TOWARD THE DEVELOPMENT OF NEW SWIR-EMITTERS FOR OPTICAL IMAGING: DESIGN, SYNTHESIS AND OPTICAL CHARACTERIZATION

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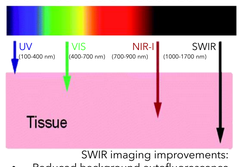
BACKGROUND

Optical imaging, an emerging imaging technique based on the use of fluorescent probes, enables non-invasive real-time diagnosis of various diseases and allows for therapeutic purposes such as **fluorescence guided surgery** (FGS).

Probes able to emit at wavelengths included in the Short-Wave Infrared Region (1000-1700 nm), also named **SWIR** or NIR-II, are advantageous compared to dyes emitting in the visible (400-700 nm) or in the NIR-I (650-900 nm) spectral region thanks to improved image quality and higher resolution.[1]

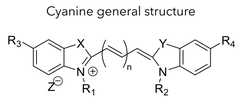


SWIR imaging improvements:
- Reduced background autofluorescence
- Reduced tissue scattering
- Increased penetration depth

The most attractive fluorophores used for optical imaging are organic dyes such as cyanines, due to their good optical properties and safety profile.

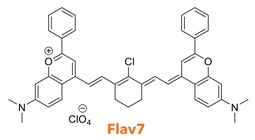
The main limitations of these probes consist in the *poor water solubility* and the tendency to *form non-emissive aggregates* in polar biological environments, thus limiting their use in vivo.[2]

Cyanine general structure



Among the SWIR emitters with a cyanine-like scaffold, **Flav7** was synthesized in 2017 and showed interesting optical properties, with an emission beyond 1000 nm in organic solvents.[3]

However, **Flav7** suffers from high lipophilicity, low solubility in polar media and the tendency to form non-emissive aggregates in these environments, limiting its in vivo applicability.

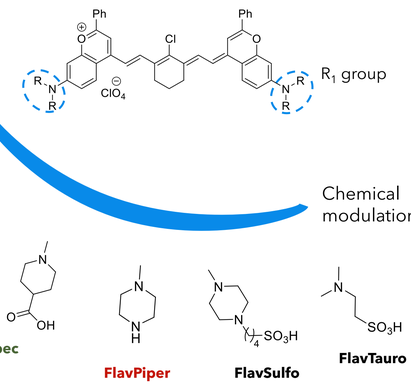


Flav7
λ_{abs}=1026 nm
λ_{em}=1045 nm

AIM OF THE PROJECT

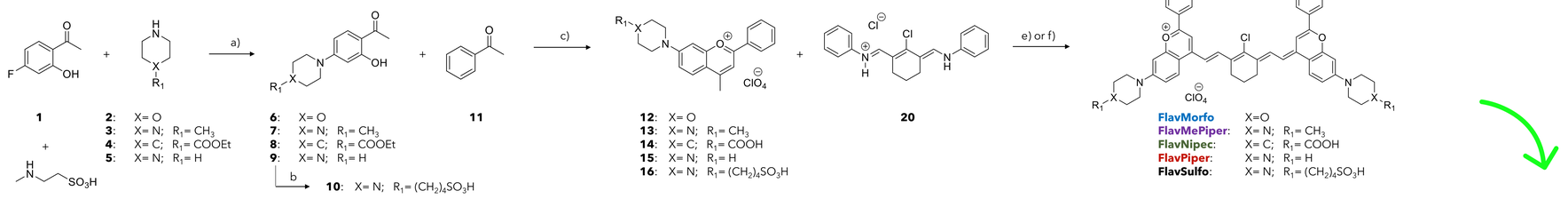
How to increase the polarity of Flav7?

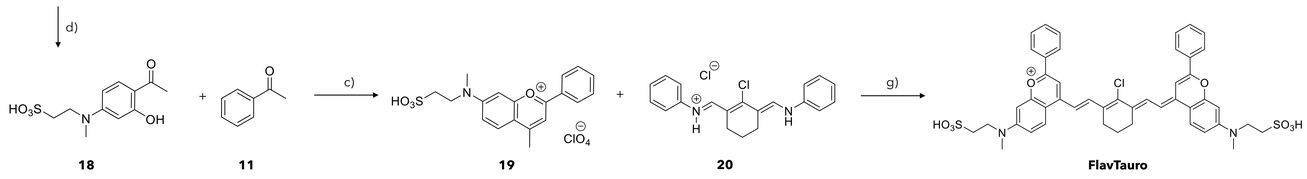
Starting from **Flav7**, the aim of our project was to design and synthesize a new series of more polar derivatives of this compound by chemical modulation of the substituent groups on the flavylium rings, while maintaining or improving the good optical properties of the original compound.



SYNTHETIC APPROACH

Nucleophilic substitution of the fluorine atom of compound **1** with different cyclic amines (**2**-**5**) afforded compounds **6**-**9** in good yields. These compounds were then cyclized with acetophenone **11** under acidic conditions to form the flavylium derivatives **12**-**16**. Last reaction with compound **20** yielded all the final compounds. An analogous synthetic route was applied to obtain **FlavTauro**, starting from the reaction of compound **1** with the linear amine derivative **17**.





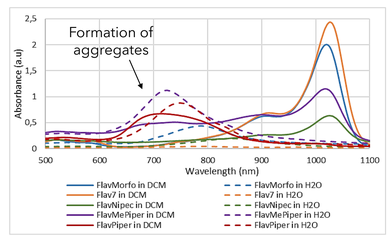
Reaction conditions: a) 110–130 °C, 2-3 h, 65-90%; b) 1,4-butanesultone, 1,4-dioxane, 95 °C, 24 h, 89%; c) $HClO_4$ 70%, CH_3COOH, 105 °C, 20 h, 30-64%; d) K_2CO_3, DMF, 110 °C, 24 h, 80%; e) 2,4,6-trimethylpyridine or NaOAc, n-BuOH/Toluene, 100 °C, 3-5h, 21-35%; f) NaOAc, Acetic anhydride, 80 °C, 2 h, 22%; g) 2,6-di-tert-butyl-4-methylpyridine, n-BuOH/ Toluene; 100 °C, 3-4 h, 7%.

OPTICAL CHARACTERIZATION

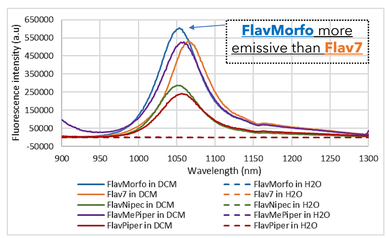
Newly synthesized compounds, as well as **Flav7**, were characterized for their optical properties. Absorption/emission spectra were recorded in different organic and polar solvents such as DCM, ACN, H_2O (spectra reported below), as well as in DMSO, MeOH, Acetone, THF and Seronorm (not shown) at the same concentration of 20 µM. Absorption/emission spectra in a mixture of ACN/H_2O were also recorded.

- New compounds behave as SWIR emitters in organic solvents, maintaining the absorption and the emission above 1000 nm as **Flav7** (panels A-D).
- One compound, **FlavMorfo**, showed a higher intensity of emission compared to **Flav7** both in DCM and ACN (panels B and D).
- The main difference between new compounds and **Flav7** is visible from the spectra acquired in water, where no absorption was recorded for **Flav7**, due to its presumable aggregation and precipitation in water, while new compounds showed a peak at around 700-800 nm, typical of weakly soluble aggregated forms (panel A).
- Even if aggregated forms of new compounds are more soluble than those generated by **Flav7**, they are still non-emissive in the SWIR region (panel A and B).
- In more polar organic solvents, such as ACN, a decrease in absorption was noted (cf. panel A to C) while only a slightly reduced emission was recorded (cf. panel B to D).
- When water was added to ACN, formation of aggregated forms was evident (panel C); however, newly synthesized compounds, which are more soluble than **Flav7**, retained a greater ability to emit in the SWIR region in presence of 50 % of water compared to the original compound (panel D).

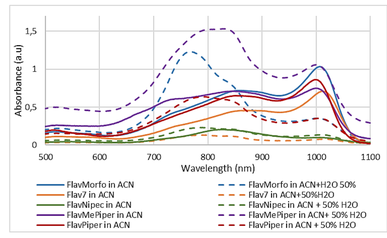
A Absorption spectra in DCM (–) and H_2O (--) of selected compounds at 20 µM.



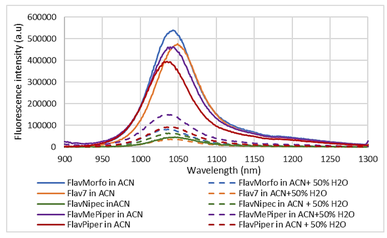
B Emission spectra in DCM (–) and H_2O (--) of selected compounds at 20 µM. λ_{exc} = 870 nm



C Absorption spectra in ACN (–) and ACN/H_2O 1:1 (--) of selected compounds at 20 µM.

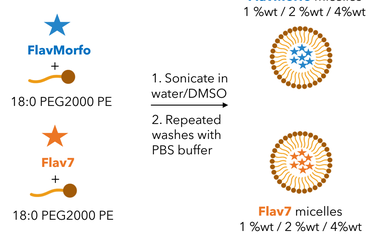


D Emission spectra in ACN (–) and ACN/H_2O 1:1 (--) of selected compounds at 20 µM. λ_{exc} = 870 nm

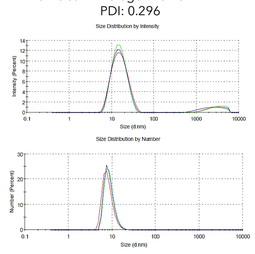


MICELLES FORMULATION & ANALYSIS

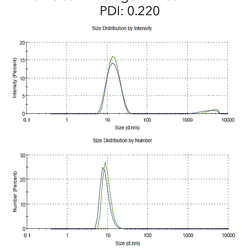
To overcome the formation of aggregates in polar environment, phospholipid micellar formulations with different loading capacity of **FlavMorfo** and **Flav7** were prepared. Micelles were characterized by DLS analysis, and the amount of inner fluorophore was quantified.



DLS measurement of **FlavMorfo** 1 %wt micelles
Particles Z-Average : 15.95 ± 7 nm
PDI: 0.296

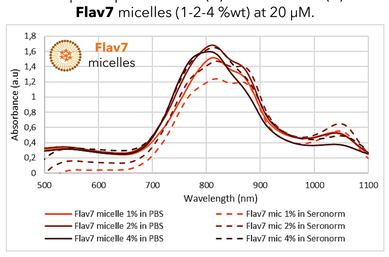


DLS measurement of **Flav7** 1 %wt micelles
Particles Z-Average : 14.85 ± 4.9 nm
PDI: 0.220

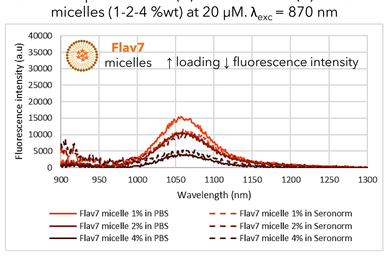


Absorption and emission spectra of **FlavMorfo** micelles and **Flav7** micelles were recorded in PBS and Seronorm.

Absorption spectra in PBS (–) and Seronorm (--) of **Flav7** micelles (1-2-4 %wt) at 20 µM.

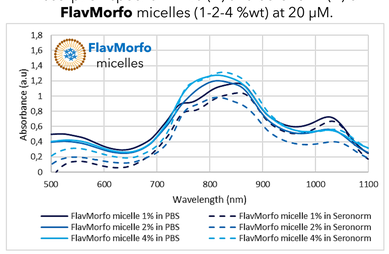


Emission spectra in PBS (–) and Seronorm (--) of **Flav7** micelles (1-2-4 %wt) at 20 µM. λ_{exc} = 870 nm

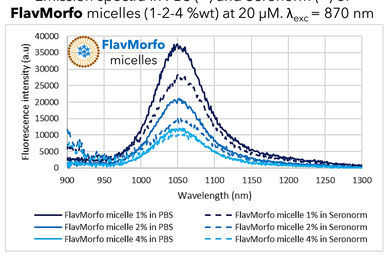


- **FlavMorfo** and **Flav7** micelles behave as SWIR emitters in polar solvents.
- As the fluorophore amount increases, quenching phenomena are observed, and the emission intensity decreases for both compounds.

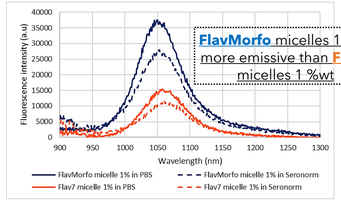
Absorption spectra in PBS (–) and Seronorm (--) of **FlavMorfo** micelles (1-2-4 %wt) at 20 µM.



Emission spectra in PBS (–) and Seronorm (--) of **FlavMorfo** micelles (1-2-4 %wt) at 20 µM. λ_{exc} = 870 nm

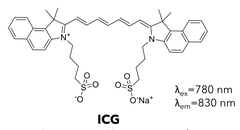


Comparison of emission spectra in PBS (–) and Seronorm (--) of **FlavMorfo** micelles (1 %wt) and **Flav7** micelles (1 %wt) at 20 µM. λ_{exc} = 870 nm



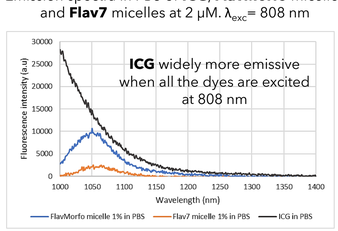
- **FlavMorfo** micelles 1 %wt are about twice as emissive as **Flav7** micelles 1 %wt, resulting in a better SWIR emitter.

Emissions of **FlavMorfo** and **Flav7** micelles (1 %wt) were also compared with the tale emission in the SWIR region of the FDA-approved dye **ICG**[4] in both PBS and Seronorm. The dyes were excited at 808 nm (typical excitation λ used for **ICG** and laser available) and at their respective maximum of absorption (1012 nm for **FlavMorfo** and 1019 nm for **Flav7**). To compare the intensity of emission, the AUC of the region between 1030-1400 nm was considered.



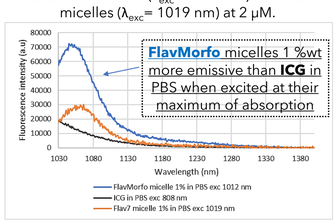
ICG
NIR-I emitter FDA-approved

Emission spectra in PBS of **ICG**, **FlavMorfo** micelles and **Flav7** micelles at 2 µM. λ_{exc}= 808 nm



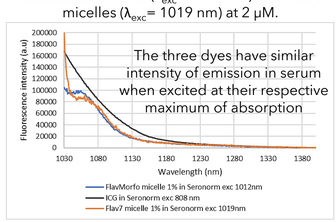
Compound	AUC
ICG	1873432
FlavMorfo	805473
Flav7	90071

Emission spectra in PBS of **ICG** (λ_{exc}= 808 nm), **FlavMorfo** micelles (λ_{exc}= 1012 nm) and **Flav7** micelles (λ_{exc}= 1019 nm) at 2 µM.



Compound	AUC
ICG	1175388
FlavMorfo	5282148
Flav7	2231661

Emission spectra in Seronorm of **ICG** (λ_{exc}= 808 nm), **FlavMorfo** micelles (λ_{exc}= 1012 nm) and **Flav7** micelles (λ_{exc}= 1019 nm) at 2 µM.

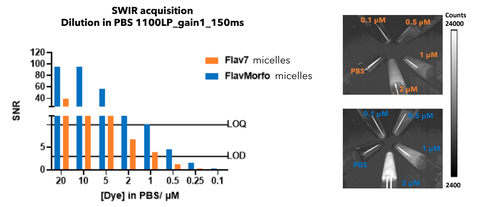


Compound	AUC
ICG	10796468
FlavMorfo	7614135
Flav7	7817829

OPTICAL IMAGING OF THE PROBES

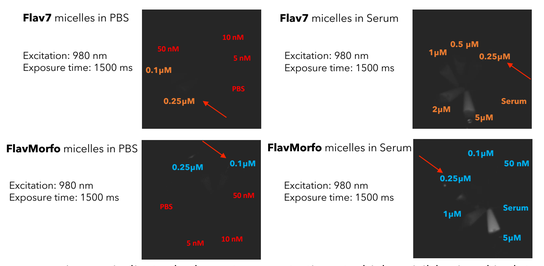
FlavMorfo and **Flav7** micelles (1 %wt) were also tested through the use of specific imaging devices to assess their visible brightness looking at their use for FGS purposes. Two excitation wavelengths, 805 and 980 nm, were used, according to currently available devices. The two dyes were tested both in PBS and Seronorm at different concentrations, and their respective Limits of Detection (LOD) were defined.

Excitation at 805 nm, collecting the emission above 1100 nm



Dye	Batch	LOD [µM]
FlavMorfo	PBS	0.5
Flav7	PBS	1.0
FlavMorfo	Serum	0.5
Flav7	Serum	0.5

Excitation at 980 nm, collecting the emission above 1100 nm.



Arrows indicate the lowest concentration at which a visible signal is detected.

- **FlavMorfo** micelles showed a lower LOD in PBS with respect to **Flav7** micelles when imaged at 805 nm, demonstrating slightly improved optical properties. A similar LOD was recorded for both compounds when analyzed in serum.
- Excitation at 980 nm did not significantly improve the detected brightness, and no advantage was observed over excitation at 805 nm.
- Probably, the use of a custom-made laser able to excite the dyes at their respective maximum of absorption could give better results and show an improved brightness of the dyes, thus opening the possibility of *in vivo* application.

CONCLUSIONS

- A new series of **Flav7** derivatives was designed and synthesized. New compounds can be classified as SWIR emitters in organic solvents.
- Although more polar than the original compound, newly synthesized dyes still suffer from the tendency to form non-emissive aggregates in polar media, analogously to **Flav7**.
- Our best compound **FlavMorfo**, encapsulated in phospholipid micelles, was more emissive in physiological-like environment than **Flav7** in micelles, resulting in a potentially better SWIR emitter.
- Optical imaging of the dyes-containing micelles at a suboptimal wavelength showed a lower LOD for **FlavMorfo** in PBS and a similar LOD in serum compared to **Flav7**.
- Our results suggest that the use of more appropriate lasers, capable of exciting **FlavMorfo** at its maximum absorption wavelength, could reveal an improved brightness compared to FDA approved **ICG** dye.

References
[1] Zhu S. J.; Tian R.; Antaris A.L.; Chen X. Y.; Dai H. J. Near-Infrared-II Molecular Dyes for Cancer Imaging and Surgery. Adv. Mater. 2019, 31, 1900321
[2] Wang S.; Li B.; Zhang F. Molecular Fluorophores for Deep-Tissue Bioimaging. ACS Cent. Sci. 2020, 6, 1302−1316
[3] Cosco, E. D.; Caram, J. R.; Bruns, O. T.; Franke, D.; Day, R. A.; Farr, E. P.; Bawendi, M. G.; Sletten, E. M. Flavylium Polymethine fluorophores for near- and shortwave infrared imaging. Angew. Chem., Int. Ed. 2017, 56, 13126−13129
[4] Marshall M. V.; Rasmussen J. C.; Tan I. C.; Aldrich M. B.; Adams K. E.; Wang X.; Fife C. E.; Maus E. A.; Smith L. A.; Sevick-Muraca E. M. Near-Infrared Fluorescence Imaging in Humans with Indocyanine Green: A Review and Update. Open Surg Oncol J. 2010;2(2),12-25