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A re-evaluation of the role of coumarin C460 as a fluorescent Pd(II) sensor

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ABSTRACT

In 2018, Ashwin and co-workers presented a method for the selective and sensitive detection of Pd^{2+} based on variations in the fluorescence of coumarin (C460). Herein, we re-evaluate the ability of C460 to probe Pd^{2+} concentration via a systematic reproduction of the experiments reported in the original publication and the performance of additional control experiments. Our analysis indicates that C460 is in fact not a fluorescence sensor for Pd^{2+}, but rather can act as a pH sensor over a restricted pH range. Furthermore, we are of the opinion that the experimental procedures described in the original paper were inaccurately or incompletely presented, particularly with regard to sample preparation. To support our conclusions, we provide a detailed assessment of the fluorescence properties of C460 in aqueous media as a function of pH. To summarize, our analysis suggests that reductions in fluorescence are not due to molecular interactions with palladium species, but instead result from the inhibition of intramolecular charge transfer (ICT) caused by the protonation of the amine group at low pH values.

1. Introduction

Fluorescence probes are a ubiquitous tool for the detection and quantitation of metal ions in a range of applications, due to their simplicity, selectivity, non-destructive nature, excellent spatial and temporal resolution and sensitivity [1–3]. Such probes commonly comprise a fluorophore linked to a receptor group that binds or interacts with the analyte under study. Receptor groups for metal ions usually contain heteroatoms capable of forming coordination bonds with the metal [1,2], which in turn can trigger, enhance or quench fluorescence emission.

Coumarins are an extensive family of fluorophores containing the 1-benzopyran-2-one motif. They have found wide application and use as fluorescence-based chemosensors, since they normally display good photostabilities and appreciable fluorescence quantum efficiencies in the visible region of the electromagnetic spectrum [4]. Their fluorescence can be controlled by introducing appropriate substituents that enhance intramolecular charge transfer (ICT) [4]. For example, many coumarin-derived fluorophores integrate an electron-donating group (such as an amino or hydroxyl group) at the 7-position and an electron-withdrawing group at the 3- or 4-position [5] to enhance charge transfer character and allow for strong emission between 400 and 550 nm [4]. Coumarin-based chemosensors have been used to target a wide-range of species, including inorganic anions, metal cations and biologically active molecules and can be either "turn-on" or "turn-off" in nature [6].

In a 2018 paper published in this journal, Ashwin and co-workers reported the use of a commercially available coumarin as a turn-off probe for Pd^{2+} quantification [7]. Specifically, 7-diethylamino-4-methylcoumarin (or C460) was used to quantify Pd^{2+} in both aqueous solutions and in living cells. On first inspection, such an approach has many merits. The probe is widely available, cheap, provides for good analytical sensitivities and detection limits sensitive, is selective towards Pd^{2+}, as well as being compatible with biological systems. Surprisingly, in this study, the authors employ $PdCl_2$ as the Pd(II) precursor. $PdCl_2$, a common starting material in palladium chemistry, is a highly stable salt that decomposes at about 800 K under an N_2 atmosphere [8]. This observation is a direct indication of the bond strength between the palladium and the chlorine atoms, and thus of the high chemical stability of the compound. It is well recognised that $PdCl_2$ is essentially insoluble in pure water [9], with Pd^{2+} ions not existing in aqueous solution. Instead, and depending on the pH and Cl^- concentration, Pd(II) precursors form various metal complexes when dissolved. A common strategy for dissolving stubborn compounds such as $PdCl_2$ in water, involves the use of an excess of Cl^- ions at pH values below 7 to convert the precursor into the stable, square planar $[PdCl_4]^{2-}$ complex. However, when dissolving other water-soluble precursors such as $Pd(NO_3)_2$ and $[Pd(NH_3)_4](NO_3)_2$ in the absence of chloride ions, $[Pd(H_2O)_4]^{2+}$ and $[Pd(NH_3)_4]^{2+}$ are formed, respectively [9,10]. Under strongly basic conditions, the

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prevalent form of all these complexes becomes $[Pd(OH)_4]^{2-}$ [9,11]. The stability of Pd(II) complexes is further supported by thermodynamic data, with the standard Gibbs free energy of formation for $[PdCl_4]^{2-}$ being negative, indicating a spontaneous process, whereas for Pd^{2+} the standard Gibbs free energy of formation is positive [12].

Herein, through a re-evaluation of the studies presented by Ashwin and co-workers, we show that C460 is in fact not a Pd^{2+} chemosensor, but rather a pH sensor that operates over a narrow range. Specifically, we investigate the fluorescence quenching of C460 in the presence of three different Pd(II) precursors within various matrices, as well as conducting a detailed study of the photophysical behaviour C460 as a function of pH. Finally, control experiments are performed to further explore C460-Pd(II) interactions, and the influence of pH on the photophysical properties of C460.

2. Materials and Methods

A stock solution of C460 (10 mм, Acros Organics) was prepared in acetonitrile (Sigma-Aldrich). Stock solutions of $PdCl_2$ (10 mм, abcr) was prepared in both HCl (0.1 м, Sigma-Aldrich) and NaCl (0.1 м, Sigma-Aldrich). Stock solutions of $Pd(NO_3)_2{\cdot}2H_2O$ (2 mм, abcr) and $[Pd(NH_3)_4](NO_3)_2$ (1 mм, abcr) were prepared in high resistivity (>18 MΩ cm^{-1}) ultra-pure water, henceforth referred to as MiliQ water. The salts used for the cation interference study are summarized in Table S2 along with a statement of their source.

UV–visible absorption (excitation slit = 1 nm, emission slit = 1 nm) and fluorescence emission (λ_{ex} = 380 nm, excitation slit = 3 nm, emission slit = 3 nm) measurements were performed at room temperature using a Fluoromax-4 Spectrofluorometer (Horiba Scientific). Samples were contained in 1.5 mL quartz cuvettes (Orient Optical) or polystyrene cuvettes (BrandTech Scientific), for absorbance and fluorescence measurements, respectively.

Samples were prepared by addition of the appropriate volumes of C460 (10 μм – absorbance, 1 μм – fluorescence) and HCl (0–10 mм) or Pd(II) precursor (0–10 μм) to a chosen medium; either MiliQ water or PBS (pH 7.4, ThermoFisher Scientific) (Table S1). The pH of all solutions was measured with a 3505 Bench pH meter (Jenway) and adjusted to the desired values by μL additions of 1 mм HCl or 1 mм NaOH (Sigma-Aldrich).

For experiments involving visual detection, MN 615 filter papers (Macherey-Nagel) were soaked in a 1 mM C460 solution and left to dry over 1 h. Then, 50 μL of the desired solutions (PBS, 10 μм $PdCl_2$/HCl/PBS, 10 mм HCl, or 10 μм $PdCl_2$/HCl) were pipetted on the papers. The papers were then left to dry and illuminated at 302 nm using a MB-16 UltraBright transilluminator (MaestroGen).

^{1}H NMR measurements were performed using a UltraShield 300 MHz NMR spectrometer (Bruker). Samples for NMR analysis were prepared by dissolving 1 mм $PdCl_2$ and 0.5 mм C460 precursors in DMSO-d_6 (Sigma-Aldrich), optionally adding concentrated HCl when required (to a final concentration of 10 mм).

Throughout this manuscript, samples are identified using the following nomenclature: Pd(II) precursor/Solvent1/Solvent2. Solvent1 refers to the medium used for dissolving the Pd(II) precursor, and Solvent2 refers to the measurement medium.

3. Results

Initially, the fluorescence intensities (at 465 nm) of C460 solutions in different matrices where measured as a function of Pd(II) concentration (Fig. 1a, Fig. S4). Contrary to the claim by Ashwin et al. [7], we were unable to dissolve $PdCl_2$ in pure water. To this end, we employed both HCl (0.1 м) and NaCl (0.1 м) as Solvent1 in this study, which allowed dissolution of $PdCl_2$. Inspection of Fig. 1a demonstrates that only two of the investigated samples showed a decrease in C460 fluorescence as a function of HCl or $PdCl_2$ concentration; namely HCl/MiliQ and $PdCl_2$/HCl/MiliQ. Significantly, these are the only two samples in which pH decreases (between 2 and 3.5), due to the addition of HCl. In addition, Stern-Volmer plots for this dataset display similar linear gradients, strongly suggesting that Pd(II) species are not involved in fluorescence quenching (Fig. 1b). Indeed, the other four samples, in which pH does not vary to any appreciable extent, do not exhibit any variation of C460 fluorescence with increasing Pd(II) concentration (Fig. 1a and Fig. S1a), which is in direct contradiction to the data presented by Ashwin and co-workers. Moreover, it is worth noting that for the $PdCl_2$/HCl/PBS sample, the volume of the $PdCl_2$ solution added (into a 3 mL volume) was kept under 30 μL, so as not to exceed the buffering capacity. The absence of quenching in this control experiment indicates that a decrease in pH is indeed responsible for the decrease in fluorescence (Fig. S1b). To further support this analysis, additional experiments were performed using "sensor strips" fabricated on paper. Visual inspection of images of the sensor strips under UV illumination (Fig. S2) are in excellent agreement with the solution phase data, with fluorescence quenching only visible for the HCl/MiliQ and $PdCl_2$/HCl/MiliQ samples. Additionally, the fluorescence emission intensity of a 1 μм C460 solution containing 10 μм of various cations (with oxidation states between +I and + IV) was recorded as an interference study. As expected, only the

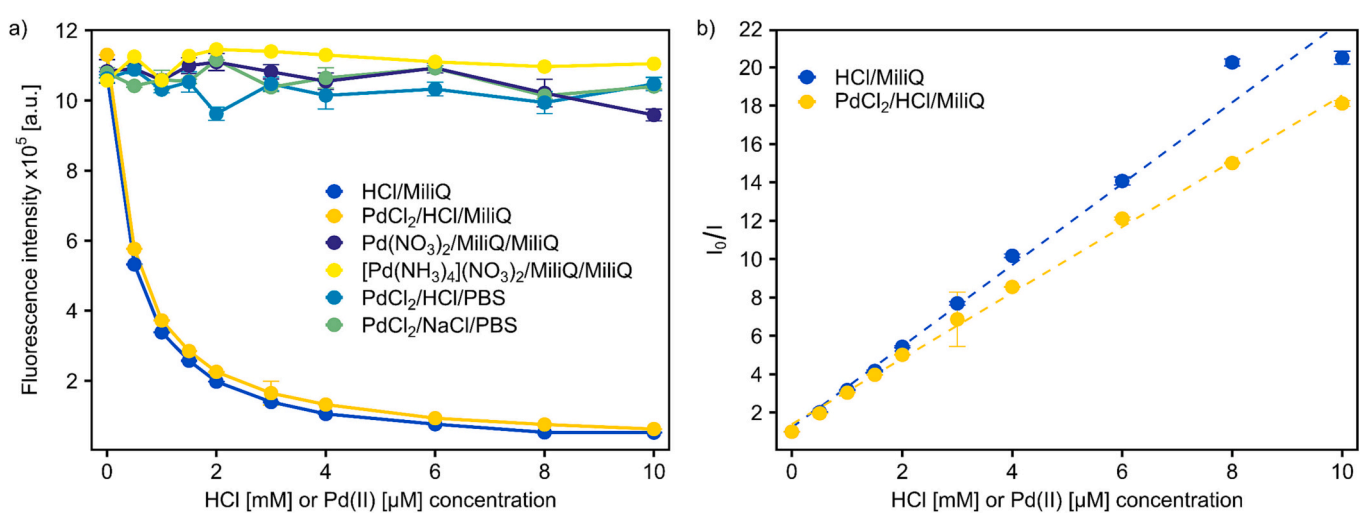

Fig. 1. (a) Variation of fluorescence intensity (at 465 nm) for a 1 μм C460 solution with increasing HCl (0–10 mм) or Pd(II) (0–10 μм) concentrations for various Pd (II) precursors, and in several matrices. (b) Stern-Volmer plots for the two acidic samples (HCl/MiliQ and $PdCl_2$/HCl/MiliQ), highlighting the existence of dynamic fluorescence quenching. Data points represent the average of three repeat measurements, with error bars representing the standard errors of three measurements.

samples containing HCl, used to facilitate dissolution of the salts, induce quenching of C460 (Fig. S5).

To further explore the observed photophysical behaviour of C460, the fluorescence intensity of C460 (1 μM) and C460 (1 μM) in the presence of $PdCl_2$ (10 μM) was measured as a function of pH between 1 and 13 (Fig. 2). It is striking that the variation of fluorescence intensity at 465 nm is almost identical for both samples. It can be seen that the fluorescence intensity remains relatively stable (and large) for pH values between 5 and 11, whereas significant quenching of the fluorescence is observed for pH values below 3 and above 12. In addition, it is interesting to note that fluorescence varies linearly with pH between pH values of 2 and 4; an observation that agrees well with the data presented in Fig. 1b. Importantly, the observed dependence of fluorescence on pH is in accordance with existing literature on related coumarin derivatives [13]. For example, two 7-diethylamino-4-methylcoumarin derivatives show similar pH dependence [14], while another imino-coumarin derivative exhibits decreased fluorescence at basic pH [15]. For completeness, we theorize that protonation of the amine group of C460 restricts ICT (necessary for fluorescence) and thus leads to significantly reduced emission. Indeed, this has been shown to be the case for other closely-related coumarin derivatives [13].

Finally, and in accordance with experiments performed by Ashwin et al., we recorded the ^{1}H NMR spectra of C460 in the presence of HCl, $PdCl_2$, and $PdCl_2$ with HCl (Fig. S3). Inspection of these spectra indicates the presence of a peak at 8.15 ppm for both the C460/HCl and C460/$PdCl_2$/HCl samples. This peak can be unequivocally attributed to the protonated amine group that exists at low pH, as per our hypothesis.

4. Conclusions

Through a re-evaluation of the study presented by Ashwin et al. [7], we have demonstrated that C460 is not a "selective and sensitive fluorescent sensor for Pd^{2+}", but rather acts as a turn-off fluorescence probe of pH that operates (linearly) within a restricted pH range (between 2 and 3.5). The absence of fluorescence intensity variations upon addition of various Pd(II) precursors to neutral C460 solutions confirms that Pd (II) does not influence C460 fluorescence. Additional experiments further confirm that Pd(II) does not molecularly interact with C460. Instead, protonation of the amine group at low pH quenches the fluorescence via inhibition of intramolecular charge transfer. In summary, our work presents a vastly different picture of the sensing capabilities of C460 than that claimed by Ashwin and co-workers. As a final comment, we would like to highlight the importance of providing an accurate and complete description of experimental methods used to generate data in scientific publications. It is not our place to speculate on the reasons for the inaccuracies and omissions in the work presented by Ashwin et al., but hope that our reanalysis of this published study highlights the consequences and repercussions of publishing inaccurate or erroneous findings in the peer-reviewed literature.

CRediT authorship contribution statement

Nika Žurga: Methodology, Investigation, Validation, Visualization, Writing – original draft. **Thomas Moragues:** Conceptualization, Methodology, Investigation, Validation, Visualization, Writing – original draft, Writing – review & editing, Formal analysis, Supervision. **Andrew J. deMello:** Writing – original draft, Writing – review & editing, Formal analysis, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

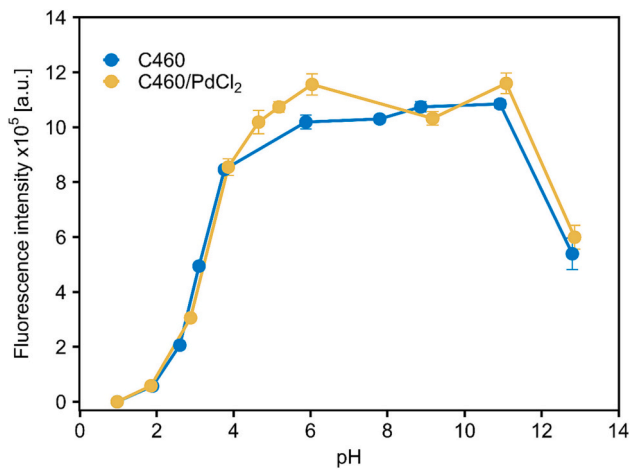

Fig. 2. Variation of the fluorescence intensity (at 465 nm) for a 1 μM solution of C460 in MiliQ water, and 1 μM C460 in MiliQ water in the presence of $PdCl_2$ (10 μM), as a function of pH. Data points represent the average of three repeat measurements, with error bars representing the standard errors of three measurements.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jphotobiol.2023.112783.

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