Uracil-Appended Fluorescent Sensor for Cu\(^{2+}\) and Hg\(^{2+}\) Ions: Real-Life Utilities Including Recognition of Vitamin B\(_2\) (Riboflavin) in Milk Products and Invisible Ink Applications

Gitanjali Jindal\(^1\) · Navneet Kaur\(^*\)

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Abstract
A simple uracil-appended fluorescent sensor (1) has been developed by one pot reaction and characterized by using common spectroscopic methods such as UV-vis, Fluorescence, HRMS and FT-IR analyses. Upon addition of various metal ions to the CH\(_3\)CN solution of sensor 1, the fluorescence was quenched in the presence of Cu\(^{2+}\)/Hg\(^{2+}\) ions. The limit of detection for Cu\(^{2+}\) and Hg\(^{2+}\) was calculated to be 3.31 and 0.316 µM, respectively. Further, the sensor was applied for real-life applications in the determination of Vitamin B\(_2\) (riboflavin) and its presence in milk products. With the incorporation of different sources of vitamin-B to acetonitrile solution of it, there was discernible fluorescence enhancement only in the presence of vitamin B\(_2\). Also, it has been successfully applied for the detection of Vitamin B\(_2\) (riboflavin) in milk and curd. Moreover, based on the fluorescent color changes, the sensor was utilized for invisible ink applications.

Keywords Uracil · Riboflavin · Biological sensing · Real sample analysis · Invisible ink

Introduction
In the present time, the expedition to devise sensitive and selective analytical systems for the recognizing environmentally and biologically related heavy and transition metal ions has been a focus for the research fraternity [1, 2]. In this context, the development of selective sensors has appeared to be an imperative research field [3, 4]. Metal ions take part in harmful biological as well as environmental procedures, and their participation has been firmly regulated by their corresponding concentration levels. Metal ions display tremendous influence in either becoming valuable or lethal depending on their presence in the appropriate or a hysterical quantity, respectively [5].

Mercury, the largely detrimental heavy metal element, create intimidations to the atmosphere by accretion owing to its non-degradable character and can lead to various health issues in the kidneys, brain, and the central nervous system [6, 7]. Additionally, its occurrence in diverse varieties such as ionic, metallic and in organic salts and complexes augments the trouble to a larger extent. Likewise, the third-most profuse transition metal, Copper, occupies an important place in different physiological processes [8–13]. Despite the biological and environmental significance, copper possesses relative abundance, economic ability, and acquire excellent malleability, electrical and, thermal conductivity, chemical stability as well as germicidal competence [14, 15]. Though, intemperance of Cu\(^{2+}\) has been extremely noxious to living creatures, it’s excessive amassing in humans can cause different syndromes involving neuro-degenerative diseases for example, Alzheimer, Wilson’s and Menkes disease, gastrointestinal and inflammatory disorders, kidney damage, amyotrophic sclerosis, lipid metabolism [16–18].

Further, Riboflavin (vitamin B\(_2\)) has been known as a micronutrient that is easily absorbed in the human and animal body by sustaining physical wellbeing escorted with broad range of cellular processes. This vitamin has an essential function in metabolism of ketone bodies, carbohydrates, proteins and fats, and energy metabolism [19]. It is disseminated within all the tissues, although at consistently minute amounts [20]. Once it is taken orally, it has not been regarded harmful attributable to its poorer water solubility...
due to which excess of this vitamin rapidly get excreted via the urine [21, 22]. Riboflavin has become renowned to be used as a medicine and is frequently used in energy drinks and related remedial tonics. Several studies have been carried out to employ riboflavin in clinical and scientific set-ups. In support of this statement, riboflavin has been utilized to reduce migraine problem when consumed in large doses [23, 24]. Also, it has been found helpful in preservation against the nuclear cataracts [25]. Taking into consideration, the nutritional and medical significance of riboflavin, there should be developed various systems for its detection and assessment.

A range of techniques have been explored for the evaluation of vitamin B₃ (riboflavin). They incorporate high-performance liquid chromatographic separation accompanied with fluorescent determination of the analytical species, ultraviolet-visible sensing and mass spectrometric detection [26–29]. Currently, the research areas that involves planning and development of highly selective and sensitive detection techniques have been of radiant accomplishment [30, 31]. Among these, the fluorescent recognition has borrowed substantial consideration due to their capability for sensing of multiple analytes alongside wide-ranging relevance in environmental chemistry, cell biology, and biochemical methods [32]. The investigations containing fluorescent sensors possess variety of benefits like trouble-free sample formation and handling, fast reaction time, reusability of analyte and highly cost-effective [33–36].

Moreover, in the today’s world, one essential information could reach anywhere in countless modes comprising social network, internet and printing records [37, 38]. Hence, it turns out to be incredibly essential for pursuing an approach to cover up some relevant data encrypted on a document [39]. In case any imperative documentation got exposed, it may possibly generate elevated threats to national security. For this reason, the conception of high security invisible ink [40, 41] has been launched now-a-days to conceal information/data and circumvent considerable menace to commercial and military rationales [42].

In the present exertion, we have demonstrated the sensing of metal ions using uracil-appended fluorescent sensor, 1. Upon addition of both the Cu²⁺/Hg²⁺ ions in the acetonitrile solution of sensor 1, the fluorescence intensity was quenched; while other metals ions imparted negligible effect on the fluorescence intensity. This paper also comprises the recognition of vitamin B₂ (Riboflavin) using the sensor 1 and its detection in milk products. The incorporation of vitamin B₂ in acetonitrile solution of sensor 1 enhanced the fluorescence intensity significantly. Furthermore, the sensor 1 acted as an invisible ink due to the fluorescent color changes in the presence of Vitamin B₂ (Riboflavin).

Experimental

Materials and methods

6-Amino-1, 3-dimethyluracil, 4-nitrobenzaldehyde, tetrafluoroacetic acid, sources of vitamin-B such as Vit.B₁ (Thiamine), Vit.B₂ (Riboflavin), Vit.B₃ (Nicotinamide) and Vit.B₁₂ (Cyanocobalamin) and perchlorate salts of different metal ions such as Al³⁺, Na⁺, K⁺, Mg²⁺, Mn²⁺, Fe²⁺, Co²⁺, Ni²⁺, Cu²⁺, Zn²⁺, Hg²⁺, and Cd²⁺ were purchased from Aldrich. All other chemicals were used as received without further purification. Acetonitrile (CH₃CN) was of HPLC grade. Melting points were determined in capillary and are uncorrected. ¹H and ¹³C NMR spectra were recorded on a BRUKER AVANCE 500 and 125 MHz instrument using tetramethylsilane as an internal standard. Various sources of vitamin-B such as B₁, B₂, B₃, B₁₂ were added for fluorescence experiments. Aliquots of metal ions and different salts under examination were then injected into the sample solution through a rubber septum in the cap. The solutions were kept for some time to get stabilized after each addition and then they were scanned.

General procedure for UV-vis and fluorescence experiments

The UV-vis and fluorescence titrations were performed with 20 μM and 10 μM solution of 1 corespondingly in acetonitrile solution. All the UV-vis experiments were conducted on JASCO UV-750 spectrometer; while fluorescence spectra were taken using HITACHI-7000 spectrophotometer equipped with 220–240 V Xe lamp with quartz cell of 1 cm width and 3.5 cm height. The excitation wavelength was taken as 375 nm for sensor 1 with 5 nm excitation as well as emission slit widths in fluorimeter. Stock solutions of the sensor 1 (1×10⁻² M) were prepared in DMSO and were diluted with CH₃CN solution for further different spectroscopic analyses. The solutions of all the vitamin B sources (1×10⁻¹ M) and above mentioned metal ions (1×10⁻¹ M) were made in water. And, 100 equivalents of each of them were added in CH₃CN solution of sensor (1) for spectroscopic measurements. All absorption scans were saved as ACS II files and further processed in Excel(tm) to produce all graphs shown.

Synthesis of 1,3-Dimethyl-6-[(4-nitro-benzylidene)-amino]-1 H-pyrimidine-2,4-dione (1)

4-Nitrobenzaldehyde (0.48 g, 3.22 mmol) was dissolved in 40 ml absolute ethanol in a round bottom flask. To this mixture, 6-amino-1, 3-dimethyluracil (0.50 g, 3.22 mmol) was added. The reaction mixture was heated at 60 °C for
Results and discussion

Fluorescence emission spectral studies

The sensing ability of sensor 1 was evaluated in the presence of various metal ions such as Al\(^{3+}\), Na\(^+\), K\(^+\), Mg\(^{2+}\), Mn\(^{2+}\), Fe\(^{3+}\), Co\(^{2+}\), Ni\(^{2+}\), Cu\(^{2+}\), Zn\(^{2+}\), Hg\(^{2+}\), and Cd\(^{2+}\) by observing UV-vis as well as fluorescence emission changes. The sensor 1 exhibited an absorption peak at 280 nm, which remained unperturbed in the presence of mentioned metal ions (Fig. S5). In the fluorescence spectrum, it displayed an emission band at 470 nm upon excitation at 375 nm. Amidst the various ions inspected, addition of only Cu\(^{2+}\) and Hg\(^{2+}\) ions to CH\(_3\)CN solution of 1 (10 µM) quenched its fluorescence intensity. All the other inspected ions exhibited negligible fluorescence responses under similar spectroscopic environment (Fig. S6). For the quantitative study of the interaction between 1 and sensed metal ions, fluorescence titration experiments were executed by the incorporation of sensed metal ions to solution of sensor 1 (10 µM). It was observed that with the gradual addition of Cu\(^{2+}\) and Hg\(^{2+}\), the fluorescence intensity was quenched progressively with slight blue shift as illustrated in Fig. 1.

The quenching of fluorescence intensity may be due to the interactions between imine group and Cu\(^{2+}\)/Hg\(^{2+}\) (Scheme 2), where paramagnetic effect of Cu\(^{2+}\) and heavy metal effect of Hg\(^{2+}\) might be responsible for this observed quenching [43, 44].

The job’s plot revealed the 1:1 binding stoichiometric ratio of sensor 1 with Cu\(^{2+}\) and Hg\(^{2+}\) ions (Fig. S7). The limit of detection was calculated to be 3.31 µM (Cu\(^{2+}\)) and 0.316 µM (Hg\(^{2+}\)) by using the equation LOD = 3σ/ρ [45],
where, $\sigma =$ standard deviation of response and $\rho =$ slope of the calibration curve.

The binding constants between sensor 1 and Cu$^{2+}$ and Hg$^{2+}$ ion were evaluated as $1.16 \times 10^5$ and $1.37 \times 10^5 \text{M}^{-1}$, respectively using following the Benesi-Hildebrand equation [46]:

$$1/[F-F_0] = 1/[F_{\text{max}}-F_0] + 1/[F_{\text{max}}-F_0]K[C].$$

here, $F_0$, $F$, and $F_{\text{max}}$ is the fluorescence of free 1, measured with Cu$^{2+}$/Hg$^{2+}$ ions and measured with an excess amount of Cu$^{2+}$/Hg$^{2+}$ ions at 470 nm correspondingly. $K$ is the association constant and $[C]$ is the concentration of Cu$^{2+}$/Hg$^{2+}$ ions added.

### Competition experiments

The selective nature of a sensor in presence of other interfering species has been a significant tool for determination of its sensing performance. The competition studies were executed in the presence of 20 equiv. of Cu$^{2+}$/Hg$^{2+}$ ion with 100 equiv. of all the other metal ions using fluorescence spectroscopic experiments. These experiments disclosed that none of the added metal ions have any kind of effect on the fluorescence intensity of the Cu$^{2+}$/Hg$^{2+}$ ions, respectively, pointing to the selective nature of sensor 1 in recognition of Cu$^{2+}$/Hg$^{2+}$ ions (Fig. S8).

![Fig. 2](image1.png) (a) Fluorescence preliminary spectrum of 1 (10 µM) in CH$_3$CN in the presence of various vitamin B sources excited at 375 nm. (b) Fluorescence titration experiment in the presence of increasing concentrations of Vit.B$_2$, Riboflavin. (Inset: fluorescence intensity plot at 511 nm versus the concentration of added Vit.B$_2$, Riboflavin)

![Fig. 3](image2.png) (a) Fluorescent color changes upon the addition of various vitamin-B sources to the sensor 1 solution; (b) Bar graph showing the fluorescence changes upon addition of milk and curd in the solution of sensor 1; (c) Fluorescence titration experiment in the presence of increasing concentrations of milk. (Inset: fluorescence intensity plot at 511 nm versus the concentration of added milk)
Real-life applications

Vitamin B₂ sensing

To ensure the feasibility of the sensor I in real-life, it was tested for the recognition of vitamin B sources such as Vit.B₁ (Thiamine), Vit.B₂ (Riboflavin), Vit.B₃ (Nicotinamide) and Vit.B₁₂ (Cyanocobalamin). Upon the addition of 100 equiv. of all the Vitamin B sources to the sensor I solution (CH₃CN), the fluorescence intensity enhanced exceedingly only in the presence of Vit.B₂ (Riboflavin) as shown in Fig. 2a. To obtain the sensing performance quantitatively, the fluorescence titration experiments were carried out and it was observed that with the progressive addition of Vit.B₂ (Riboflavin) in the CH₃CN solution sensor I, the emission intensity enhanced continuously (Fig. 2b). The enhancement of fluorescence might be attributed to the interactions of > C = O and –NH groups in flavin ring of Vit.B₂ (Riboflavin) moiety with the imine hydrogen and nitrogen atoms in sensor I which increased the conjugation owing to the formation of [I:Riboflavin] complex and hence, the emission intensity was increased [47, 48]. The association constant and limit of detection values of sensor I for Vit.B₂ (Riboflavin) were estimated to be 2.27 × 10⁴ M⁻¹ and 0.137 µM, respectively.

This change was also accompanied with fluorescent color change from light green to bright greenish-yellow (Fig. 3a). The sensor I was also examined for the determination of Vit.B₂ (Riboflavin) in real samples such as milk and curd. For this examination, about 100 µL of milk was taken, and then CH₃CN solution of sensor I (10 µM) was added. This resulted in around three-fold increase in fluorescence intensity of the sensor I. Similarly, the one small spoon of curd was taken which was then diluted with small amount of water and incorporated with the sensor (I) solution. This led to similar outcomes as obtained with milk sample (Fig. 3b). Moreover, the quantitative results for the detection of Vit.B₂ (Riboflavin) in milk were obtained by performing fluorescence titration with varying concentrations of milk (Fig. 3c). The limit of detection of sensor I for Vit.B₂ (Riboflavin) in milk was evaluated as 0.15 µM, which was found to be much lower than the previous literature reports [49–51]. Henceforth, all the consequences suggested that sensor I can be successfully utilized for the sensing of Vit.B₂ (Riboflavin) in milk products.

Invisible ink

The fluorescent color responses allowed sensor I to be utilized as invisible ink, when observed under the UV light (Fig. 4). It was seen that almost colorless ‘Vitamin B₂’ written by sensor I solution turned greenish-yellow upon the addition of Vit.B₂ (Riboflavin) solution when examined under UV lamp.

Conclusions.

A fluorescent uracil-based sensor (I) has been synthesized for detection of Cu²⁺/Hg²⁺ ions in acetonitrile. The Cu²⁺/Hg²⁺ ions when added to the solution of sensor I quenched the fluorescence intensity when irradiated at 470 nm. The association constant (LOD) values for Cu²⁺ and Hg²⁺ ion were calculated to be 1.16 × 10⁵ M⁻¹ (3.31 µM) and 1.37 × 10⁵ M⁻¹ (0.316 µM), respectively. Further, the sensor I could be applied in real-life sensing of Vitamin B₂ (Riboflavin) along with its detection in milk products. Also, the fluorescent color changes with riboflavin allowed it to be used in invisible ink applications.

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Author contribution Gitanjali Jindal contributed towards conceptualization, data curation, formal analysis, visualization, writing-original draft. Navneet Kaur gave contribution for methodology, writing-review & editing, supervision, project administration.

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Availability of data material The data provided in the manuscript is original and will be made available at any time on request basis.

Code Availability Not Applicable.

Declarations

Ethics approval This article does not contain any studies with human participants or animals performed by any of the authors.

Consent to participate The authors declare that they consent to participate.

Fig. 4 Colorless text written on glass using sensor I solution became greenish-yellow under UV light (375 nm excitation) in presence of Vit. B₂ (Riboflavin) solution

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Consent for publication The authors provide consent for the publication.

Conflict of interest The authors have no conflicts to declare.

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