LIBERTY UNIVERSITY

The Quest for the Ideal Fluorescent Cell Stain

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Abstract

As the field of fluorescence becomes more mainstream as an analytical technique in the medical field, more research is needed on discovering and creating improved fluorescent dyes (fluorophores). Some of these characteristics have to do with the inherent fluorescent properties of the dye, such as:

- (1) a **Stokes shift** of 100nm or larger (the difference between the electron's excitation wavelength and the emission wavelength),
- (2) **emission** in the near-infrared region (depending on the Stokes shift and bandgap energies E_g)
- (3) high **quantum yield** (the ratio of photons emitted from the total absorbed to give a bright signal),
- (4) high **photostability** (the stability upon light exposure)

whereas other properties include the interaction of the fluorophore with biological entities, such as cells; these properties include:

- (5) **solubility** in biological media
- (6) **staining** characteristics (live/dead cells; organelles)
- (7) other **medicinal** properties (cytotoxicity, antimicrobial, anticancer)

and finally:

- (8) low **production costs**.

The aromatic **anthraquinone core (Fig. 1)** is the structural building block of several commercially available fluorophores, including DRAQ5, DRAQ7, CyTRAK Orange, and doxorubicin.

This research aims at comparing and contrasting some known anthraquinone-based and other fluorophores (**Fig.1**) with new fluorophore developments in our lab.

Introduction and Research Question

This research seeks to identify and utilize the fundamental characteristics of anthraquinone fluorescent dyes and a selected few other ones to create a dye that optimizes the criteria mentioned above resulting in the ideal fluorophore for biomedical applications. Some of our own new fluorophores will be presented as well. Aiding in the development of the ideal fluorophore include results from computational analyses.

Methods

New anthraquinone-based compounds were synthesized in our lab by placing different substituents or functional groups R on the anthraquinone core **1 (Fig.1)**. These synthesized compounds were analyzed by thin-layer chromatography, infrared spectroscopy, melting point analysis, liquid chromatography-mass spectrometry and high-performance liquid chromatography for identity and purity. Fluorimetry was utilized to determine the fluorescent properties (Stokes shift, excitation and emission spectra). Selected few were tested for antimicrobial activity against *E. coli*.

Fluorescence microscopy was utilized to determine the ability of fluorophores to interact with and stain live buccal cells. Some information (price) and experimental values for reference compounds were found through published sources.

Gaussian 16W was implemented to calculate the HOMO and LUMO energies of selected compounds and to obtain the optimized structure for every compound. Avogadro was utilized to visualize the Gaussian output files. The Gaussian route section was as follows: #n B3LYP/6-31G(d) opt freq.

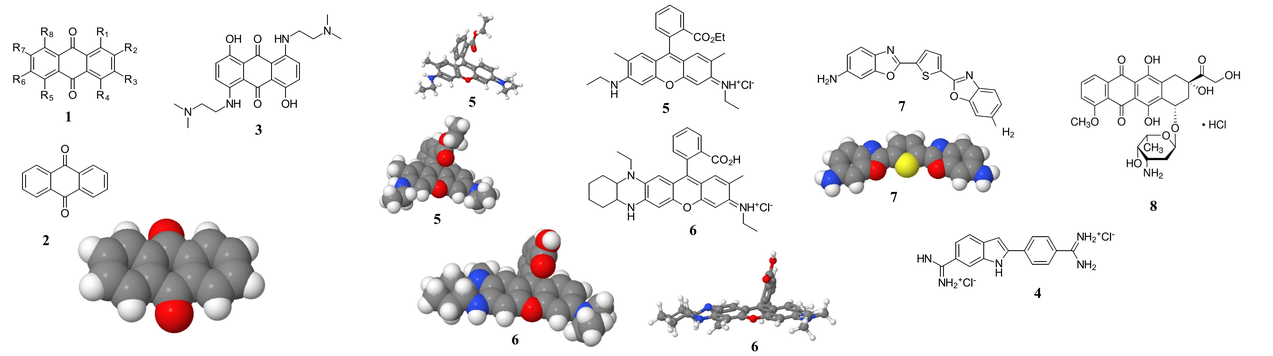


Figure 1. Structure of the anthraquinone core **1** with respective R-group positions [6]; anthraquinone **2**; DRAQ5 **3**; DAPI **4**; Rhodamine 6G **5**; DQF 570 **6**; BBTA **7**; doxorubicin **8** [8].; and respective 3-D structures for selected compounds (generate.

1. Stokes shift

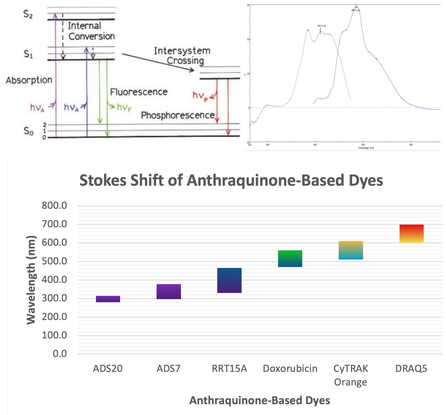


Figure 2. Jablonski diagram shows the process of light absorption and emission in fluorescence [23] (**B**) anthraquinone (λex = 400nm; λem = 484.5nm) [6]; (**C**) summary chart [6], (**D**) electromagnetic color spectrum [24].

3. Quantum yield

$$\Phi_X = \Phi_{ST}\left(\frac{Grad_X}{Grad_{ST}}\right)\left(\frac{\eta_X^2}{\eta_{ST}^2}\right) \quad [9]$$

Fluorophore	Quantum Yield	
DRAQ5	3.00 x 10^{-2}	[10]
DAPI	0.035	[21]
BBTA	0.11	[20]
Doxorubicin	3.9 x 10^{-2}	[11]
Rhodamine 6G	0.94	[19]
DQF-570	0.31	[16]

4. Photostability

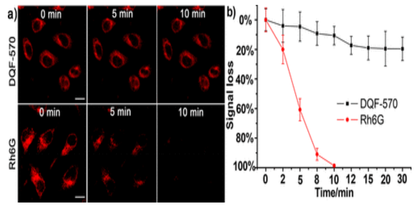
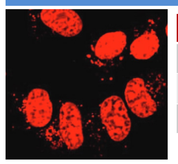


Figure 6. Images from a confocal microscope demonstrating photobleaching comparing compounds **6** and **5** from above [16]

2. NIR Emission and Band Gap Energies



Compound	HOMO	LUMO	Eg, eV	Absorption, nm
Anthraquinone	-0.25714	-0.10263	4.204435	294.89 nm
BBTA	-0.18306	-0.07048	3.063460	404.72 nm
DRAQ 5	-0.17380	-0.07971	2.560321	484.25 nm

Figure 8. (a) Confocal microscopy of DRAQ5 stained-nuclei in HeLa cells [18]; (b) calculated band gap energies. HOMO and LUMO orbital energies are in Hartrees..

7. Medicinal properties

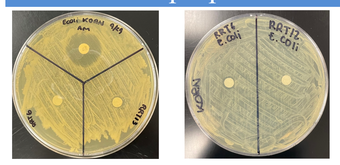


Figure 7. Images of antibiotic disk diffusion tests with ampicillin, RRT6, RRT12, and RRT13

Fluorophore	Cytotoxicity
DRAQ5	High cytotoxicity
BBTA	Low cytotoxicity
DQF-570	Low cytotoxicity
DRAQ7	High cytotoxicity
Doxorubicin	High cytotoxicity and cytostatic (anticancer)

6. Staining Characteristics

Fluorophore	Staining Capability
DRAQ5	Live or fixed cells
BBTA	Live cells
DQF-570	Live cells; mitochondria
Rhodamine 6G	Live cells [22]
DRAQ7	Dead cells [13]

5. Solubility

Fluorophore	Water Solubility
DRAQ5	Completely soluble
DAPI	Completely soluble
BBTA	Partially soluble but enhanced solubility with PEG550 [20]
DRAQ7	Completely soluble
Doxorubicin	Completely soluble

8. Cost

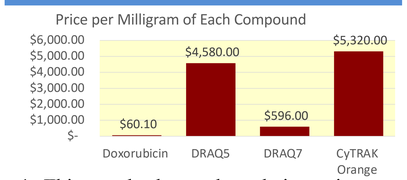


Figure 4. This graph shows the relative price per milligram of each commercial dye [12, 13, 14, 15].

Results and Conclusion

1. A significant shift of the excitation wavelength towards red can be accomplished by decorating the anthraquinone core with electron-donating groups, such as O and N, as seen in **3**.
2. The Stokes shift can be widened by increasing energy loss through the introduction of flexible groups (**6**) or by extending the conjugation (**7**).
3. Some fluorophores (**7**) exhibit a Stokes shift of 186 nm, compared to DRAQ5, CyTrak Orange, and doxorubicin which have Stokes shifts of 98, 100, and 90 nm, respectively.
4. Photostability of commercial dyes have been determined from literature data and referenced for analysis of the photostability for the development of novel anthraquinone-based dyes.
5. Low quantum yield was identified in various commercial dyes, i.e., DRAQ5 and doxorubicin, from literature data. Low quantum yields indicates a low ratio of photons emitted to photons absorbed. Thus, more photons are absorbed and translated into nonradioactive decay [10].
6. Manufacturer data was utilized to represent the prices of each commercial dye, indicating relatively higher production costs.

Future Work

1. Analyze some of the commercial fluorophores for benchmarking.
2. Determine quantum yields on our fluorophores.
3. Complete cytotoxic tests using antibiotic and antimicrobial plating methods for selected fluorophores.
4. Investigate how the degree of symmetry affects Stokes shift.
5. Compute more complex data from compounds using both the Gaussian 16W and Spartan 16 software.
6. Utilize the confocal microscope at LUCOM to image cells stained with the synthesized dyes to identify what structures/organelles are being stained.

References and Acknowledgments

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We thank Drs Winter, Rockabrand, and Passburg for their advice and any materials they provided us. We thank the Center for Research and Scholarship (CRS) at Liberty University for their financial support of this project.