A simple and easy-to-prepare imidazole-based probe for the selective chromo-fluorogenic recognition of biothiols and Cu(II) in aqueous environments

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ABSTRACT

A new simple and easy-to-prepare imidazole-based probe 1 was synthesized and used to detect Cu(II) and biothiols (Cys, Hcy and GSH) in aqueous environments. Addition of increasing amounts of Cu(II) to water (pH 7.4)-acetonitrile 90:10 v/v solutions of probe 1 induced the appearance of a red-shifted absorption together with a marked colour change from colorless to deep blue. In addition, probe 1 was fluorescent and a marked emission quenching in the presence of Cu(II) was observed. The optical response is selective and other cations tested do not induce significant chromo-fluorogenic modulations. Limits of detection for Cu(II) of 0.7 and 3.2 μM using UV–visible and fluorescence data were determined. On the other hand, addition of Cys, Hcy and GSH to the deep-blue water (pH 7.4)-acetonitrile 90:10 v/v solutions of the 1-Cu(II) complex resulted in a marked bleaching together with the appearance of a highly emissive band centred at 475 nm. Other amino acids tested induced negligible response. The limits of detection for Cys, Hcy and GSH using 1-Cu(II) and emission data are 6.5, 5.0 and 10.2 μM, respectively. These optical changes were ascribed to a biothiol-induced demetalation process of the 1-Cu(II) complex that released the free probe. Besides, probe 1 is non-toxic and can be used for Cu(II) detection in HeLa cells.

1. Introduction

Transition metal cations are involved in several vital processes and are also used as diagnostic tools in medical, physiological and environmental fields [1–3]. In this scenario, the development of techniques for monitoring transition metal cations is an active area of research. Among transition metal cations, Cu(II) is the third most abundant essential element in the human body and plays vital roles in several physiological processes [4–9]. For instance, it has been reported that Cu(II) stimulates the proliferation of endothelial cells and is necessary for the secretion of several angiogenic factors by tumour cells [10,11]. Aside from its biological and environmental importance, copper is widely used in metallurgical, pharmaceutical and agrochemical industries [12]. As a result of the extensive applications of Cu(II) in life science and industry, it has become one of the first hazard environmental pollutants [13]. Despite the important role played by Cu(II) in several biological processes, abnormal levels of this cation can cause serious health problems on humans due to its ability to displace other vital metal ions in some enzyme-catalysed reactions [14]. In addition, high concentrations of Cu(II) in cells was documented to cause toxicity and different neurodegenerative diseases such as Menkes, Wilson’s and Alzheimer [15]. Therefore, simple and rapid sensing tools to monitor Cu(II) levels in biological and environmental media is of importance.

In the past years, electrochemical methods, spectrometry and chromatography have been employed to detect Cu(II). However, these methods are limited by their relatively high costs, are time consuming and are not usually suitable for in situ and on site analysis. As an
alternative to these classical methods, the use of chemical optical probes has attracted great attention in the last years and several Cu(II) sensors have been reported [16]. Some of these probes are able to detect Cu(II) both in solution (by colour and/or emission changes) and in living cells (by using confocal microscopy) [17–21]. In spite of these interesting features, some of these probes operate in organic solvents and often presented poor selectivity [22,23]. Thus, the preparation of selective probes that can detect Cu(II) in water or water/organic solvents mixtures is still a matter of interest.

On the other hand, biothiols (GSH, Cys and Hcy) are molecules that play fundamental roles in living systems because are involved in many important biological processes (such as tissue growth and defences) [24,25]. Dysregulation of biothiol levels could induce the appearance of certain diseases such as Alzheimer and cardiovascular disorders [26,27]. For the above mentioned reasons, the sensitive and selective detection of biothiols has been a matter of concern. In this respect, in the last years, several probes for the chromo-fluorogenic detection of biothiols have been published [28–31]. Most of the published examples are designed following the chemodosimeter paradigm, which makes use of the high nucleophilic reactivity of the thiol group. For example, biothiols reaction with fluorophores containing aldehyde [32–34], and 4-methoxythiophenol moieties [35,36] are recently reported. Besides, hydrolysis reactions induced by biothiols coupled with emission changes are also used [37,38]. However, among different approaches used for the design of biothiol chemosensors the use of Cu(II) complexes is perhaps one of the most promising. These probes worked using the well-known indicator displacement assay (IDA) paradigm [39,40]. These IDA assays are based on the use of fluorescent probes that coordinate selectively with Cu(II) (a highly effective quencher). As a consequence a non-emissive complex is formed. In the presence of biothiols, the non-emissive complex is demetallated (due to the preferential binding of Cu(II) with the thiol moieties in the biothiols) restoring the fluorescence of the free fluorophore. Using this IDA approach several systems for biothiols detection have been recently published [41–46].

Bearing in mind our experience in the development of molecular probes for detection of anions, cations and neutral molecules of biological and environmental significance [47–52], we report herein the synthesis and sensing behaviour of a new imidazole-based easy-to-prepodechrome-fluorogenic probe 1 able to detect Cu(II) in water (pH 7.4)-acetonicitrile 90:10 v/v mixtures. Besides, the complex formed between probe 1 and Cu(II) was used for the selective chromo-fluorogenic detection of relevant biothiols (Cys, Hcy and GSH). Probe 1 was also successfully used for detection of Cu(II) in living cells.

2. Experimental section

Chemicals: Commercially available reagents 4-(dimethylamino) benzaldehyde (1a), 1,2-di(thiophen-2-yl)ethane-1,2-dione (1b), ammonium acetate, Na2S2O3, and I2 were purchased from Sigma-Aldrich and Acros and used as received. TLC analyses were carried out on 0.25 mm thick precoated silica plates (Merck Fertigplatten Kieselgel 60F254) and spots were visualized under UV light. Chromatography on silica gel was carried out on Merck Kieselgel (230–240 mesh). All the metal salts used for the UV-visible and fluorosence experiments are nitrates.

Materials and methods: All melting points were measured on a Stuart SMP3 melting point apparatus. IR spectra were determined on a BOMEM MB 104 spectrophotometer using KBr discs. NMR spectra were obtained on a Bruker Avance III 400 at an operating frequency of 400 MHz for 1H and 100.6 MHz for 13C using the solvent peak as internal reference at 25°C. All chemical shifts are given in ppm using 1H Me4Si = 0 ppm as reference. Assignments were supported by spin decoupling-double resonance and bi-dimensional heteronuclear correlation techniques. High resolution mass spectrometry (HRMS) data were obtained with a TRIPLETOF T5600 (ABSciex, USA) spectrometer. UV/visible titration profiles were carried out with JASCO V-650 spectrophotometer (Easton, MD, USA). Fluorescence measurements were recorded with a JASCO FP-8500 spectrophotometer.

Synthesis of probe 1 (method A): 4-(Dimethylamino) benzaldehyde (1a, 0.15 g, 1 mmol), 1,2-di(thiophen-2-yl)ethane-1,2-dione (1b, 0.2 g, 1 mmol) and NH2OAc (20 mmol) were dissolved in glacial acetic acid (5 mL), followed by stirring and heating at reflux for 8 h. Then, the reaction mixture was cooled to room temperature, ethyl acetate (15 mL) was added and the solvent was evaporated under reduced pressure. The resulting crude product was purified by column chromatography (silica gel, CH2Cl2/MeOH 100:1), giving the pure product as a pink solid: yield (70 mg, 59%). 1H NMR (400 MHz, DMSO-d6): δ = 2.96 (s, 6H, NMMe2), 6.77 (dd, J = 7.2 and 2.4 Hz, 2H, H3 and H5), 6.99 (dd, J = 5.2 and 3.6 Hz, 1H), 7.13 (dd, J = 3.6 and 1.2 Hz, 1H), 7.19 (dd, J = 5.2 and 3.6 Hz, 1H), 7.36–7.39 (m, 2H), 7.65 (dd, J = 5.2 and 1.2 Hz, 1H), 7.84 (dd, J = 7.2 and 2.0 Hz, 2H, H2 and H6), 12.46 (s, 1H, NH) ppm. 13C NMR (100.6 MHz, DMSO-d6): δ = 40.12 (NMMe2), 111.86 (C3 and C5), 117.51 (C1), 119.51, 123.08, 124.52 (C2 and C6), 125.60, 126.98, 127.24, 127.25, 128.14, 122.71, 131.27, 133.01, 137.87, 146.70 (C4), 150.45 ppm. IR (Nujol): ν = 2855, 1615, 1510, 1201, 1107, 908, 705, 841, 822, 687 cm−1. HRMS-EI m/z: calc for C30H21N1S2+ + H+: 352.0942; measured: 352.0936.

Synthesis of probe 1 (method B): 4-(Dimethylamino) benzaldehyde (1a, 0.15 g, 1 mmol), 1,2-di(thiophen-2-yl)ethane-1,2-dione (1b, 0.2 g, 1 mmol), NH2OAc (20 mmol) and I2 (5 mol %) were dissolved in ethanol (5 mL), followed by stirring and heating at reflux for 27 h. Then, the reaction mixture was diluted with water (15 mL) having a small amount of Na2S2O3 and was cooled in an ice bath. The resulting crude product which precipitated was purified by recrystallization from ethanol given the pure compound 1 as a pink solid: yield (100 mg, 84%).

Synthesis of complex 1-Cu(II): Probe 1 dissolved in acetonicitrile (1.0 mmol) was mixed with Cu(NO3)2 (1.0 mmol) followed by stirring and heating at reflux for 4 h. Then [NH4][PF6] was added and the solid product formed was collected, washed with cold acetonicitrile and dried: yield (0.8 mmol, 80%). Elemental analysis, [1-Cu(II)][PF6]2: Calculated: C, 54.98; H, 4.13; N, 10.12; Cu, 15.31; Found: C, 55.05; H, 4.06; N, 10.17; Cu, 15.25.

3. Results

Probe 1 is not completely water soluble and, for this reason, the spectroscopic behaviour was studied in water (pH 7.4)-acetonicitrile 90:10 v/v mixture. In this respect, water (pH 7.4)-acetonicitrile 90:10 v/v solutions of probe 1 (1.0 × 10−5 mol L−1) presented an absorption band centred at ca. 320 nm with a molar extinction coefficient of 28000 M−1 cm−1 (see Fig. 1). Next, UV-visible changes in probe 1 upon addition of 10 eq. of Cu(II), Pb(II), Mg(II), Ge(II), Ca(II), Zn(II), Co (II), Ni(II), Ba(II), Cd(II), Hg(II), Fe(III), In(III), As(III), Al(III), Cr(III), Ga(III), K(I), Li(I) and Na(I) was studied. As could be seen in Fig. 1, among all cations tested, only Cu(II) was able to induce the appearance of a new absorption band centred at 555 nm (e = 32000 M−1 cm−1). The marked changes in the UV-visible spectrum of probe 1 upon addition of 10 eq. of Cu(II) is reflected in a clear colour modulation from colourless to deep blue (see also Fig. 1).

Having assessed the highly selective response of probe 1 toward Cu (II) we studied, in the next step, the changes in the UV-visible spectra upon addition of increasing amounts of Cu(II). Addition of different amounts of Cu(II) to water (pH 7.4)-acetonicitrile 90:10 v/v solutions of 1 (1.0 × 10−5 mol L−1) induced the progressive decrease of the absorption centred at 320 nm together with the growth of the visible band at 555 nm. From the obtained titration profile a limit of detection of 0.7 μM was determined (see Supporting Information) which is almost 100 times lower than the limit prescribed by the World Health...
Organization (WHO) guideline for drinking water (30 mM) [53,54].

In order to understand the mode of coordination between probe 1 and Cu(II) Job’s plot were determined. As could be seen in Fig. 2, probe 1 clearly forms a 1:1 stoichiometry complex with Cu(II). From the UV–visible titration shown in Fig. 3 a logarithm of the stability constant for the formation of the 1-Cu(II) complex of 3.50 ± 0.15 was determined.

Probe 1 is also fluorescent. Excitation at 324 nm (one of the isoabsorptive points observed in the Cu(II) UV–visible titration profile of water (pH 7.4)-acetonitrile 90:10 v/v of probe 1 (1.0 × 10⁻⁵ mol L⁻¹) showed a marked emission band centred at 475 nm (quantum yield of 0.26). Among all cations tested, only Cu(II) induced emission quenching as could be seen in Fig. 3. From the emission titration profile (see Supporting Information) a linear ratio between the emission intensity and the amount of Cu(II) added was observed. Besides, a limit of detection of 3.7 μM of Cu(II) was determined. On the other hand, the emission of probe 1 in water-acetonitrile 90:10 v/v mixtures at acidic pH (5.0 and 6.0) remained nearly unchanged upon addition of Cu(II) cation (see Supporting Information).

The observed emission quenching is remarkable, especially when compared with the results previously published obtained with a structurally related probe 2 (see Scheme 1). In this respect, ethanol solutions of macrocycle-containing probe 2 presented a weak emission band that was markedly increased upon addition of Cu(II) [55]. The marked emission enhancement observed with 2 was ascribed to an increase in the rigidity of the probe upon formation of 2:1 metal-probe complexes in which one Cu(II) coordinated with the macrocycle and the other with the nitrogen atoms of the imidazole with a logarithm of the stability constant of 11.58 ± 0.01. In our case, the fluorescence experiments were carried out in a more competitive media (water-acetonitrile 90:10 v/v) and probe 1 lacks the macrocycle binding domain presented in 2.

Taking into account the non-emissive nature of 1-Cu(II) complex (quantum yield of 0.07) and the high affinity of thiol moieties for Cu(II) we tested the possible use of this complex in an IDA assay for biothiols detection. As stated above, water (pH 7.4)-acetonitrile 90:10 v/v solutions of 1-Cu(II) complex (6.2 × 10⁻⁶ mol L⁻¹) presented a marked deep blue colour due to a remarkable absorption band centred at 555 nm. In a first step, the chromogenic response of 1-Cu(II) complex was tested in the presence of amino acids (Val, Leu, Thr, Lys, Trp, His, Phe, Ile, Arg, Met, Ala, Pro, Gly, Ser, Cys, Asn, Gln, Tyr, Asp, Glu and

**Fig. 1.** UV–visible spectra of probe 1 in water (pH 7.4)-acetonitrile 90:10 v/v (1.0 × 10⁻⁵ mol L⁻¹) alone and in the presence of 10 eq. of selected metal cations. The inset shows the change in colour of probe 1 in the presence of Cu (II). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

**Fig. 2.** Job’s plot of probe 1 and Cu(II) in water (pH 7.4)-acetonitrile 90:10 v/v. Total concentration of 1 and Cu(II) of 2.0 × 10⁻⁵ mol L⁻¹.

**Fig. 3.** Fluorescence spectra of probe 1 in water (pH 7.4)-acetonitrile 90:10 v/v (1.0 × 10⁻⁵ mol L⁻¹) upon addition of increasing amounts of Cu(II) (from 0 to 10 eq.).

**Scheme 1.** Synthesis of probe 1 and structure of closely related macrocycle-containing imidazole-derivative 2.
H.E. Okda et al.

Dyes and Pigments 162 (2019) 303–308

Fig. 4. UV–visible changes of 1-Cu(II) \((6.2 \times 10^{-6}\, \text{mol}\,\text{L}^{-1})\) in water (pH 7.4)-acetonitrile 90:10 v/v in the presence of selected amino acids (0.2 eq.) and biothiols (0.2 eq.).

Fig. 5. Changes in the emission band of 1-Cu(II) complex \((6.2 \times 10^{-6}\, \text{mol}\,\text{L}^{-1})\) in water (pH 7.4)-acetonitrile 90:10 v/v upon addition of biothiols (0.2 eq.) and selected amino acids (0.2 eq.).

Complex, induced by \(\text{P}_2\text{O}_7^{4-}\) anion, that produced the free probe [56].

The selective emission quenching of 1 in the presence of Cu(II) and the recovery observed with GSH suggests that the probe can be used for the imaging of these species in living cells. Based on these observations, the cytotoxicity of 1 was first evaluated. HeLa cells were treated with 1 (5 \(\mu\text{M}\)) over half an hour period and cell viability was determined by a WST-1 assay. Moreover, the viability of probe 1 in the presence of Cu(II) (1 and 10 eq.) was also assessed. The obtained results are shown in Fig. 6. As could be seen, probe 1 was non-toxic to HeLa cells at the concentration tested. Besides, the concentrations of Cu(II) added (alone and in the presence of probe 1) were also non-toxic to HeLa cells.

Then, in order to verify the feasibility of the developed probe to detect Cu(II) and GSH in highly competitive environments, we prospectively used probe 1 for the fluorescence imaging of both species in living cells. In a typical experiment, HeLa cells were incubated in DMEM supplemented with 10% fetal bovine serum. To conduct fluorescence microscopy studies, HeLa cells were seeded in 24 mm glass coverslips in 6-well plates and were allowed to settle for 24 h. Cells were treated with probe 1 in DMSO (1%) at a final concentration of 5 \(\mu\text{M}\). After 30 min, the medium was removed and solutions of different concentrations of Cu(NO\(_3\))\(_2\) in PBS were added (5 \(\mu\text{M}\) and 50 \(\mu\text{M}\)) and cells were incubated for another 10-min period. Finally, treated cells were incubated overnight in order to ascertain the intracellular GSH effect. As seen in the confocal fluorescence microscope images shown in Fig. 7a, the control experiment (HeLa cells without probe 1) showed a weak fluorescence, and cells treated with 1 (5 \(\mu\text{M}\)) showed a marked intracellular emission (Fig. 7b). Moreover, a significant quenching in intracellular emission was observed in the Cu(II)-treated cells (Fig. 7c), clearly indicating the possible use of 1 to detect this divalent metal cation in complex biological settings. Finally, after incubation overnight, a remarkable emission enhancement could be observed probably due to a intracellular GSH-induced demetallation of complex 1-Cu(II) which generated the free probe (Fig. 7d). Besides, the emission intensity of the HeLa cells after each treatment was measured and the obtained results are presented in Fig. 7e.

4. Conclusions

In summary, we report herein an easy-to-prepare imidazole-based chromo-fluorogenic probe 1 for the selective and sensitive optical detection of Cu(II) and biothiols. Probe 1 was able to selectively detect Cu(II) in a highly competitive media (water-acetonitrile 90:10 v/v) by a marked colour change from colourless to deep blue. Besides, a significant quenching of the probe emission in the presence of Cu(II) was observed. Moreover, real-time fluorescence imaging measurements
confirmed that probe 1 can be used to detect intracellular Cu(II) at micromolar concentrations. Moreover, 1-Cu(II) complex was used for the development of an IDA assay for the selective chromo-fluorogenic sensing of biothiols (Cys, Hcy and GSH). Biothiols were able to demetallate 1-Cu(II) complex with the subsequent release of free probe 1 and Cu(II) and after incubation overnight. (e) Emission intensity quantification by the confocal images analysis.

Fig. 7. Confocal microscopy images of probe 1 in HeLa cells. HeLa cells were incubated with 1 (5 μM) for 30 min at 37 °C in DMEM. Cells images obtained using an excitation wavelength of 405 nm. (a) Fluorescence images of Hela cells incubated with probe 1 and pre-treated with Cu(II) 10 eq. (50 μM) for 10 min. (d) Fluorescence images of HeLa cells after the pre-treatment with probe 1 and Cu(II) and after incubation overnight. (e) Emission intensity quantification by the confocal images analysis.

[41–46, 57–59].

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dyepig.2018.10.017.

References


