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Egg-Citing! Isolation of Protoporphyrin IX from Brown Eggshells and Its Detection by Optical Spectroscopy and Chemiluminescence

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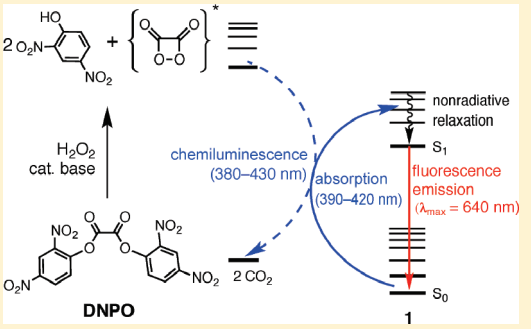
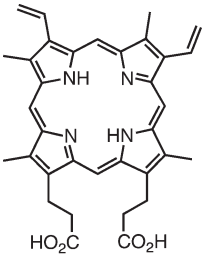
Supporting Information

ABSTRACT: A simple and cost-effective laboratory experiment is described that extracts protoporphyrin IX from brown eggshells. The porphyrin is characterized by UV−vis and fluorescence spectroscopy. A chemiluminescence reaction (peroxyoxalate ester fragmentation) is performed that emits light in the UV region. When the porphyrin extract is added as a fluor to this chemiluminescence reaction, an eye-catching deep red glow is emitted from the solution. Using a familiar object, an egg, the experiment touches upon many aspects in chemistry (natural products isolation, structure and photophysical properties of porphyrins, photochemistry). Focus is placed on the understanding of the optical properties of protoporphyrin that enable the final chemiluminescence experiment. This project is appropriate for upper-level organic chemistry students, but might also serve in the advanced physical chemistry laboratory to facilitate a more detailed discussion of photophysical phenomena. Finally, the chemiluminescence experiment is suitable as an impressive stand-alone demonstration.



KEYWORDS: Upper-Division Undergraduate, Interdisciplinary/Multidisciplinary, Laboratory Instruction, Organic Chemistry, Physical Chemistry, Dyes/Pigments, Fluorescence Spectroscopy, Natural Products, Photochemistry, UV-Vis Spectroscopy

The students' familiarity with eggs makes them an ideal teaching tool. This is particularly true when the familiar becomes a component in an unfamiliar and attention-grabbing chemiluminescent reaction that emits a deep red glow. In fact, it was recently pointed out that chemiluminescence reactions are some of the most exocharmic reactions known.[1,2] This laboratory project, divided into several modules, extracts protoporphyrin IX, **1**, from brown eggshells.



protoporphyrin IX (**1**)

Subsequently, this dye is used to study photophysical phenomena observed via UV−vis absorption, fluorescence emission, and chemiluminescence. The egg pigment extraction and chemiluminescence experiments were adapted from reports by Brandl.[3−6]

Experiments have been described using eggs in topics ranging from chemical kinetics to biological chemistry.[7−12] In addition, the distinctive and readily measured optical spectra of porphyrins and their facile synthesis of tetraarylporphyrins have made them popular study objects.[13−19] The added appeal of using brown eggshells as a naturally occurring porphyrin source lies in the simplicity of the extraction, the low cost, and their availability.[a]

This laboratory exercise is suitable for upper-level organic and physical chemistry courses and was performed with major and nonmajor second-year organic chemistry laboratory courses at a large university. Depending on the learning objectives of the class and the availability of instrumentation, some modules can be simplified, combined in different ways, or skipped altogether. The spectroscopic experiments can also be performed with synthetic or commercially available porphyrins. Modification suggestions are provided in the Supporting Information. The chemiluminescence reaction, when scaled up, is also suitable as an impressive stand-alone demonstration. Instructions and a video demonstration are also provided in the Supporting Information.

INSTRUCTOR INFORMATION

We describe five laboratory modules, A through E, that can be completed in one 3-h laboratory period. Module A extracts the eggshell pigment from brown eggshells, module B identifies the pigment, using UV−vis spectroscopy, as protoporphyrin IX by comparison of the spectrum with published data and quantifies it. Module C records the fluorescence spectrum of

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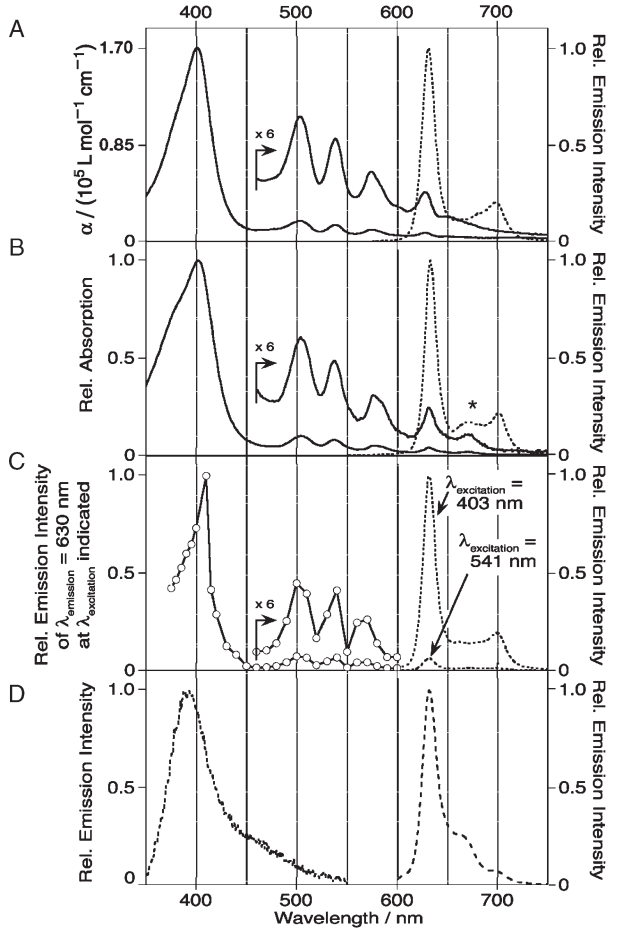

Figure 1. (A) UV−vis absorption (solid trace) and fluorescence (dotted trace) spectra of a commercial sample of protoporphyrin IX in ethyl acetate/MeOH. (B) Normalized UV−vis absorption (solid trace) and fluorescence (dotted trace) spectra ($\lambda_{excitation} = \lambda_{Soret}$) of brown egg extract prepared in Module A (in ethyl acetate/MeOH); asterisk (*) indicates an impurity. (C) Normalized excitation−emission spectrum (solid trace; recorded at $\lambda_{emission}$ = 635 nm; markers indicate the excitation wavelengths used) and emission spectra (dotted traces; $\lambda_{excitation}$ as indicated) of a brown egg extract (in ethyl acetate/MeOH). (D) Normalized chemiluminescence spectrum associated with the fragmentation of the peroxyoxalate esters generated from DNPO (left trace, $\lambda_{max-emission}$ = 393 nm) and normalized chemiluminescence spectrum of this reaction in the presence of protoporphyrin IX (brown eggshell extract in ethyl acetate/MeOH; right trace, $\lambda_{max-emission}$ = 635 nm).

the porphyrin extract. The focus of these experiments is not on the porphyrin per se, but on the photophysical connections between the UV−vis and fluorescence spectra. In module D, the emission spectrum of a well-known oxalate-based chemiluminescence reaction is recorded.[20,21] As the emission maximum falls exactly into the main absorption band of the porphyrin, the emission spectrum of a porphyrin is observed when the chemiluminescence spectrum is, in module E, recorded in the presence of the eggshell extract. This experiment illustrates the relationships between all electronic processes. Finally, the chemiluminescence reaction in the presence of the eggshell extract fluor is performed in a test tube in a dimmed room, yielding a bright red glowing solution, providing a visually appealing finale to the exercise and a "see it to believe it" moment for the students.

Table 1. Spectroscopic Data of Commercial Protoporphyrin IX

UV−vis Absorption Spectrum[a,b] λ_{max}/nm [ε/(L mol^{-1} cm^{-1})]	Fluorescence Spectrum λ_{max-em}/nm (rel. I)	Fluorescence Quantum Yield ϕ_F (%)
(in $CHCl_3$)[b]	(in MeOH)*	(in MeOH)*
407 (171,000),	635 (1.0)	15.5
505 (14,150),	698 (0.2)	
541 (11,600),		
575 (7,440),		
630 (5,380)		

[a] Data from refs 22 and.23. [b] Solvatochromic effects for the porphyrins are below ±5 nm.

STUDENT ACTIVITY AND DISCUSSION

Module A: Extraction of Protoporphyrin IX from Brown Eggshells

The students extract the pigment from the brown eggshells by placing the eggshells in an Erlenmeyer flask with a biphasic mixture of 2 M hydrochloric acid and ethyl acetate. Within 10−20 min at ambient temperature, the majority of the colorant present is extracted into the organic layer, which is filtered, washed, and dried to provide a clear, pale pink solution. Its volume is recorded. This solution is the porphyrin stock solution used in Modules B, C, and E. The extraction step can be replaced by the preparation of solutions using commercially available porphyrins (see the Supporting Information).

This experiment shows that a simple surface etching of the eggshells is sufficient to extract the eggshell pigments into an organic phase. It also demonstrates that the coloring of the eggshells is only found in the outmost layer (cuticle) of an eggshell. Depending on the background of the students, this module is an excellent way of introducing or reinforcing extraction techniques.

Module B: Identification and Quantification of the Eggshell Pigments by UV−Vis Spectroscopy

The students record the UV−vis spectrum of the extract prepared in Module A in the range from 350 to 750 nm. The UV−vis spectrum of the brown eggshell extract (Figure 1B) compares well with the spectrum of a commercial sample of protoporphyrin IX (Figure 1A, Table 1). Thus, this identifies the predominant chromophore present in brown eggshells.[b] Only the slight broadening of the Soret band, the most intense band that occurs at about 400 nm, and an unidentified absorption peak at ~670 nm indicates the presence of minor quantities of one or more colored compounds.

Using the Beer−Lambert law (A_λ = [porphyrin]$\varepsilon_\lambda d$), the students are able to calculate the concentration of the porphyrin in the eggshell extract. Revisiting some topics that the students were exposed to in general chemistry courses, the students can also calculate the absolute quantities of the pigments extracted ([porphyrin]$V_{extract} = n_{porphyrin}$) using the information provided in Figure 1 and Table 1. This number, correlated with the amount of eggs (number of eggs or grams of eggshell), provides the amount (in mol or gram) of pigment per egg or per gram of

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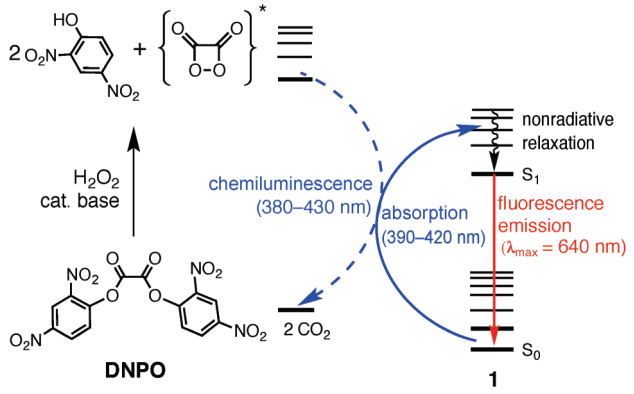

Figure 2. Modified Jablonski diagram showing the relationship between the chemiluminescence reaction and the absorption−fluorescence emission of porphyrin **1**.

eggshell. We typically found that brown eggs contained ~50 nmol protoporphyrin/egg. These values are in accord with values reported in the literature.[24−27] Of course, the amounts of the pigments may vary widely, depending on the intensity of the eggshell coloring. Indeed, even seemingly white eggs may contain traceable amounts of porphyrin.

The students are asked to compare the values they obtained with the values obtained by other students. The surprisingly low (microgram) quantities of porphyrins extracted can still be readily seen with the unaided eye, confirming the high extinction coefficient of porphyrins and the sensitivity of our eyes (and UV−vis spectrometers).

Module C: The Fluorescence Spectrum of Protoporphyrin IX

In this module, the students record the fluorescence spectrum of the eggshell extract. Figure 1B shows the typical two-band emission spectrum of porphyrins. The spectrum compares well with the corresponding spectrum of protoporphyrin IX (Figure 1A), thus, further identifying the primary constituent of the extract.

As part of this exercise, the relationship of the UV−vis spectrum and the fluorescence spectrum is highlighted. The wavelength of the shortest wavelength emission can be compared with that of the longest absorption wavelength. This identifies the Stoke shift of the emission, that is, the minimal quantity of energy that is lost in the excitation−emission process through nonradiative processes (Figure 2).[28] The emission spectra recorded at three different excitation wavelengths can be recorded and compared. Their similarity highlights that the peak position of the emission spectrum is independent of the excitation wavelengths. The emission intensity of the band at 635 nm can be plotted against a number of excitation wavelengths to produce an excitation−emission spectrum (Figure 1C) that is compared to the UV−vis spectrum of the porphyrin (Figure 1A or 1B). The exercise demonstrates to the students that the emission spectra remain the same regardless of excitation wavelength. This further reinforces the understanding of the relationship between absorption, excitation, and emission spectra.

Module D: Chemiluminescence Spectrum of DNPO/H_2O_2

The chemiluminescence spectrum of the reaction of dinitrophenyloxalate (DNPO) with hydrogen peroxide was obtained using the bio/chemiluminescence feature of our fluorometer. The spectrum recorded is shown in Figure 1D. The light given off is in the UV region ($\lambda_{max\text{-}emission}$ = 393 nm) and, thus, invisible to the unaided eye. Inspection of the UV−vis spectrum of **1** (Figure 1B) reveals that the wavelength of emission of this chemiluminescence reaction is ideally suited to be absorbed by a porphyrin, such as **1**. This should prompt the students to surmise that if the chemiluminescence reaction takes place in the presence of a porphyrin, the light emitted could be absorbed and, in turn, again emitted as a porphyrin-like fluorescence (Module C). This hypothesis is then tested in Module E. The coupling of the two reactions through energy transfer is illustrated by a modified Jablonski diagram (Figure 2).

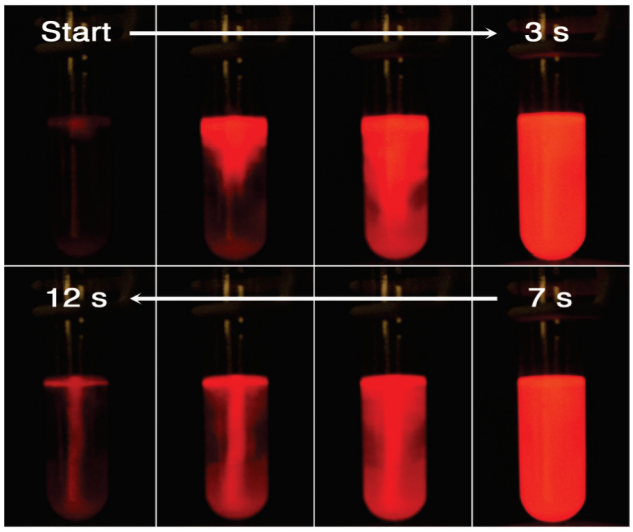

Figure 3. Time elapsed images of the chemiluminescence reaction described in Module E (stirred magnetically, and in the presence of a KOH pellet that intensifies and speeds up the light emission).

Module E: Chemiluminescence in the Presence of the Brown Egg Extract

The chemiluminescence reaction[3−6] performed in Module D is repeated in the presence of the brown egg porphyrin extract. The spectrum recorded is shown in Figure 1D. Comparison of this spectrum with that recorded for the porphyrin extract emission (Figure 1B) shows that they are identical. No sign for any emission native to the chemiluminescence reaction is seen, supporting the energy-transfer mechanism. All the components of Figure 2 should now be clear to the students.

In the finale of this experiment, the chemiluminescence reaction described above can be repeated, on a larger scale in a test tube and in a (partially) darkened room. A red glow visible to the unaided eye is observed. Depending on the particular mixing times and porphyrin concentrations in the extract, the glow may be quite intense and last up to 20 s (Figure 3 or see video in the Supporting Information). Scaled up, the red chemiluminescence emitted from this reaction also makes an impressive lecture hall demonstration.

Chemiluminescence reactions in the presence of a fluorophore can be related to a number of real-life situations. Among them are the popular glow sticks[29] and bioluminescence as observed in fireflies or glowing plankton.[30] This experiment is not only beautiful, but the generation of a deep red glow emanating from essentially colorless solutions holds some magic even for seasoned chemists.

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HAZARDS

Care must be taken and suitable eye protection must be worn when working with strong acids (2 M aqueous hydrochloric acid), bases (sodium hydroxide pellets) or flammable solvents (ethyl acetate). Both hydrochloric acid and sodium hydroxide are corrosive. Bis(2,4-dinitrophenyl)oxalate (DNPO) is a possible eye and skin irritant. Aqueous 30% hydrogen peroxide is a strong oxidant. Because of the chemicals used, the use of gloves is advised. The extraction procedures require a well-ventilated fume hood and waste containers for the organic solvents must be made available.

EDUCATIONAL ASPECTS

The study of eggshell pigmentation connects chemistry to several topics discussed in biology courses. Since a majority of the students taking laboratory chemistry classes are not chemistry majors, creating this link to other subjects promotes scientific interdisciplinary perspectives and relevance. Topics in this lab include chemical composition of eggs (organic chemistry and biology) and photophysical phenomena (physical chemistry). In addition, this series of experiments showcases porphyrins whereby the chemistry, structure, and physical properties can be discussed with a chemical or biological point of view.

When considering implementation, the desired learning objectives, student demographics, and prior background knowledge will dictate the modules to be employed. Whereas most students reviewed the lab positively (on chemiluminescence reaction performed in Module E, familiarity with brown eggs, learning new instrumentation), many of the topics covered are introduced superficially in organic chemistry and are not discussed in *great detail* until a physical chemistry course (absorption, fluorescence spectroscopy, chemiluminescence) when the students can fully comprehend what is occurring at the molecular level.

Results from pre- and post-test assessments revealed that detailed explanations of how to apply and interpret the spectra are required. Although the students could define absorption, fluorescence spectroscopy, and chemiluminescence in multiple-choice questions, they were unable to apply these concepts to spectral interpretation or explanation of the observed bright red glow in free-response questions. Because of time and instrument availability restrictions, part of Module C was eliminated, which included collection of an emission spectrum at various excitation wavelengths that would demonstrate (i) the connection between the absorption and emission spectra and (ii) only one emission spectrum exists (with varied relative intensities). Inclusion of this module would likely have improved the students' ability make connections among the spectra that were collected.

This laboratory experiment highlights how the idea of a reaction mechanism extends beyond the conventional breaking and mending of chemical bonds to the analysis of the movement of electrons in a photophysical mechanism. The students' inability to use the information collected to explain the glow could have also resulted from their inability to visualize photophysical phenomena from this mechanistic perspective. It has previously been shown that students have difficultly explaining traditional organic reaction mechanisms when the focus is on functional group transformations.[31] There are two uses of the term "reaction mechanism", one relating to the more commonly understood functional group transformations and the other to photophysical transformations. Because this experiment does not involve functional group transformations in the porphyrin structure, explicit discussions about photophysical reaction mechanisms are needed for organic chemistry students to acquire a broader understanding of the term "reaction mechanism" in general.

ASSOCIATED CONTENT

Supporting Information

Detailed experimental procedures; modification suggestions; demonstration instructions; a video demonstration. This material is available via the Internet at http://pubs.acs.org.

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ADDITIONAL NOTE

[a] All sources that traditionally were used to extract protoporphyrin, feces or blood, are generally unsuitable in a classroom setting. Synthetic porphyrins, such as *meso*-tetraphenylporphyrin (TPP), are readily synthesized. Solutions of TPP are also suitable in this project; details are provided in the Supporting Information.

[b] Because of the UV−vis spectroscopic similarities of the different naturally occurring porphyrins, their differentiation is difficult.[22]

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