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Novel anthracene and pyridine comprising schiff base probe for selective “OFF-ON” fluorescent determination of Cu\textsuperscript{2+} ions towards live cell application

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Abstract

Novel anthracene and pyridine comprising schiff base derivative 2-(2-(anthracen-9-ylmethylen)hydrazinyl)pyridine (AP) has been synthesized via one-pot reaction and its fluorescent “OFF-ON” detection of Cu²⁺ ions, via PET based mechanism was reported firstly. The 1:1 stoichiometry of AP--Cu²⁺ sensor complex was calculated from job’s plot based on PL titrations and supported by ESI (+Ve) mass analysis. In addition, the binding sites of sensor conjugate AP--Cu²⁺ was buttressed by the ¹H NMR titration. The detection limit (LOD) and association constant (Kₐ) of AP--Cu²⁺ complex was scrutinized by standard deviation and linear fittings. Furthermore, quantum yield (Φᵣ), SEM analysis, pH effect, FTIR interpretation and density functional theory (DFT) studies were investigated for the AP--Cu²⁺ sensing conjugate. More decisively, confocal fluorescence microscopy imaging from Raw264.7 cells indicated that AP could be used as an effective fluorescent probe for the analysis of Cu²⁺ ions in living cells.

KEYWORDS: Anthracene; Pyridine; Schiff base; Cu²⁺ sensors; Live cell imaging.
Introduction

Owing to the biological and environmental significance of metal ions, the chemosensors development for their detection is become a concept of interest.\textsuperscript{1-3} Among the transition metal ions, copper is the third most abundant element after iron and zinc. Divalent copper ions are more attractive, because of their crucial role in the activation of dioxygen in living organisms.\textsuperscript{4,5} On the other hand, at higher concentration levels, it will damage biomolecules leading to oxidative stress and neurodegenerative disorders.\textsuperscript{6,7} With this concern, the World Health Organization (WHO) reported that the population mean consumption of copper should not exceed 10–12 mg/day for adults.\textsuperscript{8} Similarly, copper deficiency may also lead to some serious diseases, such as Alzheimer’s, anemia, amyotrophic lateral sclerosis and prion diseases.\textsuperscript{9,10}

Considering the importance of Cu\textsuperscript{2+} ions, several methods such as inductively coupled plasma-atomic emission spectrometry, plasma-mass spectroscopy, inductively coupled atomic absorption/emission spectroscopy, and voltammetry have been reported for its determination.\textsuperscript{11-13} However, they are not suitable for on-line monitoring of Cu\textsuperscript{2+} ions due to their low selectivity, needing expensive instruments and delayed responses. In contrast, fluorescent turn-on Cu\textsuperscript{2+} detection has become more practical and favorable method in medicinal biology, and environmental chemistry.\textsuperscript{14-16}

By means of internal charge transfer (ICT), photoinduced electron transfer (PET), excimer/exciplex formation, chelation-enhanced fluorescence (CHEF) and more recently fluorescence resonance energy transfer (FRET) mechanisms, many copper selective turn-on fluorescent sensors have already been reported.\textsuperscript{17-19} However, due to its simplicity and applications in many opto-electronic and biological systems, PET mechanism is highly
appreciated.\textsuperscript{20-22} Further, PET based anthracene containing “turn-on” probes are found to be very exciting because of their faster response \textit{via} excimer/exciplex formation.\textsuperscript{23-25} Conversely, some of those probes also have synthetic difficulties,\textsuperscript{26-28} hence the development of “turn-on” probes with lesser synthetic complications for specific analyte detection is highly anticipated. By this concern, a few anthracene based schiff base derivatives have been developed with selective sensor properties.\textsuperscript{29-31} Additionally, anthracene based supramolecular dye molecules can be constructed from their sensor complexes, as reported earlier.\textsuperscript{32-34} Therefore, we aimed to develop such an anthracene containing schiff base derivative \textit{via} one pot reaction for Cu\textsuperscript{2+} ions detection.

Herein, we have successfully developed an anthracene containing fluorescent turn-on sensor probe AP with demonstrated specific selectivity to Cu\textsuperscript{2+} ions \textit{via} UV-Vis/PL spectral investigations. Further, to explore the optical properties and PET mechanism for the recognition of Cu\textsuperscript{2+} ions, B3LYP/LANL2DZ density functional calculations were adopted to investigate the electronic excitation and molecular orbitals of the probe.

**Experimental studies**

**Materials and methods**

All anhydrous reactions were carried out by standard procedures under nitrogen atmosphere to prevent the interference from ambient moisture. Solvents were dried by distillation over appropriate drying agents and reactions were monitored by thin layer chromatography (TLC). \textsuperscript{1}H and \textsuperscript{13}C NMR were analyzed on a 300 MHz Bruker spectrometer. The chemical shifts (\(\delta\)) are reported in ppm and coupling constants (\(J\)) in Hz and relative to TMS (0.00) for \textsuperscript{1}H and \textsuperscript{13}C NMR, (s, d, t, q, m, and dd means single,
double, ternary, quadruple, multiple, and doublet of doublet, respectively), and d$_6$-DMSO ($^1$H and $^{13}$C NMR at 2.49 and 39.51 ppm, respectively) were used as references. Mass spectrum (ESI) was obtained from the respective mass spectrometer. Absorption and fluorescence spectra were measured on HITACHI, U-3310 Spectrophotometer and HITACHI F-7000 Fluorescence Spectrophotometer, respectively. Identification and purity of the compound AP was characterized by NMR ($^1$H & $^{13}$C) and ESI-Mass. Buffers with various pH values were freshly prepared as per the literature. SEM studies were done by JEOL-JSM-6700. Fourier transform Infrared spectroscopy (FTIR) were analysed by Perkin Elmer - 100 FT-IR SPECTRUM ONE spectrometer. The powder XRD data of AP and AP---Cu$^{2+}$ were obtained from BRUKER AXS D2 Phaser (a26-x1-A2BOE2B). Fluorescence microscopic images were taken using Multiphoton and Confocal Microscope System, Leica, Germany, TCS-SP5-X AOBS. 

**Sensor titrations**

Sensing probe AP was dissolved in CH$_3$CN and Ba$^{2+}$, Ni$^{2+}$, Fe$^{3+}$, Cr$^{3+}$, Cu$^{2+}$, Mg$^{2+}$, Fe$^{2+}$ and Al$^{3+}$ metal ions were dissolved in DI water at $1\times10^{-3}$ M concentration from their respective chloro and perchlorate compounds. Likewise, Ag$^+$, Co$^{2+}$, Zn$^{2+}$, Pb$^{2+}$, Mn$^{2+}$ and Hg$^{2+}$ metal ions were dissolved in DI water at $1\times10^{-3}$ M concentration from their respective acetate salts.

**SEM and FTIR analysis**

For both SEM and FTIR studies, the samples were drop-casted on well cleaned silicon wafers, then annealed at 50$^\circ$C for 15 minutes and continued for the respective analysis. We used clean Silicon wafer as background reference for FTIR measurement.
ESI (+Ve) mass analysis

The AP-Cu$^{2+}$ complex was mixed at 1:1 stoichiometry in CH$_3$CN and stirred at 45°C for 12 hrs, dried in oven at 100 °C for 3 hrs. The dried complex was subjected to ESI (+Ve) mass analysis.

Procedure for the synthesis of AP$^{36}$

To 1 equiv. of anthracene-9-carbaldehyde in 50 ml of methanol, 1 equiv. of 2-hydrazinylpyridine was gradually added with constant stirring under nitrogen and then refluxed for 12 h. The reaction was periodically monitored by TLC, after completion, the reaction mixture was cooled and solvent was evaporated to obtain the crude product, which was recrystallized from ethanol to afford the pure compound AP as a dark yellow solid.

2-(2-(anthracen-9-ylmethylene)hydrazinyl)pyridine (AP): yellow solid; 93% yield; $^1$H NMR (300 MHz, d$_6$-DMSO) δ: 6.79-6.83 (dd; $J = 6.1$ Hz, 1H (-CH-pyridyl)), 7.22 (d; $J = 8.4$ Hz, 1H (-CH-pyridyl)) 7.55-7.72 (m, 5H (Aromatic)), 8.13 – 8.20 (m, 3H (Aromatic)), 8.65 (s, 1H(Aromatic)), 8.72 (d, $J = 8.7$ Hz, 2H (Aromatic)), 9.28 (s, 1H (-C=N-NH)), 11.10 (s, 1H (-CH=N)); $^{13}$C NMR (75 MHz, d$_6$-DMSO) δ: 106.59, 115.67, 125.35, 125.92, 126.67, 127.25, 128.65, 129.42, 129.68, 131.55, 138.21, 138.66, 148.45, 157.50; ESI mass: calculated: m/z = 297.13 (M$^+$, 100%); Found: m/z = 298.13 [(M+1)100%].

Fluorescence imaging and sensing

To determine the Cu$^{2+}$ ions in living cells, Raw264.7 cells were cultured in DMEM medium (Dulbecco’s Modified Eagle’s Medium with high glucose) supplemented with
10% FBS at 37°C under 5% CO₂ environment. The cells were then cultured on glass coverslips and incubated for 24 hours to promote better adherence.

The cells cultured in DMEM were treated with 30 µM of Cu²⁺ dissolved in sterilized PBS (pH 7.4) and incubated for 30 min at 37°C and then the treated cells were washed three times with 2 ml PBS to remove the remaining metal ions. 2 mL of culture media was added to the cell culture and then treated with 20 µM of AP dissolved in DMSO followed by incubation (2 hours at 37°C). Thereafter, The culture medium was removed, and the treated cells were washed with PBS (2 mL) before observation. Fluorescence imaging was determined by a Multiphoton and Confocal Microscope System, Leica, Germany, TCS-SP5-X AOBS. The cells were excited with a white light laser at λₑₓ = 350 nm at 6% output and emission images were collected between 410 and 440 nm (AP—Cu²⁺).

Results and Discussion

Synthesis and solvent selection

As illustrated in Scheme 1, AP was synthesized via one pot anthracene-9-carbaldehyde and 2-hydrazinylpyridine condensation in methanol with 93% yield and characterized with ¹H, ¹³C NMR and Mass analysis[ESI (+Ve)] (Figs. S1-S3, ESI).

\[
\text{CHO} \quad \text{NH}_2 \quad \text{MeOH} \quad \text{Reflux, 12hrs} \quad \text{93%}
\]

\[
\begin{array}{c}
\text{MeOH} \\
\text{Reflux, 12hrs} \\
\text{93%}
\end{array}
\]

\[
\begin{array}{c}
\text{AP}
\end{array}
\]

Scheme 1. Synthesis of AP probe.

In order to choose the appropriate solvent for the sensor titrations, the selectivity test
was performed in many solvents (see Fig. S4a, ESI) and established the better “off-on” selectivity (23 fold) to Cu\(^{2+}\) ions in acetonitrile rather than other solvents. Further, to describe the CH\(_3\)CN:H\(_2\)O ratio for efficient selectivity, we have also conducted PL spectral titrations (not shown) with different ratio (0-90\% with an equal span of 10\%) of H\(_2\)O. In which, the AP probe evidenced the greater “off-on” sensory response to Cu\(^{2+}\) ions only in pure acetonitrile and observed that, the presence of water content will affect the turn-on response rapidly. Hence, we did the UV-Vis/PL titrations of AP (\(\lambda_{\text{abs}}=350\) nm and \(\lambda_{\text{em}}=430\) nm;) in acetonitrile and \(^1\)H NMR titrations in [\(\text{d}_6\)-DMSO] by adding metal ions in pure H\(_2\)O and D\(_2\)O, respectively. Further, 15 fold of PL enhancement was also noticed for AP in DMSO (see Fig. S4a & b, ESI), which allow us to do the cellular imaging studies by dissolving AP in DMSO with 2 hours incubation.

**Fluorescence titrations on metal ions**

Initially, AP probe (20 \(\mu\)M in CH\(_3\)CN) was investigated towards 30 \(\mu\)M (1.5 equiv.) of metal ions (Ba\(^{2+}\), Ni\(^{2+}\), Fe\(^{3+}\), Cr\(^{3+}\), Cu\(^{2+}\), Mg\(^{2+}\), Fe\(^{2+}\), Al\(^{3+}\), Ag\(^{+}\), Co\(^{2+}\), Zn\(^{2+}\), Pb\(^{2+}\), Mn\(^{2+}\), and Hg\(^{2+}\)) in H\(_2\)O. Upon the addition of 1.5 equiv. of metal ions, AP shows better selectivity towards Cu\(^{2+}\) ions, as shown in Fig 1a and 1b. Impressively, the absorption peak of AP---Cu\(^{2+}\) complex was blue shifted and exhibited at 296 nm than that of AP probe obtained at 405 nm. The above 109 nm blue shifted absorption peak of AP---Cu\(^{2+}\) complex was an added advantage for Cu\(^{2+}\) ions detection. Similarly, contrast to other metal ions, the PL spectra of AP---Cu\(^{2+}\) complex was also evidenced the high fluorescence intensity peak at 430 nm. Remarkably, the PL intensity changes [\(I/I_0\)] of AP---Cu\(^{2+}\) was found to be 23 fold. Likewise, the quantum yield of AP probe [\(\Phi_f = 0.01\)] was also increased for AP---Cu\(^{2+}\) complex [\(\Phi_f = 0.23\)]. This indicated that the CHEF of
**AP**--**Cu**$^{2+}$ was found to be enhanced with 23 fold of quantum yield values than that of **AP** probe. The photograph of **AP**--**Cu**$^{2+}$ (visualized under UV- light irradiations at $\lambda_{em} = 365$ nm) strongly verified its sensitivity through intense blue emission, as observed in Fig. 2.

**Fig. 1** (a) UV and (b) PL spectra ($\lambda_{ex} = 350$ nm) of **AP** (20 µM) in CH$_3$CN with various other metal ions (30 µM) in H$_2$O.

**Fig. 2** Photograph of **AP** probe in presence of different metal ions.

**Fluorescence titrations on Cu$^{2+}$ sensor**

By increasing the concentrations of Cu$^{2+}$ (0-30 µM with an equal span of 2 µM in H$_2$O) the sensitivity of **AP** (20 µM in CH$_3$CN) towards Cu$^{2+}$ ions were clearly witnessed as shown in Fig. 3a. The PL spectra of **AP**--**Cu**$^{2+}$ ($\lambda_{em} = 430$ nm) demonstrated the turn-
on responses of AP to Cu\(^{2+}\) ions and the respective fluorescence intensity changes as a function of Cu\(^{2+}\) concentration is exposed in Fig. 3b. Similarly, the UV-Vis titration on

![Graphs showing fluorescence intensity changes and UV titrations](image)

**Fig. 3** (a) PL titrations (\(\lambda_{\text{ex}} = 350\) nm), (b) intensity changes as a function of Cu\(^{2+}\) concentration and (c) UV titrations of AP (20 \(\mu\)M) in CH\(_3\)CN with Cu\(^{2+}\) ions in H\(_2\)O.
Cu$^{2+}$ ions also observed the quenched absorbance at 405 nm along with increased absorbance intensity at 296 nm, as publicized in Fig. 3c. Further, an isosbestic point at 325 nm was evidenced, which supports the possible 1:1 stoichiometry of AP--Cu$^{2+}$ complex. In addition, the selectivity of AP probe was further evaluated by single and dual metal studies as follows. In order to establish the specific selectivity of AP to Cu$^{2+}$, we executed the single and dual metal competitive analysis as noticed in Fig. 4. In single metal system (black bars), except Cu$^{2+}$ ions (30 µM; 1.5 equiv.), all the metal ions (Ba$^{2+}$, Ni$^{2+}$, Fe$^{3+}$, Cr$^{3+}$, Mg$^{2+}$, Fe$^{2+}$, Al$^{3+}$, Ag$^+$, Co$^{2+}$, Zn$^{2+}$, Pb$^{2+}$, Mn$^{2+}$, and Hg$^{2+}$) concentrations were kept as 150 µM (7.5 equiv.) towards AP. However, for dual-metal (red bars) studies, two equal amounts of aqueous solutions of Cu$^{2+}$ and other metal ions (30 µM + 150 µM) were combined. Moreover, 180 µM of Cu$^{2+}$ was also considered for its effect

![Image](https://via.placeholder.com/150)

**Fig. 4** Histograms on single and dual metal studies of AP (20 µM in CH$_3$CN) probe towards Cu$^{2+}$ ions selectivity.
and the obtained results suggested specific selectivity of AP probe towards Cu\textsuperscript{2+} ions as shown in Fig. 4. Similar to the above observed results, the Cu\textsuperscript{2+} selectivity was also been demonstrated by single and dual metal studies in DMSO, THF and Methanol as shown in Figs S4b-c (ESI).

**Stoichiometry and binding sites**\textsuperscript{37-39}

Stoichiometry calculations were carried out through job’s plot as noticed in Fig. 5 to ensure the binding site of AP---Cu\textsuperscript{2+} conjugate. The emission intensity at 430 nm was plotted against molar fraction of AP under a constant total concentration. Maximum emission intensity was notified at 0.5 molar fractions, which confirmed the 1:1 stoichiometry of AP---Cu\textsuperscript{2+} complex. Further, the paramagnetic nature of Cu\textsuperscript{2+} ion also influenced on \textsuperscript{1}H NMR proton signals (not shown) of AP probe that are close to Cu\textsuperscript{2+} binding site. Likewise, the ESI-mass peak at m/z = 361.06 [AP---Cu\textsuperscript{2+} +1] also supported the 1:1 sensor complex as noticed in Fig. 6. More importantly, the high

![Job plot of AP---Cu\textsuperscript{2+} complex passes through X = 0.5. Hence 1:1 stoichiometry of AP---Cu\textsuperscript{2+} sensor complex; X = [Cu\textsuperscript{2+} ] / [Cu\textsuperscript{2+} ] + [AP]; where X = mole fraction, [Cu\textsuperscript{2+} ] and [AP] are concentrations of Cu\textsuperscript{2+} and AP. The total concentration of AP and Cu\textsuperscript{2+} was 50.0 M. The monitored wavelength was 430 nm.](image)

**Fig. 5** Job plot of AP---Cu\textsuperscript{2+} complex passes through X = 0.5. Hence 1:1 stoichiometry of AP---Cu\textsuperscript{2+} sensor complex; X = [Cu\textsuperscript{2+} ] / [Cu\textsuperscript{2+} ] + [AP]; where X = mole fraction, [Cu\textsuperscript{2+} ] and [AP] are concentrations of Cu\textsuperscript{2+} and AP. The total concentration of AP and Cu\textsuperscript{2+} was 50.0 M. The monitored wavelength was 430 nm.
resolution mass data (HRMS) was confirmed 1:1 stoichiometry of \( \text{AP}--\text{Cu}^{2+} \) complex. As shown in Fig. S5 (ESI), the HRMS data was well verified the complex formation via its isotopic pattern as well. Hence, based on job’s plot, \(^1\text{H} \) NMR titrations and mass analysis, we proposed the possible binding of \( \text{AP}--\text{Cu}^{2+} \) complex as shown in Scheme 2.

![Mass spectrum of \( \text{AP}--\text{Cu}^{2+} \) complex.](image)

**Scheme. 2** Illustration of \( \text{Cu}^{2+} \) binding with \( \text{AP} \) probe.
As noticed in Scheme 2, the formation stable five member ring was induced by Cu\(^{2+}\) ions, which also well buttressed by DFT studies next. Further, the above statement was well supported by many reports of two dentate Cu (II) complexes with stabilized five membered ring.\(^{36a,40}\)

Next, the FTIR investigations of AP probe and AP---Cu\(^{2+}\) well confirmed the 1:1 ratio complex formation of AP probe with Cu\(^{2+}\) metal ions as exposed in Fig. S6 (ESI). Upon formation of AP---Cu\(^{2+}\) complex, the \(-\text{NH}\) stretch peak became narrower. Further, the \(-\text{NH}\) bend peak at 1500 cm\(^{-1}\) was also shifted to 1700 cm\(^{-1}\) and the double-bond absorption bands were newly appeared at 1629 cm\(^{-1}\), 1552 cm\(^{-1}\) and 1494 cm\(^{-1}\). This indicated the binding of Cu\(^{2+}\) with nitrogen of imine and pyridine groups. Hence, possible illustrated binding of AP---Cu\(^{2+}\) complex (scheme. 2) was well verified. Subsequently, the morphological and crystallinity changes were established as explained next. The SEM image of AP probe reveals its scattered morphology. But, upon the addition of Cu\(^{2+}\) ions, the aggregated crystalline nature of AP---Cu\(^{2+}\) complex was noticed as publicised in Fig. S7 (ESI).

![Fig. 7 XRD Analysis of (a) AP probe and (b) AP---Cu\(^{2+}\) complex.](image-url)
In addition, the XRD results evidenced that AP---Cu\(^{2+}\) loses crystallinity nature compared with the pure AP probe (Fig. 7a), which indicates the binding of AP with Cu\(^{2+}\) complex. The amorphous nature AP---Cu\(^{2+}\) of the XRD peak are shown in the Fig. 7b.

In order to prove the detection capability of AP towards Cu\(^{2+}\) ions, the detection limit (LOD)\(^{41}\) calculation was performed through standard deviation and linear fittings as illustrated in Fig. 8a. By plotting the fluorescence intensity changes at 430 nm as a function of concentration of Cu\(^{2+}\) ions, the detection limit of AP---Cu\(^{2+}\) complex was estimated as 8.63 x 10\(^{-8}\) M. Assuming a 1:1 complex formation, the association constant (K\(_a\)) of AP---Cu\(^{2+}\) was obtained on the basis of following equation (1).\(^{42}\)

\[
\frac{\alpha^2}{(1-\alpha)} = \frac{1}{2K_aC_F [M]} \quad \text{------------------- (1)}
\]

Where C\(_F\) is the total concentration of probe AP in the system and \(\alpha\) is defined as the ratio between the free probe AP and the total concentration of probe AP. The value “\(\alpha\)” was obtained using Eq. (2)

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**Fig. 8** (a) Detection limit calculation by plotting PL intensity changes at 430 nm vs Cu\(^{2+}\) concentration and (b) Association constant calculations by plotting \(\frac{\alpha^2}{(1-\alpha)} \) vs \(1/\text{Cu}^{2+}\) via standard deviation and linear fittings.
\[ \alpha = \frac{F - F_0}{F_1 - F_0} \]  

(2)

\( F \) is the fluorescence intensity at 430 nm at any given \( \text{Cu}^{2+} \) concentration, \( F_1 \) is the fluorescence intensity at 430 nm in the absence of \( \text{Cu}^{2+} \) ions, \( F_0 \) is the maxima fluorescence intensity at 430 nm in the presence of \( \text{Cu}^{2+} \). The association constant (\( K_a \)) was estimated graphically by plotting \( \alpha^2/(1 - \alpha) \) against \( 1/ [\text{Cu}^{2+}] \). The plots \( \alpha^2/(1 - \alpha) \) vs. \( 1/ [\text{Cu}^{2+}] \) are shown in Fig. 8b. Data were linearly fitted with respect to Eq. (1) and the \( K_a \) value was obtained from the slope. The \( K_a \) value of \( \text{AP}--\text{Cu}^{2+} \) was estimated as \( 2.12 \times 10^6 \) M\(^{-2}\).

Following, the pH titration of \( \text{AP} \) probe was performed to investigate a suitable pH range for \( \text{Cu}^{2+} \) sensing. As depicted in Fig. S8 (ESI), the emission intensities of metal-free \( \text{AP} \) probe was very low. After mixing \( \text{AP} \) probe with \( \text{Cu}^{2+} \), the emission intensity at 430 nm was suddenly increased at pH 6.0 and reached maximum between pH 6 – 8. However, when pH exceeded higher than 8.5, the emission intensity started to drop towards zero. This indicates poor stability of the \( \text{AP}--\text{Cu}^{2+} \) complex at higher pH ranges. For pH <6, the emission intensity is very low due to the protonation of the amine group, which prevents the formation of \( \text{AP}--\text{Cu}^{2+} \) complex.

**DFT studies**

To better understand the nature of coordination in \( \text{AP}--\text{Cu}^{2+} \), the energy-minimized structures of \( \text{AP} \) and its corresponding \( \text{Cu}^{2+} \) complexes (Fig. 9) were obtained with the B3LYP hybrid functional using the LANL2DZ basis set.\(^{43}\) All the calculations have been performed using the Gaussian 09 suite of programs.\(^{44}\)
Fig. 9 (a) Optimized structure of AP probe (b) Optimized structure of AP---Cu$^{2+}$ complex. (c) Electrostatic potential surface (ESP) of AP.

The optimized geometry, the ESP of the probe and the AP---Cu$^{2+}$ complex are shown in Fig 9. The distances between Cu and two N atoms are observed to be 1.99 and 2.11 Å (see Fig 9). The ESP in Fig 9(b) shows that the possible position of the metal binding site in red color. The spatial distributions and orbital energies of the highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO) of AP and its corresponding Cu$^{2+}$ complex were generated and the general PET mechanism for the probe and its Cu$^{2+}$ complex has been reported in Fig.10. Further, the DFT optimized structures (HOMO, HOMO-1, HOMO-2 and HOMO-3; LUMO, LUMO+1, LUMO+2 and LUMO+3) of AP and AP---Cu$^{2+}$ complex were supplemented in Fig. S9 (ESI).
**Fig. 10** General representation of PET based mechanism for (a) AP probe and (b) AP---Cu$^{2+}$ sensor system.
Calculated HOMO, LUMO and further excitation of electrons in the probe and its complex using the state-of-the-art density functional theory have provided us important information on electron migration at the molecular level for sensor applications. The PET mechanism for the probe shows that the HOMO (donor) is higher than that of the HOMO-1 (acceptor) (See Fig. 10a). The energy level of HOMO is -5.08 eV and the energy level of HOMO-1 is -6.15 eV. Remarkably, the HOMO-1 (donor) energy in the AP-\(\text{Cu}^{2+}\) complex is lower than the HOMO (acceptor) value. It suppress the PET process, resulting in fluorescence intensity enhanced as observed in the experiment.

**Live cell application**

The potential of AP probe for imaging Cu\(^{2+}\) in live cells were obtained using a confocal fluorescence microscope.

**Fig. 11** Fluorescence images of Raw264.7 cells treated with AP and AP-\(\text{Cu}^{2+}\). Bright Field image (Left); Fluorescence image (middle); Merged image (right). The scale bar is 25 µM.
When Raw264.7 cells were incubated with AP 20 µM), no fluorescence was observed (Fig. 11). After treatment with Cu$^{2+}$, bright blue fluorescence was observed in the Raw264.7 cells (Fig. 11). An overlay of fluorescence and bright-field images shows that the fluorescence signals are localized in the intracellular area, indicating a subcellular distribution of Cu$^{2+}$ ions with good cell-membrane permeability of AP. Further, the cytotoxicity studies of AP, showed more than 70% cell viability at 40 µM concentration as exposed in Fig. 12.

**Fig. 12** Cell viability of AP probe.

**Conclusion**

In conclusion, a simple anthracene comprising schiff base derivative AP was successfully obtained via one-pot reaction. The AP probe showed high selectivity and sensitivity towards Cu$^{2+}$ in a 1:1 stoichiometric manner which was confirmed by UV/PL titrations, FTIR, and ESI (+Ve) mass analysis. The LOD and $K_a$ values of AP---Cu$^{2+}$ conjugate were estimated as $10^{-8}$ M and $10^6$ M$^{-2}$, respectively. Furthermore, AP---Cu$^{2+}$
sensor complex formation was well characterised by \( \Phi \), SEM, pH effect and DFT studies. Delightfully, the sensor selectivity of AP to Cu\(^{2+} \) ions were successfully applied in cell imaging studies with cytotoxic studies. The cell permeability of the AP probe with less toxicity enables its further application towards drug delivery into the cell.

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**References**


Graphical Abstract:

A simple anthracene based AP probe was synthesized to detect Cu$^{2+}$ ions, via PET mechanism in live cell.