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Quick accessible dual mode turn-on red fluorescent chemosensor for Cu(II) and its applicability in live cell imaging†

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A novel fluorescent chemosensor 9,10-Diphenyl-7*H*-benzo[d,e]imidazo[2,1-a]isoquinolin-7-one (BIDQ-1), which exhibits rapid colorimetric and fluorimetric response for copper ions over other competitive metal ions under mild conditions has been synthesized easily by a simple one pot condensation method. On addition of Cu(II), BIDQ-1 shows a distinct color change from yellow to green, thus permitting the probe to be used for naked eye detection of Cu(II). The new fluorescent probe exhibits a turn-on fluorescence response towards Cu^{2+} under physiological conditions with high sensitivity and selectivity. The turn-on fluorescence behaviour of BIDQ-1 with Cu(II) was found to be *via* an intramolecular charge transfer (ICT) mechanism, by TD-DFT calculations. The probe was found to be applicable for imaging intracellular Cu^{2+} in living cells.

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Introduction

Copper is one of the essential trace elements required for many biochemical and physiological functions.[1] Copper ions contained in proteins act as cofactors for many enzymes such as cytochrome oxidase, zinc-copper superoxide dismutase, lysyl oxidase and catechol dioxygenase.[2] It is involved in critical biological processes in living organisms, such as respiration, antioxidant defense and iron metabolism.[3] However, free copper ions are toxic to the cells since they catalyze the generation of highly reactive hydroxyl radicals, which cause cellular damage.[4] Copper ions are highly toxic to organisms such as certain algae, fungi, many bacteria and viruses. The interruption of the copper balance in the body results in severe disorders such as Menkes syndrome, Wilson's disease, amyotrophic lateral sclerosis, prion diseases and Alzheimer's disease. Excessive intake of copper into the body leads to tumours, new blood vessel growth, kidney and liver damage, gastrointestinal disturbance and Non-Indian Childhood Cirrhosis (NICC).[5] In view of these effects it is worth developing a suitable technique to monitor Cu(II) within the body.The conventional methods for detection of copper ions such as photometric methods, atomic absorption spectroscopy (AAS), inductively coupled plasma emission or mass spectrometry (ICP-ES, ICP-MS), total reflection X-Ray fluorimetry (TXRF) and anodic stripping voltammetry (ASV)[6] provide good detection limits. However, these methods are not preferred as not only do they require high cost analytical instrumentation but they also have operational difficulties. These techniques, though applicable for biological samples, usually result in destruction of cells. As an alternative to these methods fluorescent sensors have been demonstrated to be powerful tools for the non-destructive imaging of intracellular distribution in single cells.[7] Due to the biological significance of Cu(II), colorimetric and fluorimetric detection of copper ions have been widely documented.[8] Cu(II) is a notorious fluorescence quencher due to its paramagnetic nature and de-excitation pathways involving electron transfer/excited-state energy transfer and as such, most of the earlier reports of Cu^{2+} sensors are based on quenching of fluorescence.[9] Subsequently, the design of highly sensitive and selective turn-on fluorescence probes for copper ions has been reported.[10] Near-infrared (NIR) or red detection of metal ions is preferable due to its applicability in biological systems as they minimize auto-fluorescence, cytotoxicity and photo damage of living cells.[11] Nevertheless only very few reports of NIR/red fluorescent turn-on sensors for Cu^{2+} are available.[12] In the present work the receptor BIDQ-1, synthesized by a new procedure,[13] has been shown to be an excellent, sensitive and selective turn-on sensor for Cu(II). This is the first report of using an imidazoquinone derivative as a fluorescent sensor.

Results and discussion

One pot condensation of acenaphthoquinone, benzil with ammonium acetate in ethanol in the presence of acetic acid

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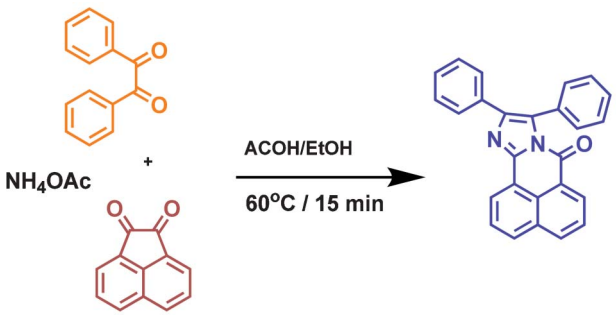

Scheme 1 Synthesis of BIDQ-1.

under refluxing conditions furnished the product in an excellent yield (ESI†) (Scheme 1).

The structure of BIDQ-1 was confirmed by spectro-analytical techniques. The response action of BIDQ-1 towards different metal ions was investigated by UV-vis and fluorescence spectrophotometric techniques. The UV-visible absorption spectrum of BIDQ-1 in CH_3CN:H_2O (1 : 1, v/v; PBS buffer, pH = 7.54) shows bands at 423, 347, 333, 290 nm. In order to check the selective sensing ability of BIDQ-1 metal ions such as Na^+, K^+, Mg^{2+}, Ca^{2+},Ba^{2+}, Zn^{2+}, Cd^{2+}, Hg^{2+}, Pb^{2+}, Ni^{2+}, Co^{2+}, Fe^{3+}, Mn^{2+}, and Ag^+ were added to BIDQ-1 and found no observable changes in its uv-vis spectrum. On addition of Cu^{2+} ions the band at 423 nm is suppressed along with the appearance of a new broad absorption band at 397 nm with the isobestic point at 429 nm. (Fig. 1).

The appearance of the isobestic point centred at 429 nm was reliable with equilibrium between BIDQ-1 and copper complexes in solution. UV-vis studies clearly show that the probe BIDQ-1 shows a selective and high binding affinity for Cu^{2+} ions even in the presence of other metal ions. The fluorescence spectra of the probe BIDQ-1 (1 μM) in the presence of tested metal ions other than Cu(II) ions exhibit a delicate emission at 588 nm, in CH_3CN:H_2O (1 : 1, v/v; PBS buffer, pH = 7.54) medium when excited at 440 nm. (Fig. 2).

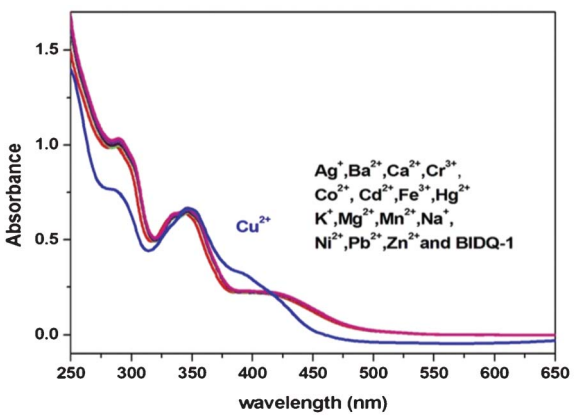

Fig. 1 UV-vis absorption spectra of BIDQ-1 (10 μM) in the presence of Cu^{2+}, Ag^+, Ba^{2+}, Ca^{2+}, Cd^{2+}, Co^{2+}, Fe^{3+}, Hg^{2+}, K^+, Mg^{2+}, Mn^{2+}, Na^+, Ni^{2+}, $Pb^{2+}$and Zn^{2+} (10 μM) in aqueous PBS buffer (10 mM PBS pH 7.54).

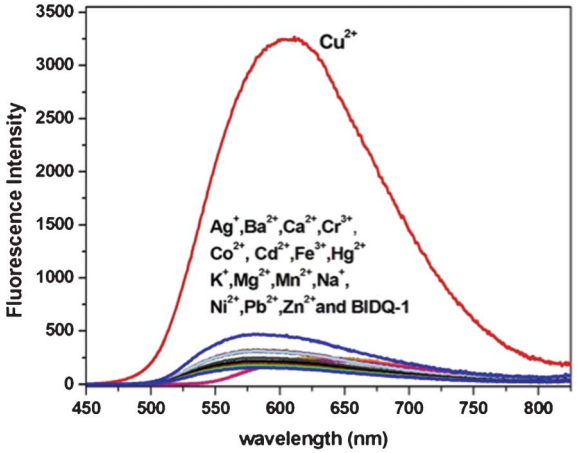

Fig. 2 Fluorescence spectra of BIDQ-1 in the presence of Fe^{3+}, Ag^+, Ca^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Hg^{2+}, K^+, Mg^{2+}, Mn^{2+}, Na^+, Ni^{2+}, $Pb^{2+}$and Zn^{2+} (10 μM) in aqueous PBS buffer (10 mm PBS pH 7.54). Excitation was performed at 440 nm. Excitation/Emission Slit width: 5/5.

The weak fluorescence band of BIDQ-1 is probably due to the ICT-type excited state. The gradual incremental addition of Cu^{2+} ions led to a gradual enhancement of the fluorescence intensity at 610 nm with a slight red shift whereas bare BIDQ-1 shows an emission band at 588 nm (Fig. 3). This intense enhancement of the fluorescence intensity of BIDQ-1 induced by Cu^{2+} ions is attributed to the improved ICT effect.

The binding constant[14] of BIDQ-1 with Cu^{2+} ions was estimated to be 5.5 × 10^2 mol L^{-1} from the fluorescence titration and the limit of detection (LoD)[15] was found to be 2.1 × 10^{-9} mol L^{-1}. The other metal ions such as Na^+, K^+, Mg^{2+}, Ca^{2+},Ba^{2+}, Zn^{2+}, Cd^{2+}, Hg^{2+}, Pb^{2+}, Ni^{2+}, Co^{2+}, Fe^{3+}, Mn^{2+}, and Ag^+ were added to BIDQ-1 but insignificant changes were observed in the fluorescence spectra. To elucidate the binding stoichiometry between BIDQ-1 and Cu^{2+}, a continuous varia-

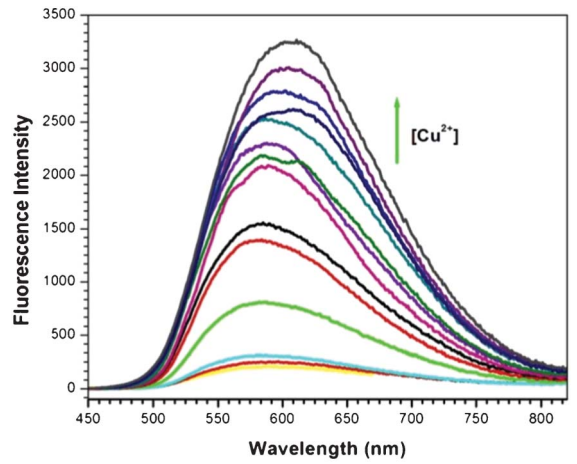

Fig. 3 Fluorescence emission spectrum of BIDQ-1 (1 × 10^{-5} mol L^{-1}) upon addition of Cu^{2+} (0–1 equivalent) in aqueous PBS (10 mM PBS, pH = 7.54) Excitation was performed at 440 nm. Excitation/Emission Slit width: 5/5.

tion method (Job's) was employed using the fluorescence emission data. The maxima was observed when the mole fraction of Cu(II) reached 0.5 exposed 1 : 1 binding. (ESI†) This stoichiometry was further supported by the nonlinear fit of the fluorimetric titration and ESI† mass spectral analysis. The peak at m/z = 436.29 clearly indicates the formation of 1 : 1 BIDQ-1 − Cu^{2+} complex (ESI†). In order to achieve excellent recognition of the analyte over potential competing species, selectivity of the probe is a key factor. The turn-on fluorescence recognizing efficiency of BIDQ-1 for Cu(II) is not affected in the presence of common cations, such as Na^{+}, K^{+}, Mg^{2+}, Ca^{2+}, Ba^{2+}, Zn^{2+}, Cd^{2+}, Hg^{2+}, Pb^{2+}, Ni^{2+}, Co^{2+}, Fe^{3+}, $Mn^{2+}$and Ag^{+}. The effect of pH on the probe BIDQ-1 was tested and the probe was found to be unaffected by pH. The fluorescence enhancement of BIDQ-1 in the presence of Cu(II) was studied at different time intervals and quick response times were revealed (ESI†). In order to investigate their molecular structure and possible electronic transitions in BIDQ-1 and BIDQ-1 + Cu^{2+}, DFT calculations were carried out using the Gaussian03 program.[16] The ground state structure optimization of BIDQ-1 and BIDQ-1 + Cu^{2+} were carried out using B3LYP and LANL2DZ functionalised with 6-31G* basis set respectively.

The lowest energy minimized structures for BIDQ-1 and BIDQ-1 + Cu^{2+} are shown in Fig. 4. The TD-DFT/B3LYP/6-31G* calculations based on the optimized structure of BIDQ-1, predict that electron density in HOMO is spread over the whole π-system while that of LUMO is localised on the acenaphthene ring The transition between HOMO to LUMO excitations (490.29 nm) has very low oscillator strength, f = 0.00624. This suggests that the probability of HOMO to LUMO transition is very low. Furthermore, the first excited state optimization of BIDQ-1 was carried out using the CIS/B3LYP-6-31G* method.

The TD-DFT results using excited state optimised geometry of BIDQ-1 clearly shows weak HOMO to LUMO transition (585.88 nm, f = 0.0962). This non radiative excited state transition leads to the weak fluorescence nature of the probe.

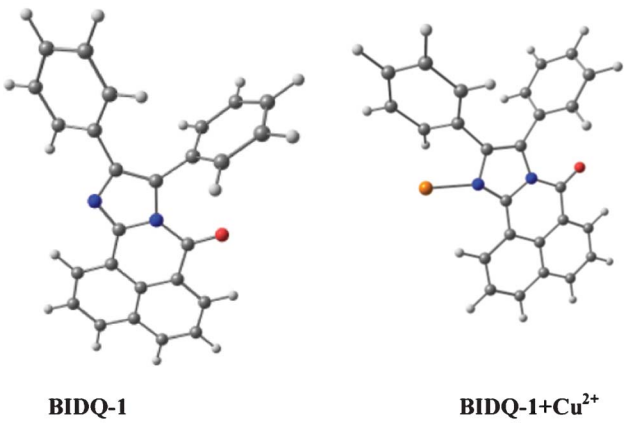

Fig. 4 Energy optimized geometry of BIDQ-1 and BIDQ-1 + Cu^{2+} by the B3LYP/6-31G and LANL2DZ methods respectively.

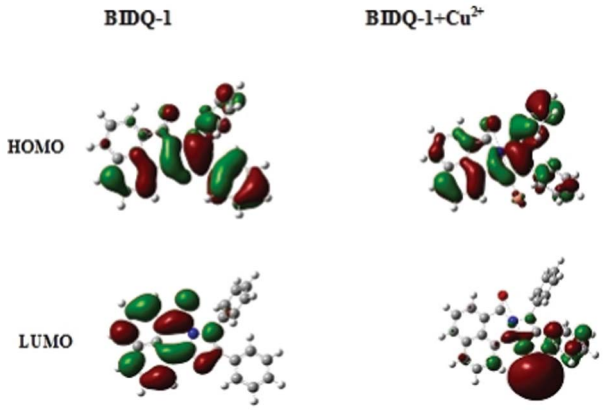

Fig. 5 Frontier molecular orbital diagram of BIDQ-1 and BIDQ-1 + Cu^{2+}.

Both ground states as well as the excited state DFT calculations predict the low probability for HOMO to LUMO transition. This in-turn leads to the inference that fluorescence will also unlikely be observed. The large degree of electron transfer between the phenyl unit and acenaphthoquinone in the first excited state was observed.(Fig. 5) The fluorescence enhancement of BIDQ-1 by Cu^{2+} could be illustrated by means of frontier orbitals obtained from the TD-DFT calculations. The HOMO–LUMO excitation (462.25 nm, f = 0.3164) was found to be relevant to the charge transfer, and the contribution to the lowest energy excitation was 40%. This excitation corresponds to charge transfer from the excited imidazo quinoline moiety to the Cu^{2+} center. It is clearly evidencing that the internal charge transfer, occurred during the addition of Cu(II), leads to the enhancement of fluorescence. TD-DFT and frontier molecular orbital calculations clearly support the observed fluorescence intensity enhancement is due to the ICT mechanism. The applicability of bio sensing probes for selective detection of guest species in live cells is of great interest and importance for biological application in the present scenario. Due to the quick response and high sensitivity of BIDQ-1 towards Cu^{2+} ion, its wide application in imaging Cu^{2+} in HeLa cells was studied. The HeLa cell lines were incubated with the probe BIDQ-1 for 30 min at 37 °C (5.0 μM in H_2O/DMSO (2 : 1, v/v) buffered with PBS, pH = 7.54) in a Dulbecco's Modified Eagle's Medium for 30 min at 37 °C.

In order to remove the extra cellular probe BIDQ-1, the HeLa cells was again washed with a phosphate-buffered saline buffer. The fluorescence images of HeLa cells were recorded. BIDQ-1 shows a hardly detectable fluorescence signal in living cells. The cells were incubated again with the Cu^{2+} (10.0 μM in H_2O) for 10 min to observe a bright red fluorescence in living cells.(Fig. 6) The fluorescence imaging results suggested that BIDQ-1 can permeate the cell membrane and can be applied for *in vitro* imaging of Cu^{2+} in living HeLa cells.

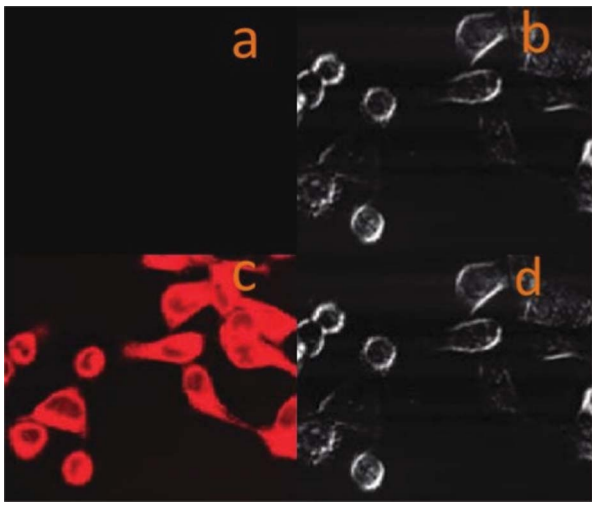

Fig. 6 (a) Fluorescence microscopic image of HeLa cells treated with BIDQ-1 (5.0 μM) for 20 min at 37 °C; (b) Bright field image of BIDQ-1 treated HeLa cells. (c) Fluorescence microscopic image of BIDQ-1 (5.0 μM) treated HeLa cells then Cu^{2+} (d) bright field image of HeLa cells treated with BIDQ-1 (5.0 μM) and Cu^{2+} (10.0 μM) λ_{ex} = 440 nm λ_{em} = 550–650 nm.

Conclusions

In conclusion, an imidazoquinoline BIDQ-1 has been synthesized very easily by a simple one pot condensation method and was well characterized. BIDQ-1 acts as a highly selective fluorescent and colorimetric sensor for Cu^{2+} in an aqueous acetonitrile medium. It is observed that BIDQ-1 displays ''Turn-on'' fluorescence and color change towards Cu^{2+}, which may be due to the ICT mechanism. The DFT, TD-DFT calculations on ground and excited state structures of BIDQ-1, BIDQ-1 + Cu(II) by 6-31G and LANL2DZ methods obviously indicate that the fluorescence enhancement can be attributed to the ICT process. The probe BIDQ-1 has great potential applications in live cell imaging of Cu^{2+} due to its excellent sensitivity and good selectivity.

Materials and methods

Acenaphthoquinone obtained from Aldrich were used as such. Benzil, ammonium acetate and metal chloride salts were procured from Merck. Absorption spectra were recorded using a JASCO V-650 spectrophotometer while fluorescence analyses were done using F-4500 Hitachi fluorescence spectrofluorimeter. The excitation and emission slit width were kept constant as 5 nm. NMR spectra were recorded on a Bruker (Avance) 300 MHz NMR instrument. Electrospray ionisation mass spectral (ESI-MS) analysis was performed in the positive ion mode on a liquid chromatography-ion trap mass spectrometer (LCQ Fleet, Thermo Fisher Instruments Limited, US). Microanalysis (C, H, and N) was performed using a Perkin-Elmer 4100 elemental analyser. Fluorescence microscopic images were taken using Zeiss LSM 510 META confocal fluorescence microscope.

Computational details

Density functional theory (DFT) calculations Were carried out with 6-31G* basis set using Gaussian 03 program in order to understand the fluorescence enhancement performance of BIDQ-1 on complexation with Cu^{2+}. The ground state geometries of BIDQ-1 and BIDQ-1 + Cu^{2+} complex were optimized by DFT-B3LYP using 6-31G* and LANL2DZ basis sets respectively. The excited state geometry of BIDQ-1 was optimised using CIS-B3LYP using 6-31G* method. Using the ground state and excited state optimized geometries absorption behaviour, emission behaviour and corresponding transitions of BIDQ-1 and BIDQ-1 + Cu^{2+} complex obtained from TD-DFT using above basis sets.

Synthesis of BIDQ-1

1 mmol of acenaphthoquinone, 1 mmol of benzil and 4 mmol of ammonium acetate was refluxed in ethanol in an oil bath for 15 min in the presence of catalytic amount of acetic acid. The reaction mixture color was suddenly changed to red. The reaction mixture was allowed to reach room temperature. The precipitated red colour solid was filtered. Further purification of the crude product was carried out by column chromatography using Petether-EtOAc (9 : 1) as eluent yields 9,10-Diphenyl-7*H*-benzo[d,e]imidazo[2,1-a]isoquinolin-7-one (BIDQ-1) as a reddish yellow solid. ^{1}H-NMR (300 MHz,$CDCl_3$): ppm δ 8.777–8.750 (dd, 1H, J = 1 Hz), 8.600–8.572(dd, 1H, J = 1 Hz), 8.241–8.213 (d, 1H, J = 8.5 Hz), 8.052–8.025 (d, 1H, J = 8.5 Hz), 7.79–7.69(m, 3H), 7.624–7.597 (dd, 3H, J = 9.6 Hz), 7.532–7.497 (m, 4H),7.304–7.211(m, 2H). ^{13}C-NMR (75 MHz, $CDCl_3$): ppm δ 160.3, 141.1, 135.3, 135.1, 133.1, 132.0, 131.9, 131.2, 130.8, 130.3, 128.5, 128.2, 128.194, 127.7, 127.5, 127.3, 126.6, 125.8, 125.6, 123.2, 120.925. MS (ESI): 373.1707($M + H^+$) calculated: 372.0412.

Cell culture and fluorescence imaging

HeLa cells were grown in modified Eagle's medium supplemented with 10% FBS (fetal bovine serum) at 37 °C. The cells were washed with PBS buffer. The HeLa cells were then incubated with BIDQ-1 (5.0 μM in H_2O/DMSO (2 : 1, v/v) buffered with PBS, pH = 7.54) in the culture medium for 30 min at 37 °C. After washing with PBS three times to remove any excess of the probe BIDQ-1 in the cells, the cells were further incubated with $CuCl_2$ (10.0 μM in H_2O) for 10 min at 37 °C and imaged with a Zeiss LSM 510 META confocal fluorescence microscope.

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