complexes, with increasing of DNA concentration. The binding constant K_b is determined from a plot of [DNA]/(ε_a–ε_f) versus [DNA] using the 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, the molar absorption coefficients ε_a, ε_b and ε_f represent the apparent absorption coefficient for the complex, the extinction coefficient for the complex in the fully bound form and the extinction coefficient for the free mononuclear complex, respectively. K_b is given by the ratio of slope to the intercept [35]. The plot of [DNA]/(ε_a–ε_f) versus [DNA] is shown in Fig. 2. The K_b value for complex 1 is $18 \times 10^5\ M^{-1}$, which is approximately 1.7 times greater than the K_b value ($10.5 \times 10^5\ M^{-1}$) for complex 2, suggesting a significant stronger affinity of complex 1 to DNA than that of complex 2. The K_b values of these complexes is comparable with [Cu(phen)(phen-dione)Cl]Cl [20] and [Cu(4,7-biphphen)(phen-dione)Cl]Cl [21], in this order: 4,7-biph > 4,7-dmp > phen > 2,9 – dmp (Table 1). The higher affinity of complex [Cu(4,7-biphen)(phen-dione)Cl]Cl towards DNA may be due to its more extended area of the ligand (biphen-phen) which greatly facilitates intercalation between a set of adjacent base pairs.

For the phen complex, a partial intercalation of the phen ring inserted between the adjacent base pairs of DNA occurs in [Cu(-phen)(phen-dione)Cl]Cl complex [20] ($K_b = 12.5 \times 10^5\ M^{-1}$); the less steric incompatible and favorable hydrogen bonding of the phen-dion ligands to the oxygens and nitrogens of adjacent bases as well as to neighboring phosphate groups of DNA provide additional stability to the interaction [36,37]. The incorporation of methyl groups on phen ring as in 4,7-dmp complex would be expected to sterically hinder the partial insertion of phen ring into DNA base pairs and weaken the binding of the complexes with DNA and hence decrease the K_b values. Nevertheless, the higher values observed, rule out the possibility of partial intercalation of the 4,7-dmp ring as the DNA binding mode. The present 4,7-dmp complex is then expected to exhibit DNA groove binding involving hydrophobic interactions, supported by the hydrogen bonding interactions of the carbonyl groups in phen-dion ligand with the N7 and O6 atoms of the adjacent guanine bases. Thus, the highest K_b value for the 4,7-dmp complex reveals that it binds to DNA by locating the hydrophobic methyl groups on the floor of the DNA grooves. However, the lower negligible value of K_b for 2,9-dmp complex reveals that arranging of methyl groups in 2,9 positions on phen ring causes partially intercalation of phen ring into adjacent bases. Thus, the shape and size length and depth of the diimine ligands, as modified by the methyl substituents, determine the mode of bindings. However, a detailed molecular modeling study is needed to provide support for these implications.

The K_b values obtained here are lower than that of reported for classical intercalators (for ethidium bromide whose binding constants have been found to be in the order of 10^6–$10^7\ M^{-1}$) [38]. In comparing the intrinsic binding constant (K_b) of these complexes with some DNA groove binders and partially intercalators, as observed in the literature, we can deduce that these complexes (1 and 2) bind to CT-DNA via groove binding and partially intercalating, respectively [39–44].

Neutral red (NR) displacement study

Competitive binding studies with NR were carried out to provide another support for the mode of binding of the Cu complexes with DNA. Absorption spectra of the NR dye in the absence and presence of DNA (pH 7.4) showed a maximum at 454 nm (Fig. 3). This absorption gradually decreased while increasing the concentration of DNA, and a new band at 541 nm was developed. An isosbestic point at 500 nm provided an evidence for the new NR–DNA complex formation. These spectral results indicate that NR is an intercalator whose chromophore inserts into the base pairs [45]. If a molecule could insert between DNA bases and be able to replace the NR, the absorption peak in 538 nm will decrease and the peak in 450 nm will increase again, (which is related to free NR). It was shown in Fig. 3A and Fig. 3B after addition of the complexes to NR-DNA solution; there was no complete recovery in the NR peaks (450 and 538 nm). This finding indicated that these complexes could not insert between DNA bases to be replaced by NR molecules, this phenomenon is indicative of their non-intercalative mode of binding [45].

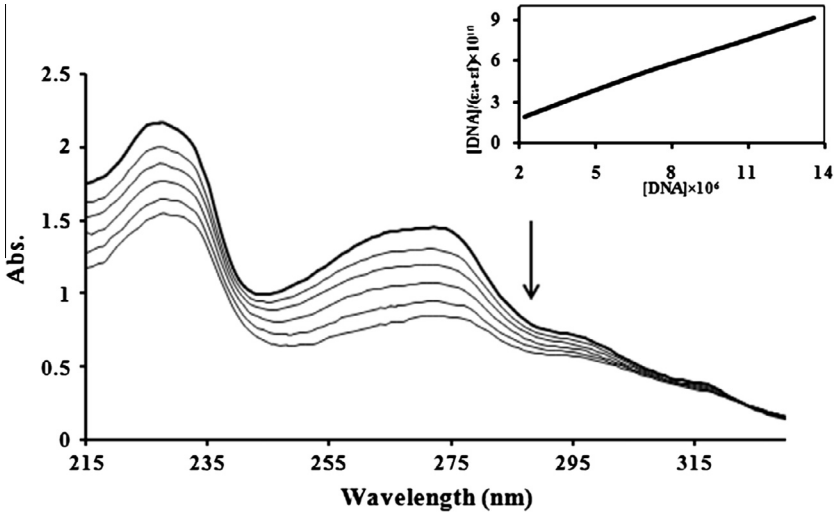

Fig. 2. Absorption spectra of a [Cu(2,9-dmp)(phen-dione)Cl]Cl in 10 mM Hepes buffer (pH 7.2) in the presence of increasing amounts of calf thymus DNA (CT-DNA) (1×10^{-4} M Cu complexes, 0–15 μM DNA). Arrows show the change in absorbance upon increasing the DNA concentration. Inset: Plot of $(\varepsilon_a-\varepsilon_f)/(\varepsilon_b-\varepsilon_f)$ versus [DNA] for the titration of DNA to Cu complex.

Table 1
Binding constants for interaction of Cu complexes with DNA.

Complex	$K_b \times 10^{-5}$ (M^{-1} bp^{-1})
[Cu(4,7-biphphen)(phen-dione)Cl]	27.5
[Cu(4,7-dimethylphen)(phen-dione)Cl]	18
[Cu(phen)(phen-dione)Cl]	12.5
[Cu(2,9-dimethylphen)(phen-dione)Cl]	10.5

Influence of DNA denaturation

Denatured DNA was produced by heating a native calf thymus DNA solution in a water bath at 100 °C for 5 min, followed by rapid cooling in an ice bath to 0 °C before being brought back to room temperature [46]. CT-DNA was split into two string-like polynucleotide chains from the original rigid double-helix structure. Similar to UV titration at above experiment, here 2.5 mL solutions of complexes [Cu(dmp)(phen-dione)Cl]Cl were transferred into cuvettes. Absorbance titration was recorded after adding solutions of native and denatured CT-DNA directly to each cuvette. Figs. 4A and Fig. 4B, present the characteristic of Cu complexes bound to native and denatured DNA. One may consider that these complexes interacted with both native and denatured DNA.

However, it is notable that the decrease in the absorbance intensity of Cu complexes binding to denatured DNA is more than that of native DNA. Difference between absorbance intensity of native and denatured DNA for complex 1 is more than that of complex 2. It could be predictable that about complex 2 interaction with dsDNA to be stronger than ssDNA due to partially intercalation. In spit of our prediction, we observed a bit stronger interaction with ssDNA, and just the difference between ds and ss-DNA interaction is smaller. So we can conclude, rather than partially intercalation due to 2,9-dimethyl phenanthroline moiety, we are encountered with another interaction. The latter is mainly hydrogen interactions in phen-dion moiety which occurs on the surface. Therefore, about complex 2, interactions occur on the surface and partially inside DNA structure, simultaneously.

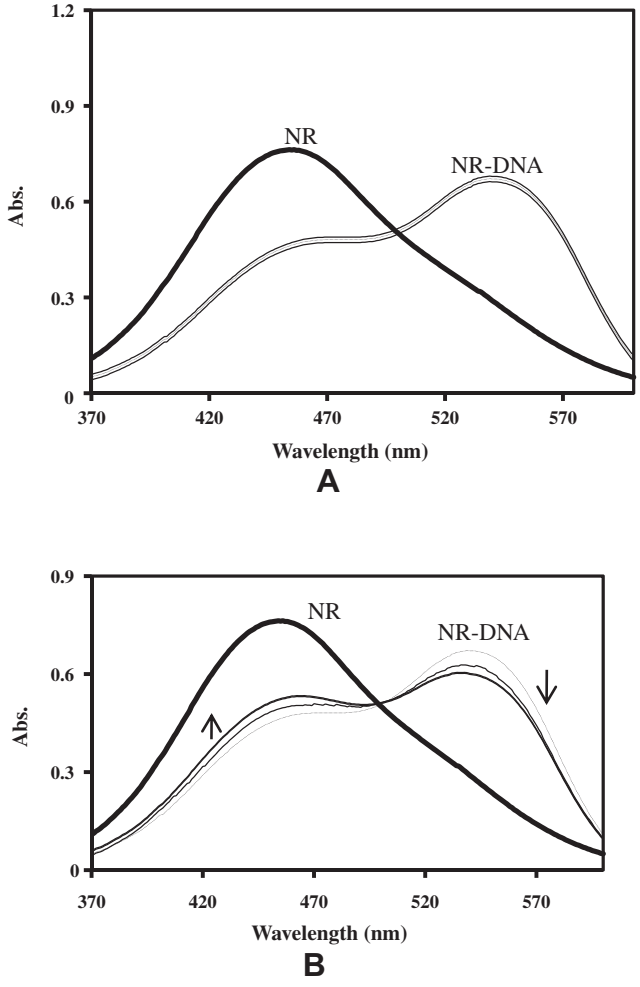

Fig. 3. Effect of Cu complexes [Cu(4,7-dmp)(phen-dione)Cl]Cl (A) and Cu(2,9-dmp)(phen-dione)Cl]Cl (B) on absorption spectra of NR-DNA: [DNA] = 5×10^{-5} M, [NR] = 1.00×10^{-5} M, and [Cu complexes] = 0–15×10^{-5} M.

Effect of the ionic strength on the fluorescence properties

DNA is an anionic polyelectrolyte with phosphate groups. Monitoring the spectral change with different ionic strength is an efficient method to distinguish the binding modes between molecules and DNA. NaCl is used to control the ionic strength of the solutions. Due to the competition for phosphate groups, the addition of Na^+ would weaken the electrostatic interaction between molecules and DNA. The effect of NaCl on the fluorescence of DNA–Cu complexes system was studied and the changes of fluorescence inten-

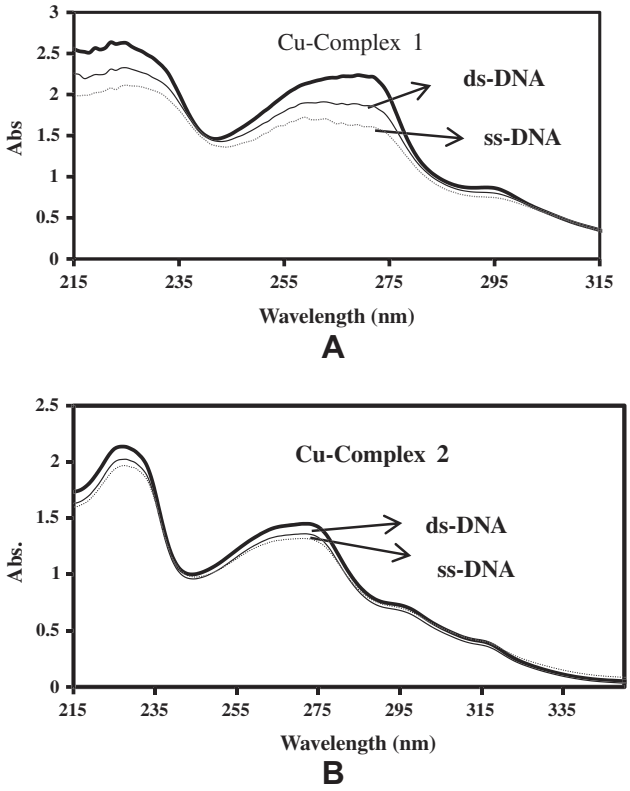

Fig. 4. Absorbance spectra of a [Cu(4,7-dmp)(phen-dione)Cl]Cl (A) and Cu(2,9-dmp) (phen-dione)Cl]Cl (B) after adding native DNA (ds-DNA) and denatured DNA (ss-DNA) directly to the cuvette, (r_i = [DNA]/[Comp]=1.0).

sity were presented in Fig. 5, which indicates no dependence of fluorescence intensity on ionic strength. The result suggested that the binding of Cu complexes to DNA could exclude electrostatic binding mode [21,23].

Voltametric studies

Electrochemical methods provide a useful complement to study metallointercalation and coordination of transitional metal complexes to DNA [47,48]. Fig. 6 shows the cyclic voltammograms of the complexes in the absence and presence of DNA. It can be seen that the cathode and anode peak currents decreased gradually with the addition of DNA. This suggested an interaction between Cu(II) complexes and DNA [49,50]. Decreases in the peak currents are ascribed to the stronger binding between the complexes and DNA [51]. In addition, the peak potentials, E_{pc} and E_{pa}, as well as $E_{1/2}$ had a shift to more positive potentials. The shift of the redox potential of the complexes in the presence of DNA to more positive values indicates a binding interaction between the complex and DNA which makes the complexes readily reducible [51].

To prove the above findings, the competitive study was done with Hochest (HO). The synthetic dye Hochest is known to bind strongly to the minor groove of double stranded DNA [52,53]. HO has a quasi-reversible electrochemical reaction on GCE surface. It shows two oxidation peaks at +0.51 and +0.7 V potentials. Whereas the HO reaction is irreversible and it is adsorbed on the electrode surface, so after each potential operation, the electrode surface must be cleaned. By addition of DNA to HO solution, the intensity of electrochemical peak decreases due to entrance of HO into the DNA groove and consequently accessibility of HO to the electrode surface could be decreased [53]. By addition of two complexes to the HO-DNA solution, an obvious increase in peak intensity for 1 (complete recovery of HO) and a slight increase for 2 were observed. This phenomenon is indicative that, HO has been replaced

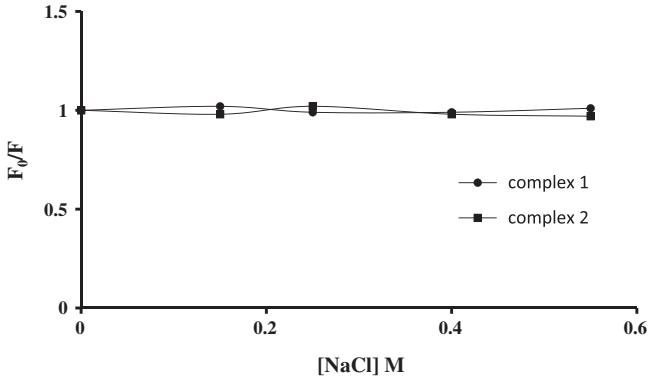

Fig. 5. Effect of NaCl on the fluorescence intensity of [Cu(dmp)(phen-dione)Cl]Cl-DNA ((1) –●– and (2) –■–). [Cu(dmp)(phen-dione)Cl]Cl = 5×10^{-5} M; [DNA] = 2.5×10^{-4} M.

only by complex 1 in groove sites and so HO molecules have been released into the solution (Fig. 7). Thus, it was concluded that complex 1 and HO have similar binding modes.

Interaction of Cu complexes with DNA on CS-CNT modified electrode

Electrochemical characterization of the biosensor

The redox peak currents in the Cyclic voltammograms (CV) of the redox probe 5 mM $[Fe(CN)_6]^{3-/4-}$ at the bare, CS–CNTs, ds/CS-CNT modified GCE (Fig. 8) were evaluated as basic parameters to characterize the electrode surface. Cyclic voltammograms were recorded at a scan rate of 100 mV/s. At the bare GCE, a pair of reversible redox peaks assigned to the electron transfer between

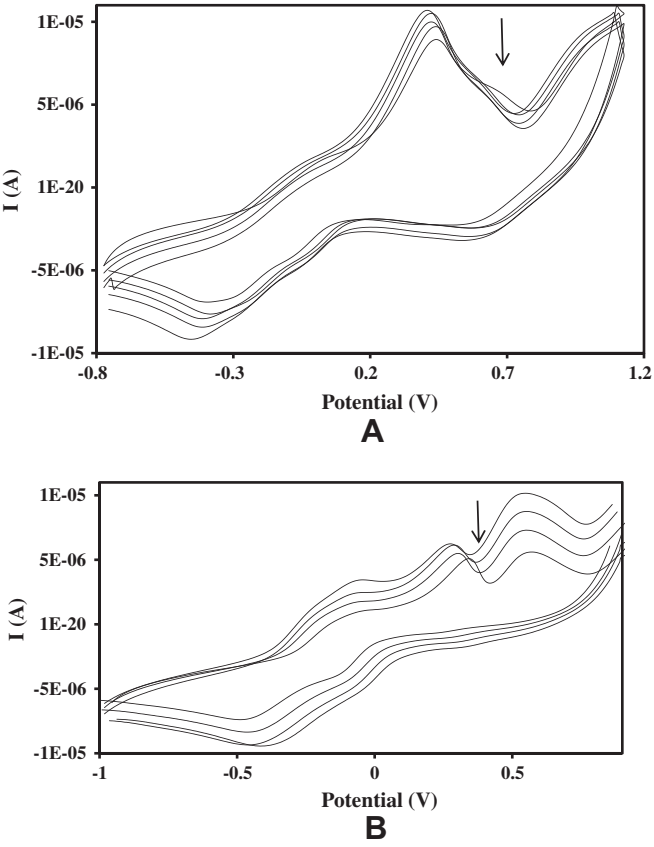

Fig. 6. Cyclic voltammograms for [Cu(4,7-dmp)(phen-dione)Cl]Cl (A) and Cu(2,9-dmp) (phen-dione)Cl]Cl (B) in the presence of DNA at different concentrations. [Cu complexes] = 5×10^{-4} M; [DNA] = ($0.00–5 \times 10^{-5}$).

$Fe(CN)_6^{3-}$ and $Fe(CN)_6^{4-}$ were clearly observed (curve a). When the electrode was modified with the CS–CNTs nanocomposite, the redox peak currents of $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ increased sharply, as expected. Also, the peak to peak separation decreased (curve b), which reveals that the presence of CNTs in the modification layer dramatically promoted the electron transfer of the electroactive molecules at the electrode surface. When DNA was adsorbed on the chitosan–CNT film, the cathodic peak current of the $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ redox probe decreased (curve c) because DNA with a negative charge could retard the redox probes to the electrode surface. Thus, the decrease of the peak current of ferricyanide indicated the formation of the DNA/CS–CNT film at the electrode surface, which is indicative of a lesser access of the redox probe to the modified electrode surface [54].

Interaction of Cu complexes with ssDNA and dsDNA confined at CS–CNTs/GCE surface

Fig. 9 shows the CVs of the [Cu(2,9-dmp)(phen-dione)Cl]Cl at the bare-GCE, CS–CNTs/GCE, ssDNA/CS–CNTs/GCE and dsDNA/CS–CNTs/GCE (data was shown for complex 2). It was obvious the redox peaks of both complexes at the CS-CNTs/GC were enhanced sharply with respect to those at the bare electrode due to the electroconductive properties of modifiers. Following the same trend, these peaks at dsDNA/CS–CNTs/GCE were obviously enhanced with respect to those at the CS–CNTs/GCE, suggesting that the copper complex has been concentrated by dsDNA at the electrode surface. According to the literature [55], this phenomenon could be explained by the good electron transmission ability of dsDNA. As a control, the electrochemical behavior of the mentioned complexes at the ssDNA/CS–CNTs/GCE was also explored. The result shows that, in contrast to the dsDNA/CS–CNTs/GCE, the electrochemical response of the copper complexes at the ssDNA/CS–CNTs/GCE is seriously weaker. These findings might be caused by the less conductive ssDNA film on the electrode surface. In summary, the significant differences in the electrochemical signals obtained at the ssDNA- and dsDNA-modified electrodes suggested that these copper complexes could be electroactive probes to differentiate between ssDNA and dsDNA in biosensing analysis [56].

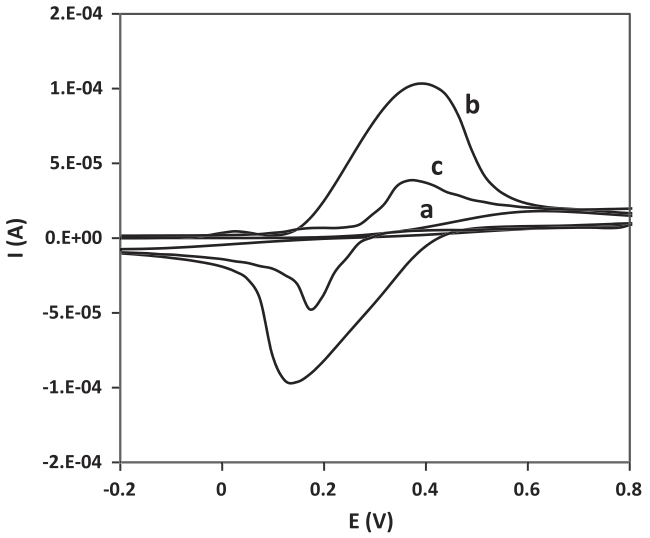

Fig. 8. CVs of 5.0 mM$Fe(CN)_6^{4-/3-}$ in 0.02 M Tris-HCl buffer obtained at the bare GCE (a), CS–CNTs/GCE (b), and dsDNA/CS–CNTs/GCE (c). Scan rate: 100 mV s^{-1}.

Effect on leukemia cells: cell viability, cell proliferation

K562 cells were exposed to increasing doses of complex 1 and 2 (0.00, 1.28, 3.20, 8.00 and 20.00, 50.00 μM) and resulting viability and proliferation measured by the TB assay at 24 and 48 h. As shown in Fig. 10A, the cytotoxicity effect of Cu(II) complexes sharply increases in a dose- and time-dependent manner. IC_{50} values for each complex species are reported in Table 2. Cell proliferation is also compromised in the presence of both species of Cu(II)-DNA complexes (Fig. 10B). In cell culture studies, low IC_{50} values indicate the considerable cytotoxic effect exerted by these compounds. Also, No significant difference in antiproliferative activity was observed between the two complexes. It was concluded cytotoxicity effect of complex 1 is more than that of 2, which was predictable through instrumental methods.

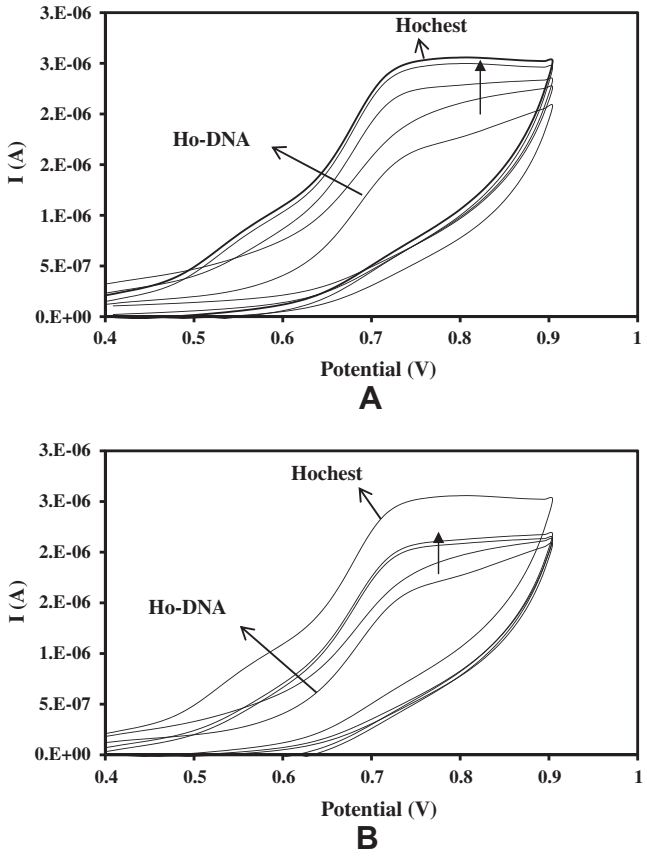

Fig. 7. Effect of Cu complexes [Cu(4,7-dmp)(phen-dione)Cl]Cl (A) and Cu(2,9-dmp)(phen-dione)Cl]Cl (B) on HO-DNA CV curve: [DNA] = 5×10^{-5} M, [HO] = 1.00×10^{-5} M, and [Cu complexes] = 0–15×10^{-5} M.

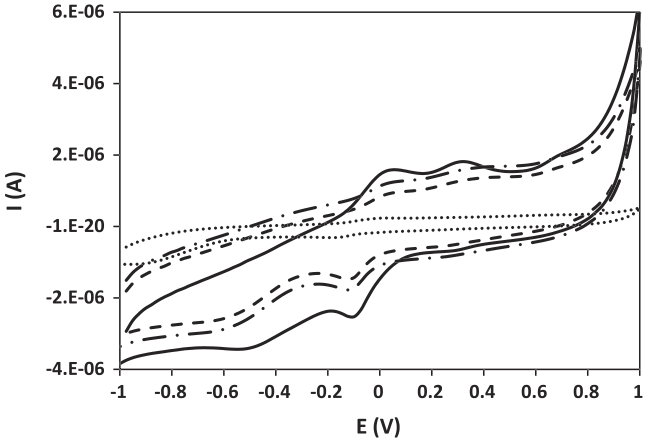

Fig. 9. CVs of 20 μM [Cu(2,9-dmp)(phen-dion)Cl]Cl at the bare GC(. . .), CS–CNTs/GCE (——), dsDNA/CS–CNTs/GCE (–) and ssDNA/CS–CNTs/GCE(– –) in 0.02 M, pH 7.1 Tris-HCl. Scan rate: 100 mV s^{-1}.

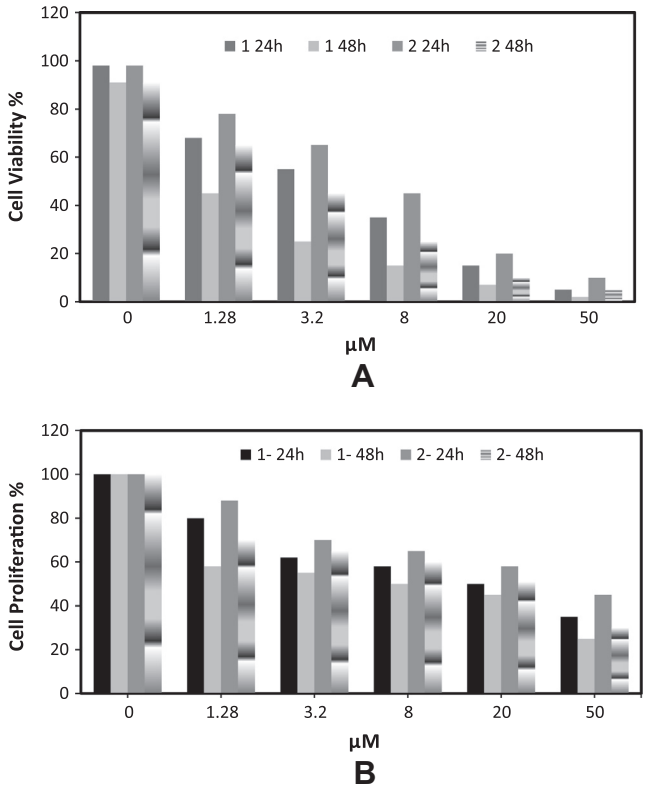

Fig. 10. Plot of cell viability% (A) and proliferation (B) versus increase of Cu complexes concentration (0.00, 1.28, 3.20, 8.00, 20.00 and 50.00 μM) displayed after 24 and 48 h.

Table 2
Cytotoxic activity of the two Cu complexes [Cu(4,7-dmp)(Phen-Dione)Cl]Cl and [Cu(2,9-dmp)(Phen-Dione)Cl]Cl was expressed as IC50 (μM) after 24 and 48 h of incubation with K562 cell line.

Compound	24 h	48 h
[Cu(4,7-dmp)(phen-dione)Cl]Cl	4.2	1.1
[Cu(2,9-dmp)(phen-dione)Cl]Cl	6.5	2.5

Conclusions

In conclusion, the DNA binding behavior of [Cu(4,7-dmp)(phen-dione)Cl]Cl and [Cu(2,9-dmp)(phen-dione)Cl]Cl was studied with a series of techniques including UV–Vis absorption spectroscopy and cyclic voltametry in solution and at the surface of modified electrode. From UV–Vis spectral titration, it is observed that the binding mode between the mononuclear complexes and CT-DNA might be partially intercalative and groove binding, which was confirmed by ionic strength effect investigation and competition studies with HO and NR as groove binder and intercalative probes, respectively. Furthermore, difference in DNA interaction of these complexes can be explained by position of methyl groups on phen ligand. It can be added, the shape and size length and depth of the diimine ligands, as modified by the methyl substituents, determine the mode of bindings. Also we fabricated a DNA biosensor using modification with chitosan and carbon nanotube as a support for DNA immobilization and increase in electron transfer. We studied interaction between these complexes with immobilized DNA, which was concluded that despite of their non-intercalative nature, they could be good candidates for electroactive hybridization probes. Finally, it should be added the considerable cytotoxicity of both complexes may be due to their effective interactions with DNA.

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