

Investigating the Structure-Property Relationships of Croconaine Dyes

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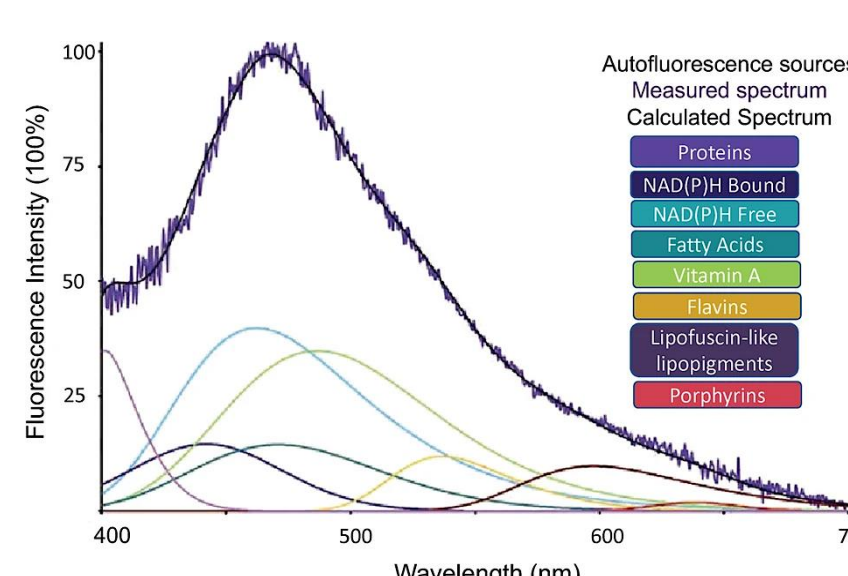
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Near-infrared polymethine dyes have become useful imaging agents to help cells and tissues visualization at the molecular level. Cyanines, a class of polymethine dyes with an odd-numbered polyene chain connecting two-nitrogen containing heterocycles, are particularly noteworthy. Within this family of dyes, squaraines and croconaines are particularly intriguing due to their absorption and emission maxima located in the near-infrared window (650–1350 nm). In biological systems, the presence of endogenous chromophores and optical scattering limit the depth of light penetration into tissues.

Nonetheless, using light in the spectral range of the first near-infrared window (NIR-I; 600–950 nm), where these effects are minimized, ensures a broad scope of biological applications, including fluorescence-based imaging. So far, only one dye – Indocyanine Green – possessing absorption and emission in the NIR-I window, has been approved by both FDA and EMA for several clinical applications. Croconaines, a sub-group of polymethine dyes, are donor–acceptor–donor (D-A-D) type zwitterionic compounds with extended π -conjugation, exhibiting narrow and intense absorption in the NIR-I region with high molar absorption coefficient ($\epsilon \sim 10^5 \text{ mol}^{-1} \text{ dm}^3 \text{ cm}^{-1}$).¹ Their absorption and emission wavelengths in the NIR-I region make croconaines promising fluorescent probes for fluorescence-based imaging. As little is known about these cousins of squaraines, we aimed at elucidating the structure-property relationship of a small library of semicroconaine and croconaine dyes. By introducing various functional groups to the indolenine ring we aimed at determining their potential influence on the photophysical, acid-base and aggregation properties.

Steady-state spectroscopy and femtosecond transient absorption spectroscopy are techniques employed for the investigation of the ground and excited-state dynamics of this family of dyes.

Figure 1. Absorption of biological molecules in Vis-IR region and the tissue transparent window (TTW; 650–900 nm).



Femtosecond time-resolved spectroscopy is a powerful technique which can be employed to investigate the excited-state dynamics of many fluorescent dyes. We used this technique to decipher the de-excitation pathways of semicroconaines (e.g. proton transfer, fluorescence, rotational isomers). Since one of the suggested major de-excitation pathways involves rotation around the croconic acid as a key step, we tested various solvent mixtures with different viscosity to test this hypothesis.

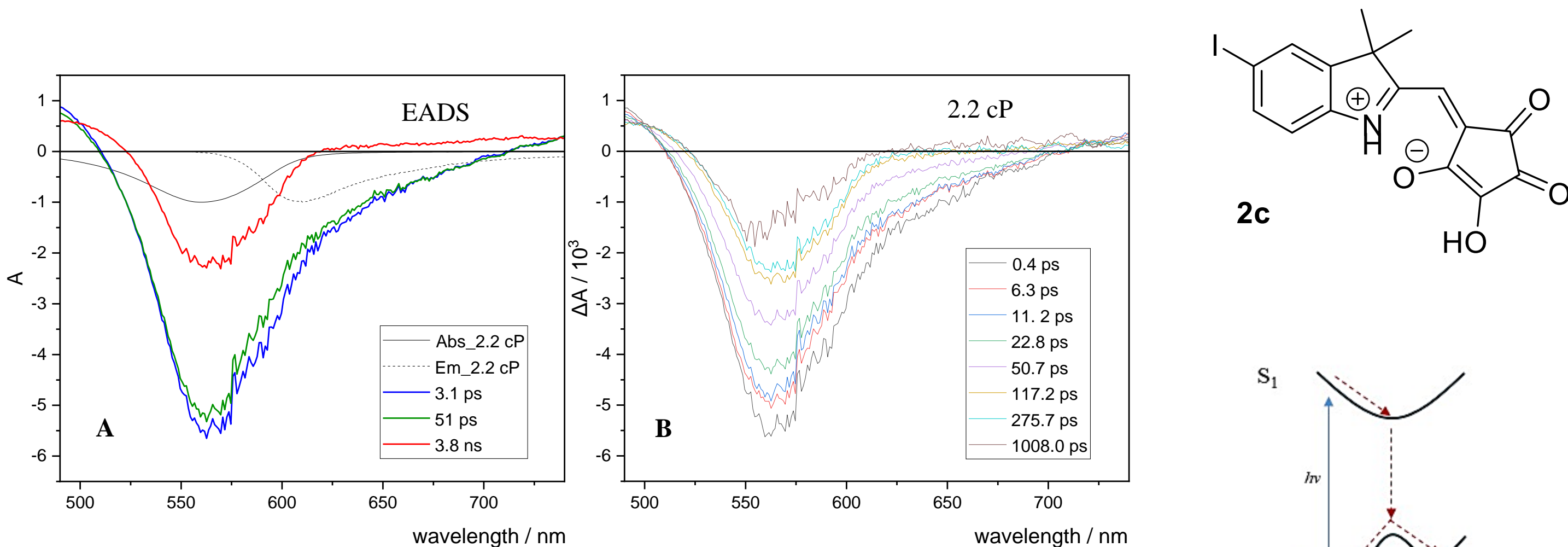


Figure 5. Transient-absorption experiment performed in a mixture of DMSO/Glycerol 2.2 cP. A) Evolution-associated decay spectrum and B) decay spectra of semicroconaine 2c.

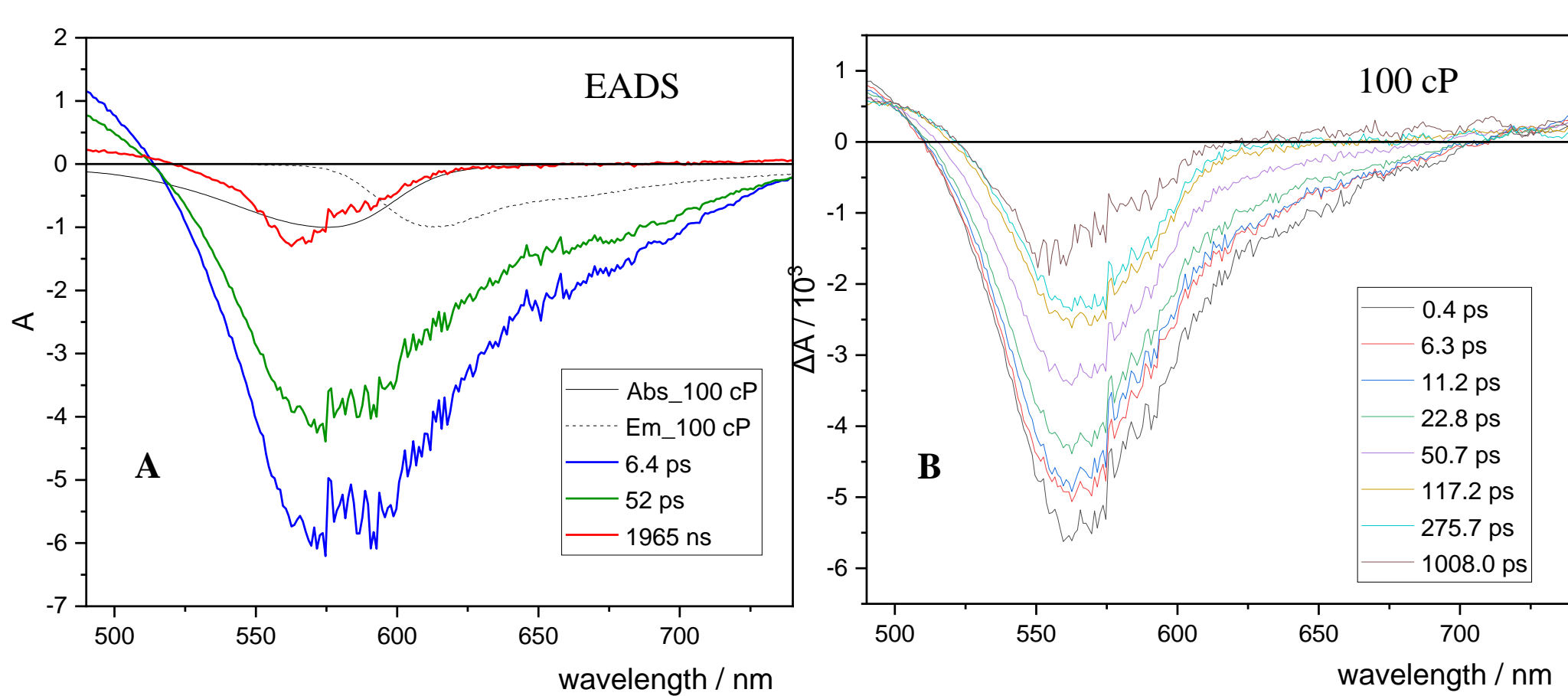


Figure 6. Transient-absorption experiment performed in a mixture of DMSO/Glycerol 100 cP. A) Evolution-associated decay spectrum and B) decay spectra of semicroconaine 2c.

Croconaine 3 and semicroconaine dyes 2 show acid-base behavior (Figure 4). The acid-base equilibrium of the dyes consists in a protonation-deprotonation of the acidic nitrogen (Scheme 2).³ Furthermore, croconaines are poorly soluble in water, which facilitates the formation of aggregates (Figure 3).

Scheme 2. Schematic representation of the existing relationship between aggregates, monomers, zwitterionic and monoanionic forms.

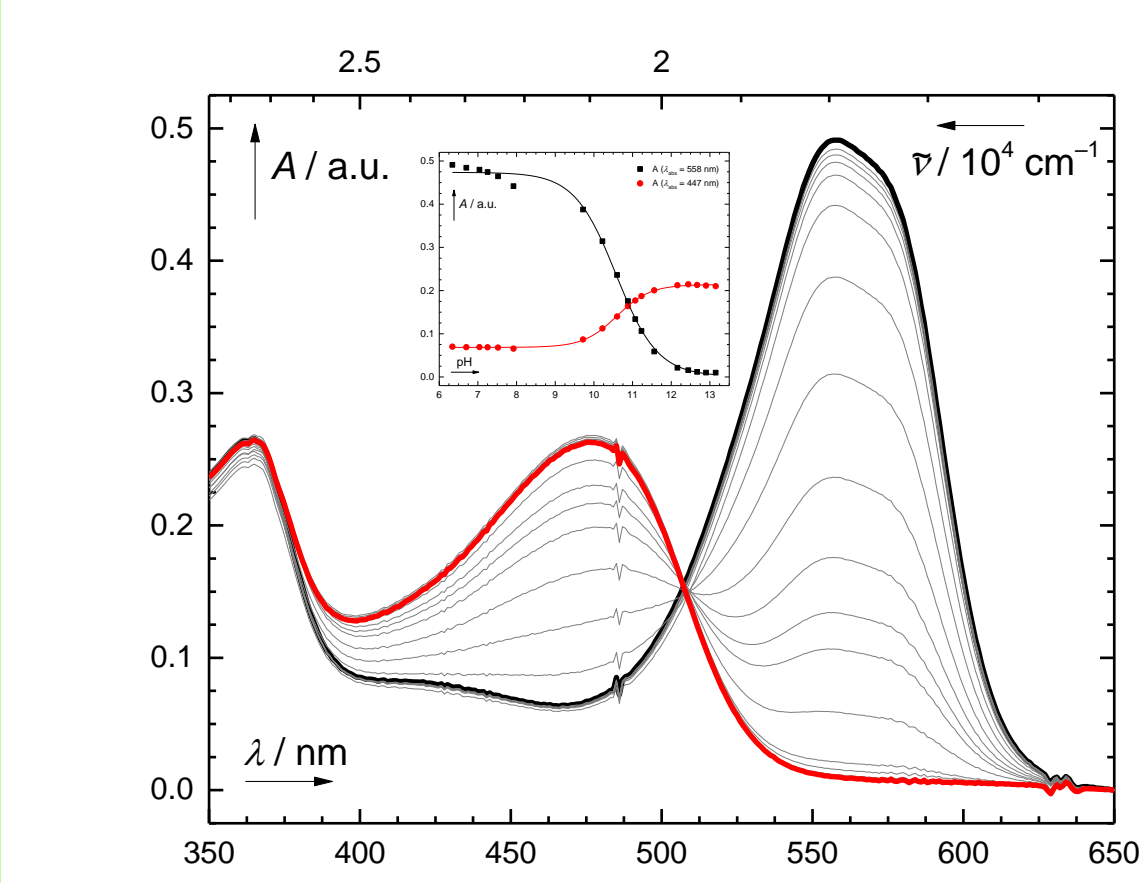
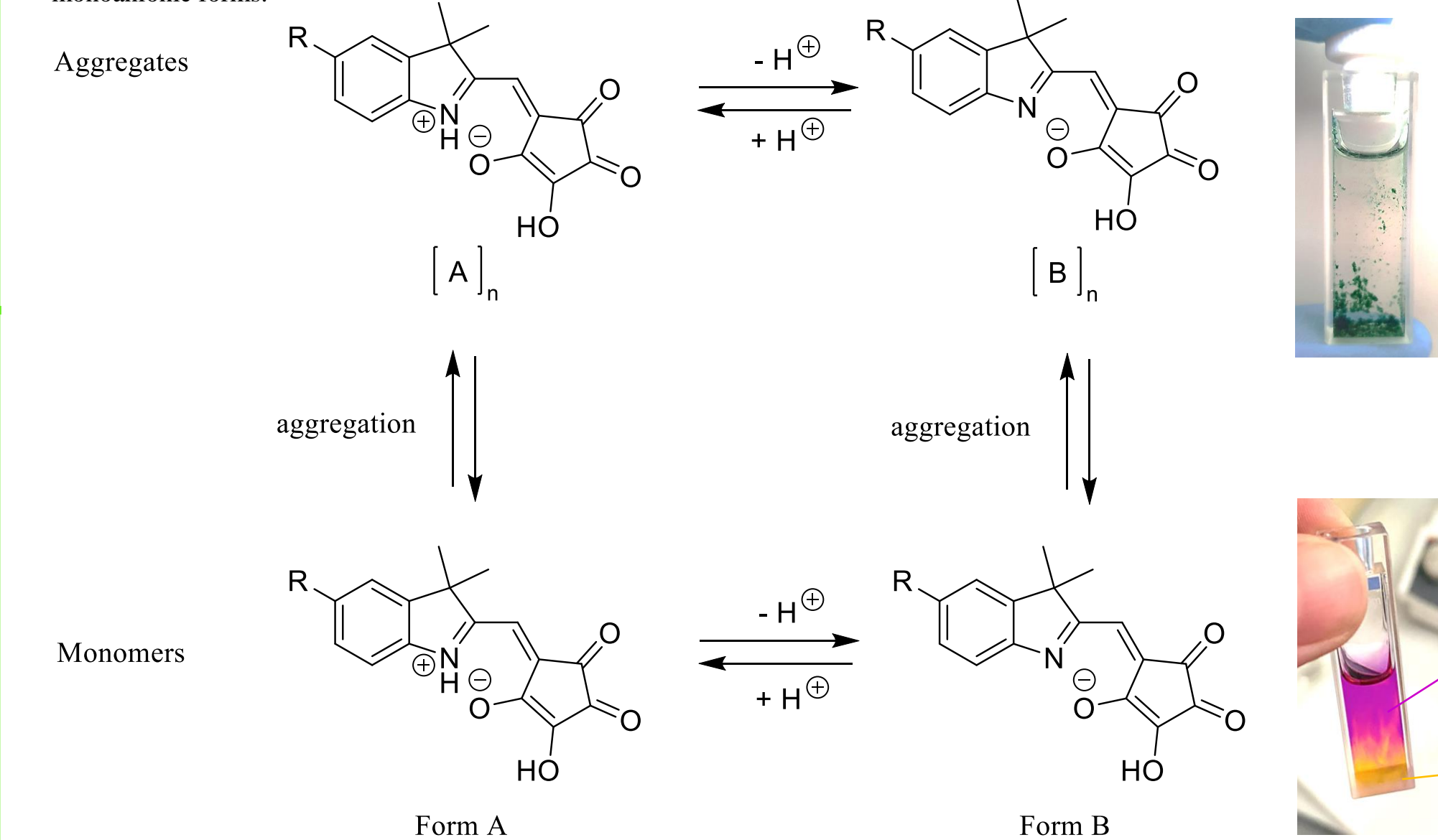


Figure 4. A pH dependence of UV-Vis absorption spectrum in a 0.1 M KCl solution (with 10% DMSO as co-solvent) of semicroconaine 2c.

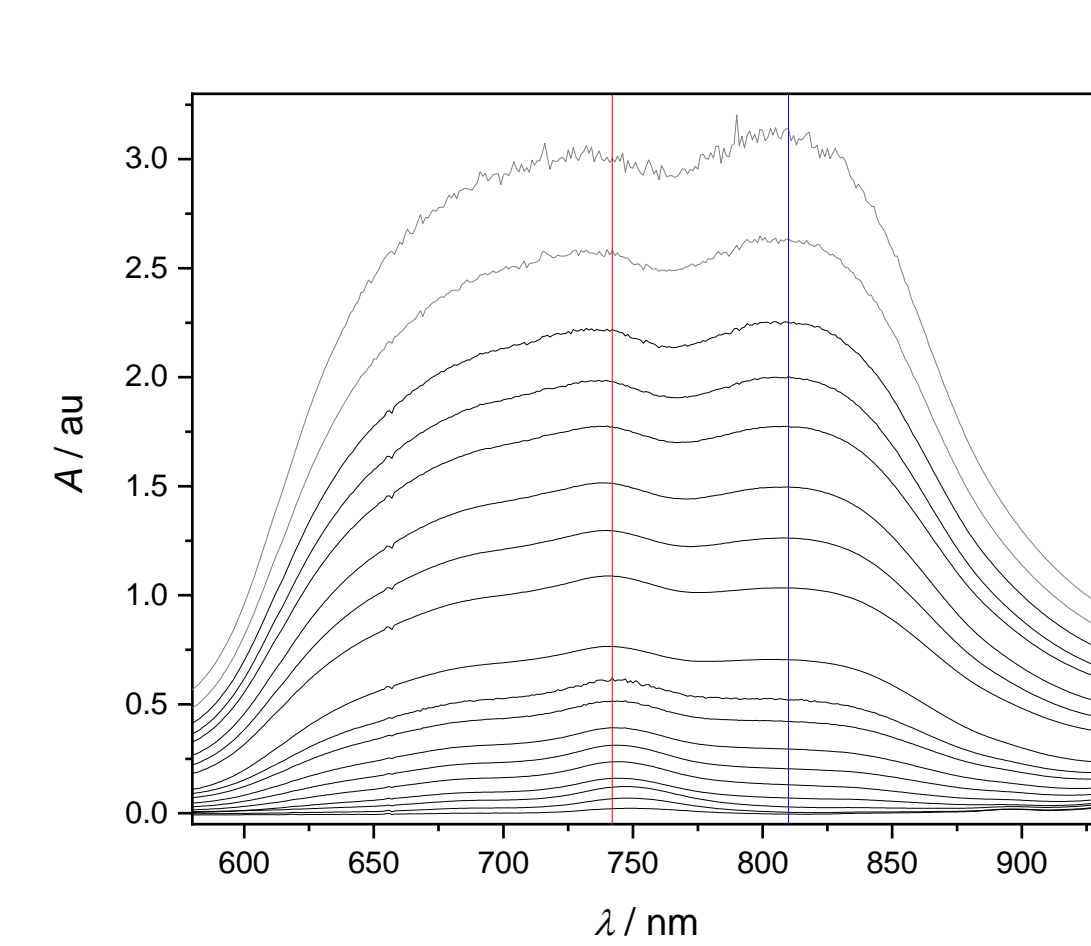
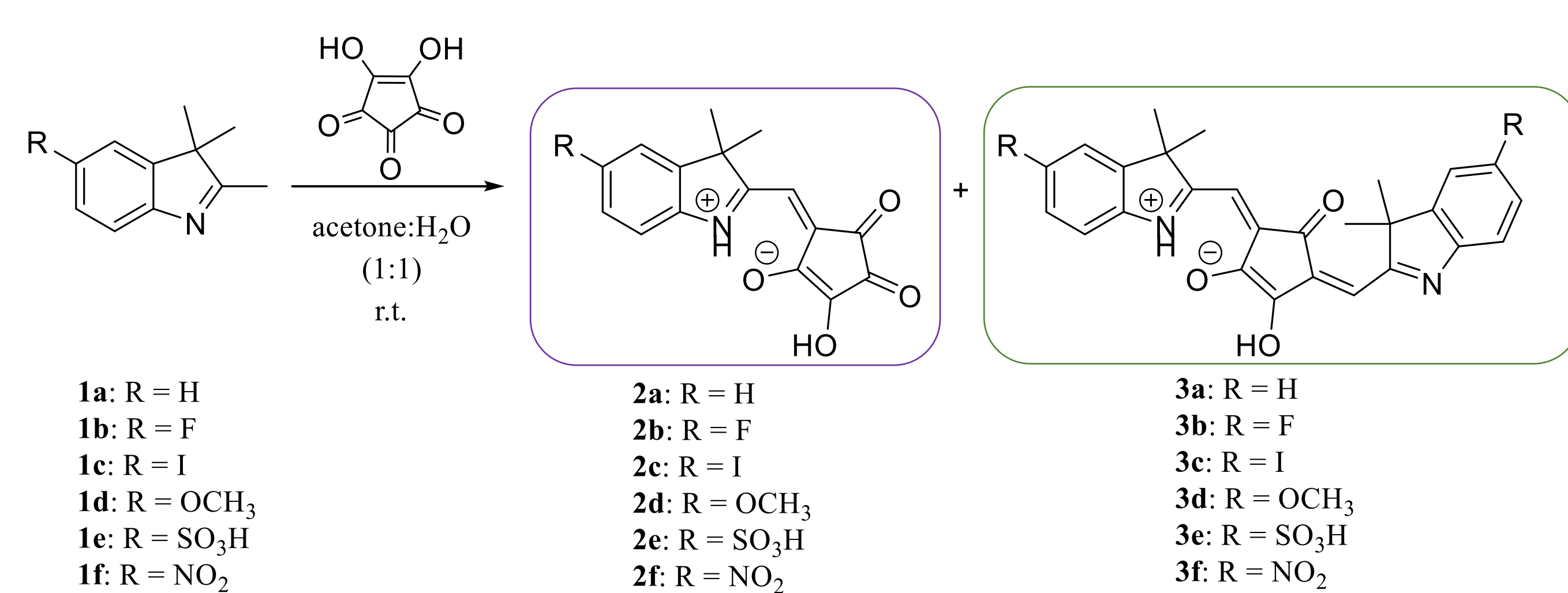


Figure 3. Aggregation data obtained in PBS (with 10% DMSO as co-solvent) for croconaine 3a.

Croconaines and semicroconaines are synthesized in a one-pot condensation reaction of croconic acid (1.0 eq) and an indolenine (2.0 eq) moiety (Scheme 1).⁴ To examine the influence of different functional groups on the photophysical properties of the dyes, we incorporated indolenine end-groups with electron-accepting (e.g., F, NO₂) and donating (e.g., OCH₃) groups, and iodine, which is known to promote intersystem crossing, and sulfonate to improve the water solubility.



Scheme 1. Synthesis of hemi-croconaines (2a-e) and croconaines (3a-e).

IV. Time-resolved spectroscopy

I. Synthesis

III. Acid-Base/Aggregation Properties

II. Steady-state spectroscopy

The characterization of our croconaine 3 and semicroconaine 2 dyes involves determination of photophysical properties. Steady-state measurements e.g., absorption and emission spectra, fluorescence emission quantum yields were performed in methanol (Table 1) and PBS with 10% DMSO as co-solvent (Table 2).

Table 1. Photophysical properties of semicroconaines (2) and croconaines (3) measured in methanol.

Cmpd	$\lambda_{\text{abs}}^{\text{max}}$ (nm)	$\log \epsilon_{\text{max}}$	$\lambda_{\text{em}}^{\text{max}}$ (nm)	ν (cm ⁻¹)	Φ_{f}	$\epsilon_{\text{max}} \Phi_{\text{f}}$
2a	567	4.52	607	1162	0.00013±0.0001	4.3
2c	565	4.62	614	1412	0.0004±0.0001	17
2d	571	3.18	649	2105	n.d.	n.d.
2e	565	4.69	607	1225	0.0009±0.0001	44
3a	755	5.01	878	538	0.0027±0.0002	276
3b	756	4.88	794	633	0.0023±0.0002	174
3c	773	4.23	803	483	0.0024±0.0002	41
3d	792	4.88	828	549	n.d.	n.d.
3e	766	4.66	799	539	0.0037±0.0001	169

Table 2. Photophysical properties of semicroconaines (2) and croconaines (3) measured in PBS (with 10% (v/v) DMSO as co-solvent).

Cmpd	Form*	$\lambda_{\text{abs}}^{\text{max}}$ (nm)	$\log \epsilon_{\text{max}}$	$\lambda_{\text{em}}^{\text{max}}$ (nm)	ν (cm ⁻¹)	pK _a
2a	A	549	4.12	704	1470	11.02
	B	465	3.88	538	2826	
2c	A	558	4.15	625	1412	10.74
2d	A	560	3.21	682	3194	n.d.
2e	A	566	4.80	603	1084	n.d.
3a	A	743	4.06	786	809	9.7 ²
3b	A	743	4.63	770	611	10.15
3c	A	733	n.d.	784	887	10.38
3d	A	740	n.d.	795	935	9.6 ³
3e	A	752	4.71	7.88	607	8.35
	B	641	4.02	7.23	2323	

*The zwitterionic form is indicated with A, while the monoanionic form with B.

Croconaine 3 and semicroconaine 2 dyes exist in different isomers, which are formed by the rotation of the methine bridge around the croconic acid. To assess the effect of such rotation on the fluorescence intensity we employed a mixture of glycerol/DMSO with increasing viscosity (Figure 2 and Table 3). Increasing the viscosity of the solvent mixture is expected to improve the fluorescence intensity.

Table 3. Variation of the fluorescence quantum yield of semicroconaine 2c as function of viscosity.

Cmpd	Solvent mixture	$\lambda_{\text{abs}}^{\text{max}}$ (nm)	$\lambda_{\text{em}}^{\text{max}}$ (nm)	Φ_{f}
2c	2.2	559	604	0.332%
	6.0	564	605	0.386%
	20	569	606	0.402%
	50	572	607	0.462%
	100	574	606	0.518%

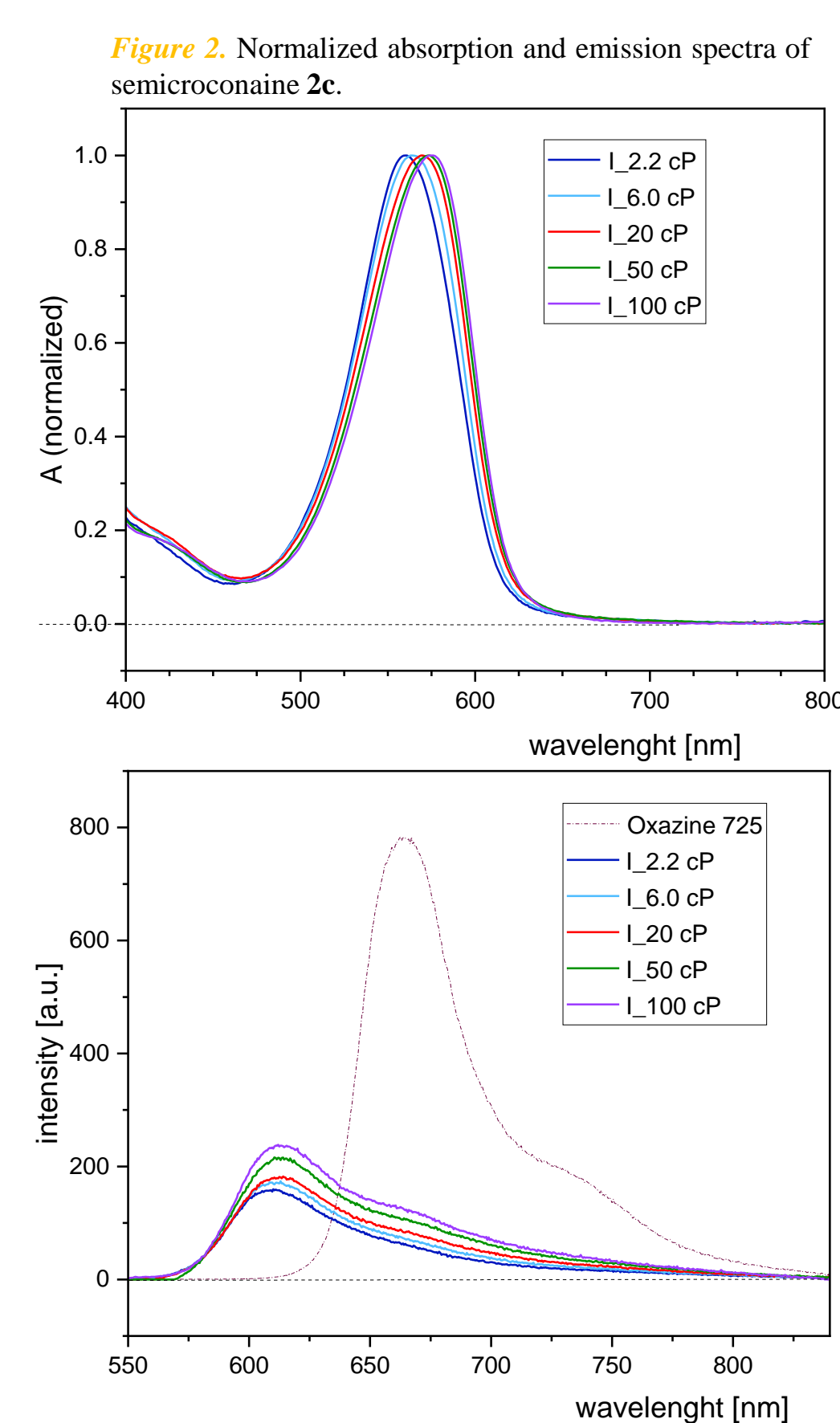


Figure 2. Normalized absorption and emission spectra of semicroconaine 2c.

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Conclusion and Future Work

In this work we proved that the flexibility of semicroconaine dyes could be the cause of the low fluorescence quantum yield, leading to de-excitation through the rotation of the donor moiety around the croconic acid core. We proved our hypothesis by a careful selection of solvent mixtures with increasing viscosity and taking advantage of steady-state, transient absorption and fluorescence upconversion spectroscopy. Additional experiments prove the existence of an acid-base equilibrium between the zwitterionic and monoanionic semicroconaines and the formation of aggregates, especially for the croconaine dyes.