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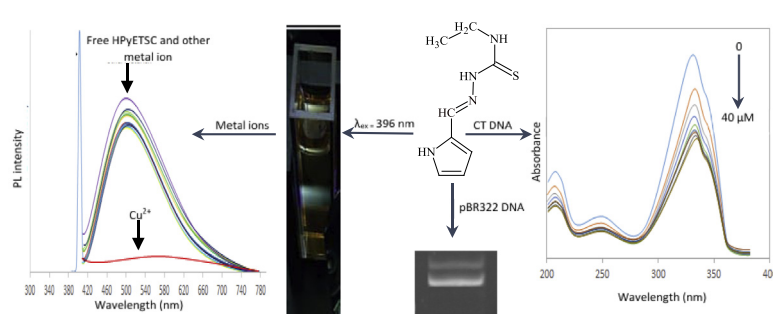
A new thio-Schiff base fluorophore with copper ion sensing, DNA binding and nuclease activity

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HIGHLIGHTS

- A white light-emitting Schiff base.
- An excellent fluorescence probe for Cu²⁺ detection.
- Binds DNA electrostatically.
- Mimics nuclease behavior.

GRAPHICAL ABSTRACT



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ABSTRACT

Copper ion recognition and DNA interaction of a newly synthesized fluorescent Schiff base (HPyETSC) were investigated using UV–vis and fluorescent spectroscopy. Examination using these two techniques revealed that the detection of copper by HPyETSC is highly sensitive and selective, with a detection limit of 0.39 μM and the mode of interaction between HPyETSC and DNA is electrostatic, with a binding constant of $8.97 \times 10^4 \text{ M}^{-1}$. Furthermore, gel electrophoresis studies showed that HPyETSC exhibited nuclease activity through oxidative pathway.

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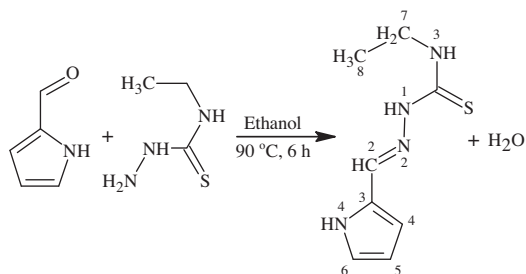
Introduction

Developing molecular receptors for heavy metal ions is a priority of many analytical scientists [1–4]. Fluorescent signaling receptors draw much attention, because fluorescence-based assays are relatively more convenient and cost-effective than conventional

techniques; such as atomic absorption spectroscopy (AAS), gas chromatography (GC), high performance liquid chromatography (HPLC) and capillary electrophoresis (CE) [5–7]. Fluorescent molecules based on rhodamine, fluorescein, anthraquinone, coumarin, pyrene, azobenzene and 1, 8-naphthalimide are efficient fluorescent probes for metal ions [8,9]; however due to their high cost and complex preparation, the search for possible alternatives is actively underway. Schiff bases qualify as potential sensors for metal ions on the basis of their established chelating nature [10–12]. Upon binding to a particular metal ion, a fluorescent

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Scheme 1. Synthesis of HPyETSC.

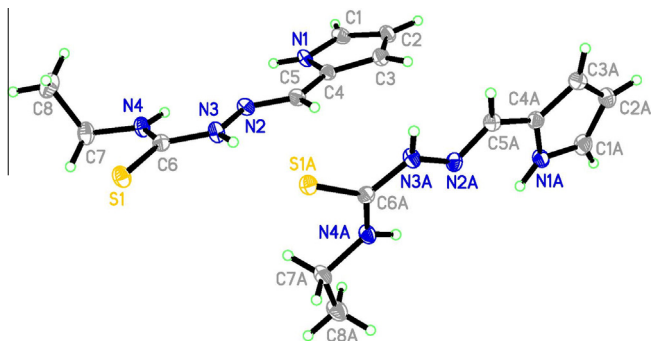


Fig. 1. ORTEP plot of HPyETSC with 50% of thermal ellipsoids.

Schiff base will respond either by stopping or noticeably reducing the intensity of emission. There are sufficient reports in the literature to support the claim that Schiff bases are efficient in detecting metal ions [13–16]. Due to the combination of analytical and biological properties [17–19], Schiff bases have a broad scope of potential applications. However, despite being a prominent class of molecules in the field of bioorganic chemistry, studies concerning DNA interaction of Schiff base are still rare. DNA binding studies are of vital importance in the process of designing chemotherapeutic agents because information from these studies is required to improve aspects such as toxicity, efficacy and target specificity of a drug [20–22]. Hence in this paper, we report the copper ion recognition and DNA binding properties of a new fluorescent thio-Schiff base (HPyETSC) studied by UV–vis spectroscopy

and fluorescent spectroscopy. The superiority of the work lies in the simplicity of the approach and the potency of the results.

Experimental

Materials

The materials were purchased from following sources: 4-ethyl-3-thiosemicarbazide, pyrrole-2-carboxaldehyde, dimethyl sulfoxide- d_6 (DMSO- d_6), tris(hydroxymethyl)aminomethane (Tris), calf thymus DNA (CT-DNA) and ethidium bromide (EB) from Sigma–Aldrich (Malaysia); supercoiled pBR322 DNA from Fermentas Fisher Scientific (Malaysia); agarose from Vivantis (Malaysia); sodium azide (NaN_3), sodium chloride (NaCl), potassium iodide (KI) from Bendosen Laboratory Chemicals (Malaysia); metal salts from R&M Chemicals (Malaysia); ethanol, hydrochloric acid (HCl) and dimethylformamide (DMF) from Friendemann Schmidt-Thermo-line (Malaysia). All supplies were analytical grade and used without further purification. For spectrophotometric experiments, methanolic HPyETSC and aqueous metal salts were used. All the experiments involving DNA were carried out in Tris–HCl buffer (5 mM Tris–HCl, 50 mM NaCl, pH 7.2) containing 5% DMF. Concentration and purity of CT-DNA were assessed according to literature methods [23,24]. The ultrapure water with 18.2 $\text{M}\Omega$ cm specific resistance was produced by a Cascada LS Ultrapure water system (Pall Corp., USA).

Instruments

Elemental analysis was carried out on a Perkin Elmer CHNS/O 2400 Series II CHN Analyzer (PerkinElmer, Malaysia). Infrared spectrum was recorded on a Thermo Scientific Nicolet iS10 spectrophotometer (Thermo Scientific, Malaysia). ^1H NMR spectra were acquired on a Bruker Avance III-400 MHz spectrometer (Bruker (M) Sdn. Bhd., Malaysia). UV–vis absorption spectra were obtained on a Shimadzu UV-3101PC spectrophotometer (Shimadzu (M) Sdn. Bhd., Malaysia). Fluorescence spectra were measured on a Horiba FluoroMax-4 spectrophotometer (ALV Technologies Sdn. Bhd., Malaysia). Mass spectrum was obtained using an Agilent Technologies 6530 Accurate-Mass Q-TQF LC/MS instrument (Agilent Technologies (M) Sdn. Bhd., Malaysia). Gel imaging was performed with a Syngene gel documentation system (V-BioScience Sdn. Bhd., Malaysia).

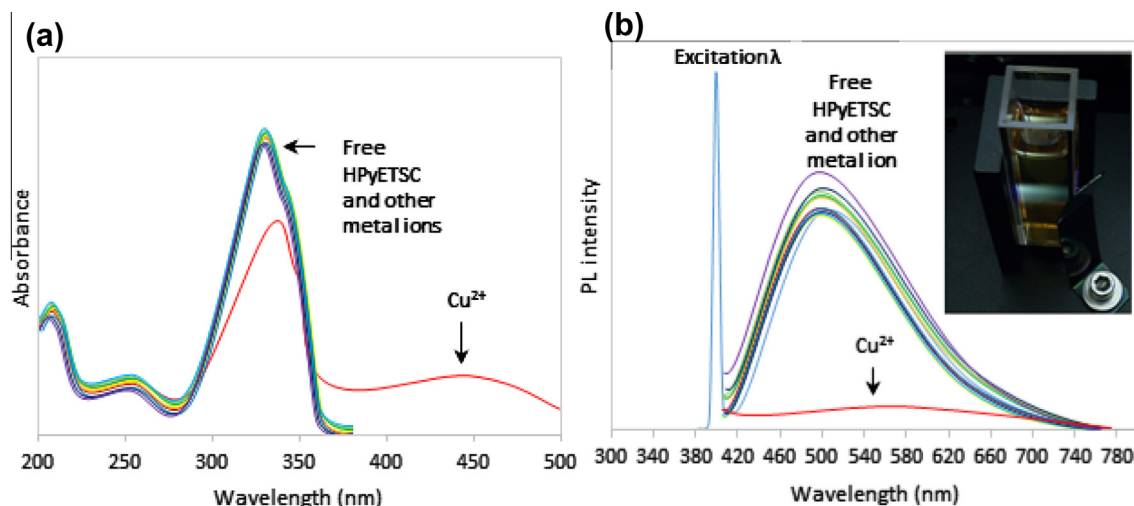


Fig. 2. (a) UV–vis absorption changes of HPyETSC (10 μM) upon addition of 10 equiv. of each metal ions. (b) Emission changes of HPyETSC (5 μM) upon addition of 10 equiv. of each metal ions. Inset: the photograph of HPyETSC solution upon excitation at 396 nm.

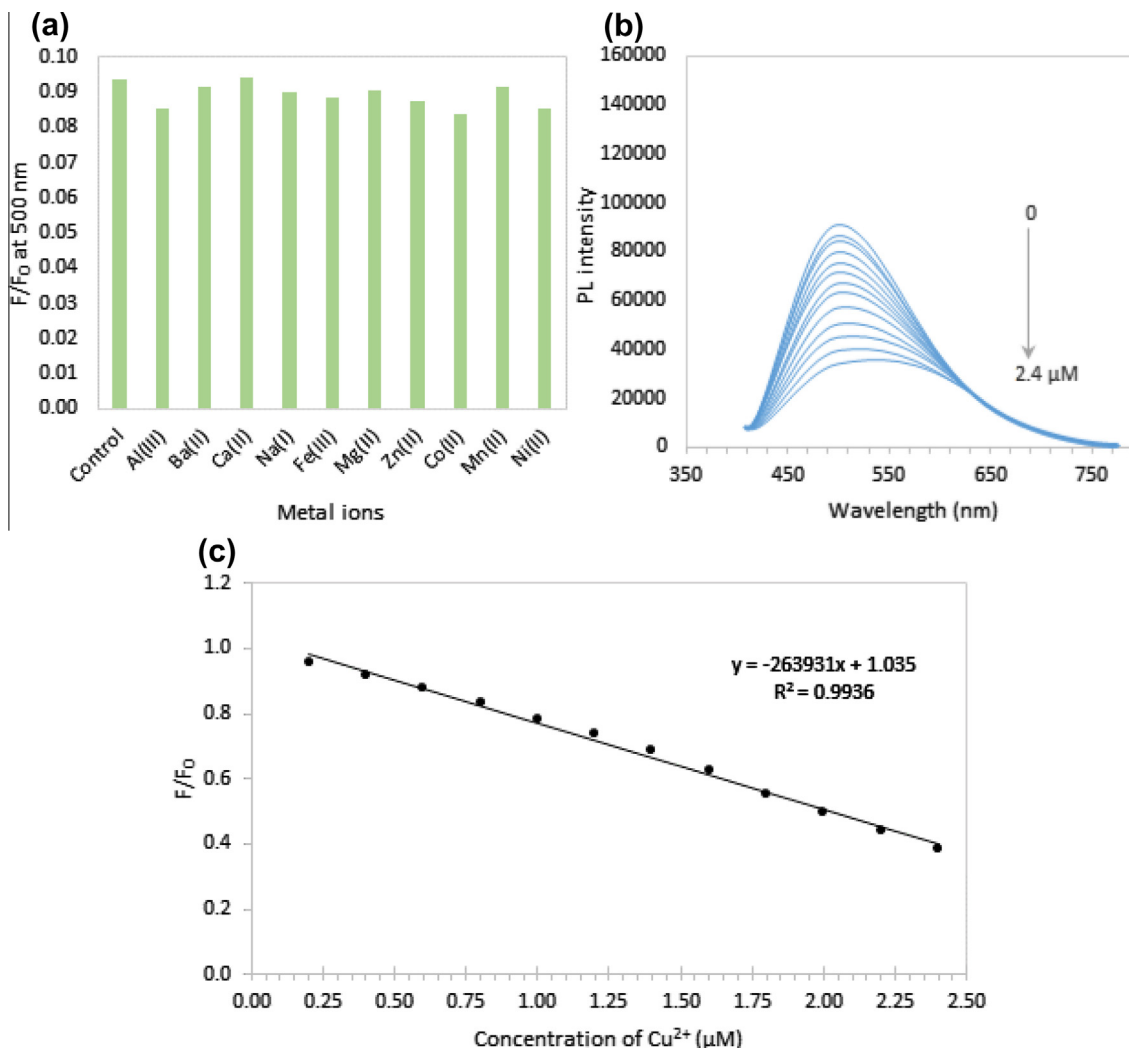


Fig. 3. (a) Relative fluorescence of HPyETSC (5 μ M, emission intensity at 500 nm) with 50 equiv. of Cu^{2+} and 50 equiv. of the metal ion stated. (b) Fluorescence spectra of HPyETSC (5 μ M) in the presence of different concentration of Cu^{2+} (0–2.5 μ M). (c) The linear relationship between fluorescence quenching efficiency and Cu^{2+} concentration from 0.20 to 2.40 μ M. F_0 and F are the emission intensities at 500 nm of HPyETSC in the absence and presence of Cu^{2+} respectively.

Synthesis

A 20 ml ethanolic solution of pyrrole-2-carboxaldehyde (0.095 g; 1 mmol) was mixed with a 20 ml ethanolic solution of 4-ethyl-3-thiosemicarbazide (0.119 g; 1 mmol) and the resulting mixture was heated at 90 °C under reflux for 6 h. The precipitate formed was filtered out, washed with cold ethanol and recrystallized with hot ethanol to yield HPyETSC.

Yield: 67%, dark brown crystal, Anal. Calc. for $[\text{C}_8\text{H}_{12}\text{N}_4\text{S}]$: C, 48.96; H, 6.16; N, 28.55%. Found: C, 48.89; H, 6.12; N, 28.64%; IR (KBr, v/cm^{-1}): 1618 $\text{v}(\text{C}=\text{N})$, 1085 $\text{v}(\text{C}=\text{S})$ (thiocarbonyl), 1493 $\text{v}(\text{C}=\text{C})$ (aromatic), 3270 $\text{v}(\text{NH})$. ^1H NMR (400 MHz, $\text{DMSO}-d_6$, δ/ppm , numbering as in Scheme 1): 11.35 (1H, s, N4H), 11.25 (1H, s, N1H) 8.41 (1H, t, N3H), 7.85 (1H, s, $\text{C}2\text{H}=\text{N}$), 7.00 (1H, d, C6H), 6.24 (1H, d, C4H), 6.12 (1H, dd, C5H), 3.59 (2H, q, C7H), 1.17 (3H, t, C8H). MS(ESI): $[\text{M}+\text{H}]^+$ calcd 197.28; found 197.02.

Crystallographic data for HPyETSC (CCDC No.: 1019355)

Crystal size: $0.15 \times 0.2 \times 0.3 \text{ mm}^3$, $M = 196.28$, Monoclinic, space group: P 21/n, $a = 10.8050(6) \text{ \AA}$, $b = 16.6463(7) \text{ \AA}$, $c = 11.0303(6) \text{ \AA}$, $\alpha = \gamma = 90$, $\beta = 93.011(5)$, $V = 1981.21(18) \text{ \AA}^3$, $T = 100(1) \text{ K}$, $d_{\text{calcd}} = 1.316 \text{ g}/\text{cm}^3$, $F(000) = 832$, 11,573 reflections

measured, 4547 unique ($R_{\text{int}} = 0.034$) 3725 with $I > 2\sigma(I)$. The final $R_1 = 0.0397$, 0.0526 (all data), $\omega R_2 = 0.0919$, 0.1013 (all data) (see Fig. 1).

Results and discussion

General aspects

The synthesis of HPyETSC involves a condensation reaction at 90 °C for 6 h between pyrrole-2-carboxaldehyde (1 mmol) and 4-ethyl-3-thiosemicarbazide (1 mmol) in 40 ml of ethanol (Scheme 1). The thio-Schiff base was characterized by FTIR, ^1H NMR and ESI-MS spectroscopic methods and the structure was confirmed by single crystal X-ray diffraction. The solubility of HPyETSC in water is moderate but it dissolves readily in polar solvents. Both the crystals and solution of HPyETSC are stable under normal laboratory conditions.

Copper ion sensing studies

The recognition profiles of HPyETSC were investigated by UV-vis and fluorescence spectroscopy. In the UV-vis spectrum of HPyETSC (Fig. 2a), three absorption bands were observed at 330,

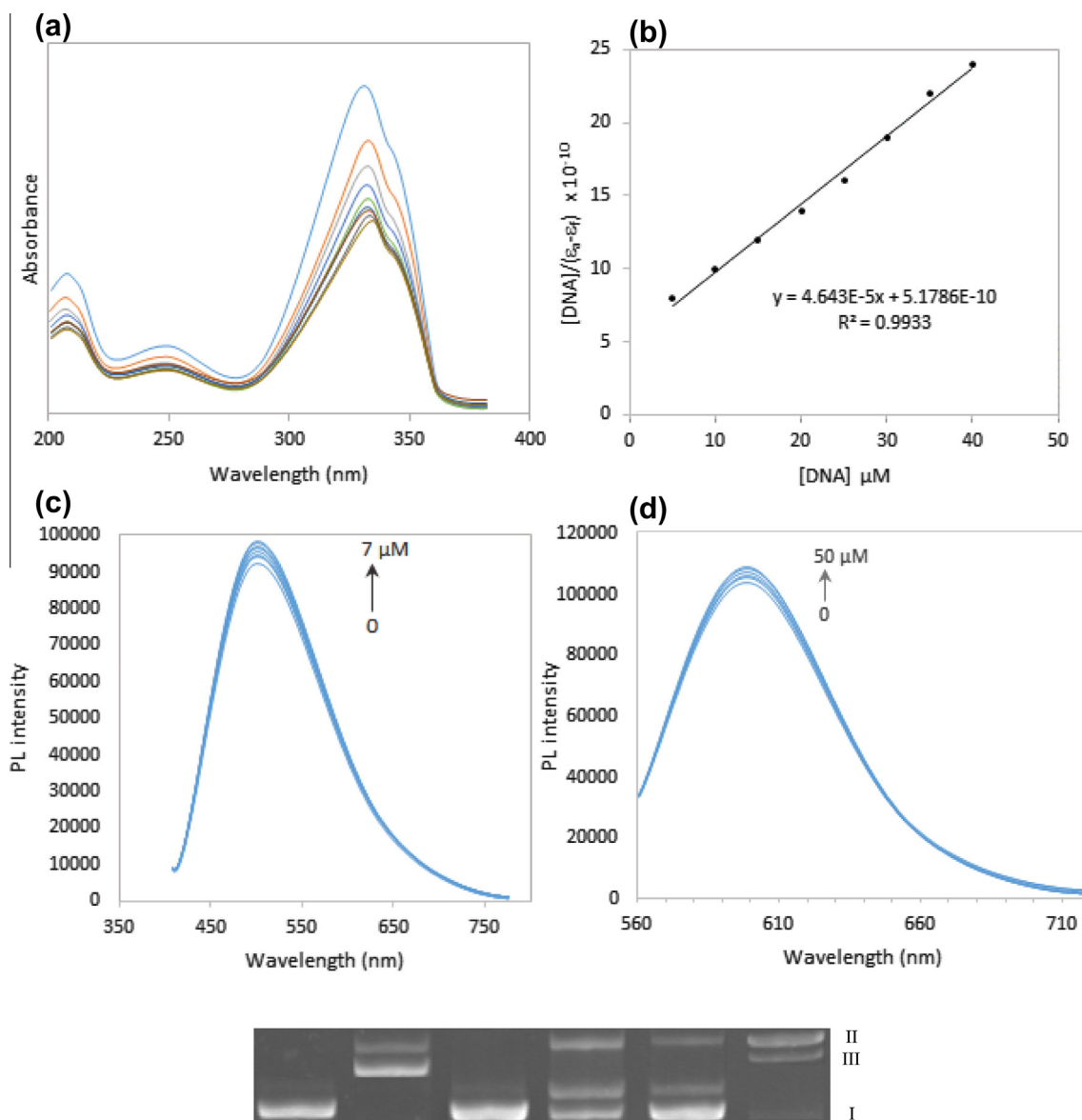


Fig. 4. (a) Absorption spectra of the HPyETSC in the absence and presence of increasing amounts of CT-DNA. [HPyETSC] = 50 μM . (b) Linear plot for the calculation of the intrinsic DNA binding constant (K_b). (c) The emission spectra of HPyETSC (5 μM) with increasing concentrations (0–7 μM) of DNA. (d) The emission spectra of the DNA–EB system in the absence and presence of increasing amounts of HPyETSC; [DNA] = 26 μM , [Complex] = 0–50 μM , [EB] = 20 μM . (e) Cleavage profile of pBR322 supercoiled DNA (0.5 $\mu\text{g}/\mu\text{l}$) by HPyETSC (1.4 mM). All reaction mixtures contained hydrogen peroxide except lane 1 and lane 3. Lane 1, DNA control; lane 2, DNA + HPyETSC; lane 3, DNA + HPyETSC (anaerobic condition); lane 4, DNA + HPyETSC + DMSO (5 mM); lane 5, DNA + HPyETSC + NaN_3 (5 mM); lane 6, DNA + HPyETSC + KI (5 mM).

255 and 208 nm assignable to $n \rightarrow \pi^*$ transition of thione moiety, $\pi \rightarrow \pi^*$ pyrrole ring transition and $n-\sigma^*$ transition of azomethine nitrogen [25], respectively. These bands remained almost unchanged upon the addition of various metal ions (Al^{3+} , Ba^{2+} , Ca^{2+} , Na^+ , Fe^{3+} , Mg^{2+} , Zn^{2+} , Co^{2+} , Mn^{2+} and Ni^{2+}) except for Cu^{2+} which resulted in intensity decay and red shift of the band at 330 nm as well as formation of a new band at 445 nm. The hypochromism and bathochromism observed at 330 nm is highly indicative of the formation of a charge transfer complex between Cu^{2+} and HPyETSC. This discriminatory detection is primarily due to the high affinity of Schiff bases towards the Cu^{2+} ion [26].

As shown in Fig. 2b, HPyETSC exhibited a broad emission band, covering the entire visible region, upon excitation at 396 nm. White light emission from HPyETSC is shown in the inset of Fig. 2b. Among the various metal ions surveyed, only Cu^{2+} was able to quench the strong emission of HPyETSC. This further proves the sensitivity of HPyETSC towards copper ions over other metal ions.

We note that the absorption band centered at 445 nm overlaps with the excitation and emission wavelengths of HPyETSC. Thus, the fluorescent quenching by Cu^{2+} is based on inner filter mechanism [27], i.e. the Cu^{2+} –HPyETSC complex formed absorbs the excitation and emission light of HPyETSC.

Interference of cations was studied by treating HPyETSC with Cu^{2+} in the presence of other metal ions. Usually, detection of copper is damped due to interference of other paramagnetic ions (mainly from Fe^{3+} and Co^{2+}) [27]. From the bar diagram (Fig. 3a), it can be clearly seen that the response of HPyETSC for Cu^{2+} was unaffected in the presence of competing metal ions (including the aforementioned paramagnetic ions). In order to confirm the fluorescence recognition phenomenon, the emission of HPyETSC (5 μM) was monitored upon titration with different amounts of Cu^{2+} . As shown in Fig. 3b, the emission intensity decreased gradually with increasing concentration of Cu^{2+} , revealing that HPyETSC is sensitive to Cu^{2+} concentration. Fig. 3c shows calibration plot

wherein linearity is observed between fluorescence quenching efficiency and Cu^{2+} concentration in the range of 0.20–2.40 μM . The detection limit of 0.39 μM was calculated according to the IUPAC 3σ criterion [28], which compares favorably with recently reported Cu^{2+} detection data [29–31]. Being highly selective and sensitive, HPyETSC could function as a fluorescent sensor for Cu^{2+} .

DNA interaction studies

Absorption and emission titration are routinely conducted experiments to characterize DNA interaction of molecules. The 50 μM absorption spectra of HPyETSC in the absence and presence of increasing amount of CT-DNA are shown in Fig. 4a. The increase in DNA concentration caused hypochromism and a red shift of 4 nm of the band at 330 nm. Bands at 208 and 255 nm, however, showed hypochromism with negligible changes in the wavelength. In order to demonstrate quantitatively the DNA interaction, the intrinsic binding constant (K_b) was determined from the following equation [32],

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

where [DNA] is the concentration of DNA in base pairs, the apparent absorption coefficient ε_a , ε_f and ε_b corresponds to $A_{\text{obs}}/[\text{HPyETSC}]$, the extinction coefficient of free HPyETSC and the extinction coefficient of HPyETSC when fully bound to DNA, respectively. From the plot of $\text{DNA}/(\varepsilon_a - \varepsilon_f)$ versus [DNA] (Fig. 4b), K_b was calculated by the ratio of slope to intercept. The intrinsic binding constant (K_b) is a commonly used scale to evaluate the DNA binding strength. The intrinsic binding constant (K_b) for HPyETSC is $8.97 \times 10^4 \text{ M}^{-1}$, a value comparable to various DNA binding and bio-relevant metal complexes [33–35].

In the fluorescence spectra, the emission intensity increased without any shift in wavelength upon addition of increasing amount of DNA solution (Fig. 4c). The enhanced emission intensity observed for HPyETSC hinted electrostatic binding mode of HPyETSC–DNA. In order to confirm the binding mode, EB displacement experiment was carried out. Ethidium bromide is a classical intercalator with poor emission intensity. It displays a dramatic enhancement of its emission intensity when intercalated into DNA. Reports in the literature have shown that the emission could be quenched by the addition of molecules that displaces EB from DNA ($K_{\text{EB}} = 1.0 \times 10^7 \text{ M}^{-1}$) [36,37]. As shown in Fig. 4d, the emission intensities at 600 nm of the DNA–EB complex displayed an increasing trend with increasing concentration of the HPyETSC. This observation confirms that displacement of EB and intercalation by HPyETSC do not occur and therefore it is evident that HPyETSC binds to DNA via surface/electrostatic interaction.

Next, the cleavage of supercoiled pBR322 DNA by HPyETSC was studied using agarose gel electrophoresis. As illustrated in Fig. 4e (lane 2), HPyETSC complexes displayed efficient cleavage activity, where the supercoiled DNA (form I) was cleaved to the nicked circular form (NC form; form II) and linear circular form (LC, Form III) over a reaction period of 5 h. Subsequently, the DNA cleavage experiment was carried out under an argon blanket. Under anaerobic conditions, nuclease activity of HPyETSC was totally retarded, implying that the DNA cleavage is only feasible through oxidative mechanism [38,39]. To gain information about the reactive species involved in the oxidative DNA cleavage reaction, we investigated the DNA cleavage in the presence of hydroxyl radical scavenger (DMSO), singlet oxygen quencher (NaN_3), and hydrogen peroxide scavenger (KI). It was found that DMSO (lane 4) and NaN_3 (lane 5) significantly reduce the nuclease activity of HPyETSC, indicating the participation of hydroxyl radicals and singlet oxygen in the cleavage process [40,41].

Conclusion

In summary, we synthesized a pyrrole-based heterocyclic thiosemicarbazone, of analytical and biological relevance, with various merits such as: (a) can be prepared easily at low cost (b) stable and polar solvent friendly (c) exhibits bright white light fluorescence (d) displays sensitive and selective copper ion detection, and (e) binds to DNA avidly and promotes DNA cleavage. Certainly, with these features, this molecule would be worthy of being investigated in more detail as a new candidate for multifunctional molecular materials.

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