

Photoactive Bile Salts with Critical Micellar Concentration in the Micromolar Range

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The aggregation behavior of bile salts is strongly dependent on the number of hydroxyl groups. Thus, cholic acid (CA), with three hydroxyls, starts forming aggregates at 15 mM, while deoxycholic, chenodeoxycholic or ursodeoxycholic acids, with two hydroxyls, start aggregating at 5-10 mM; for lithocholic acid, with only one hydroxyl group, aggregation is observed at lower concentration (2-3 mM). Here, the singular self-assembling properties of dansyl and naproxen derivatives of CA (3 β -Dns-CA and 3 β -NPX-CA, respectively) have been demonstrated on the basis of their photoactive properties. Thus, the emission spectra of 3 β -Dns-CA registered at increasing concentrations (25-140 μ M) showed a remarkable non-linear enhancement in the emission intensity accompanied by a hypsochromic shift of the maximum and up to three-fold increase in the singlet lifetime. The inflection point at around 50-70 μ M, pointed to the formation of unprecedented assemblies at such low concentrations. In the case of 3 β -NPX-CA, when the NPX relative triplet lifetime was plotted against concentration, a marked increase (up to two-fold) was observed at 40-70 μ M, indicating formation of new 3 β -NPX-CA assemblies at *ca.* 50 μ M. An additional evidence supporting formation of new 3 β -Dns-CA or 3 β -NPX-CA assemblies at 40-70 μ M was obtained from singlet excited state quenching experiments using iodide. Moreover, to address the potential formation of hybrid assemblies, 1:1 mixtures of 3 β -Dns-CA and 3 β -NPX-CA (2-60 μ M, total concentration) were subjected to steady-state fluorescence experiments, and their behavior compared to that of the pure photoactive derivatives. A lower increase in the emission was observed for 3 β -NPX-CA in the mixture, while a huge increase was experienced by 3 β -Dns-CA in the same concentration range (up to 60 μ M total). A partial intermolecular energy transfer from NPX to Dns, consistent with their reported singlet energies, was revealed, pointing to the formation of extremely fluorescent hybrid assemblies at 5-10 μ M (total concentration). The morphology of the entities was investigated by means of confocal microscopy. At 90 μ M, 3 β -Dns-CA showed disperse assemblies in the μ m range.

Introduction

Self-assembled biocompatible molecules constitute an emerging research area owing to their potential applications as nanomaterials in technology or medicine.¹⁻³ Bile salts (BS) are water-soluble natural steroids with a high tendency to aggregate in solution due to the *cis* fusion between rings A and B, the hydroxyl groups on the α -face and the carboxylic acid at the end of the lateral chain. The aggregation behavior is strongly dependent on the number of hydroxyl groups.⁴ Thus, cholic acid (CA), with three hydroxyls, starts forming aggregates at 15 mM, while deoxycholic, chenodeoxycholic or ursodeoxycholic acids, with two hydroxyls, start aggregating at 5-10 mM; for lithocholic acid, with only one hydroxyl group, aggregation is observed at lower concentration (2-3 mM).^{5,6}

Among applications, BS mainly act as biological surfactants^{7,8} and have great potential as biomolecules for the preparation of nanostructures, since they combine their unique and peculiar structural features with the fact that they are inexpensive and readily available starting materials. In this context, in the last few years it has been proven that even small modifications of the molecular structure of BS lead to derivatives with uncommon self-assembly properties.⁹⁻¹² For instance, lithocholic acid and some different artificial derivatives of CA have been reported to self-assemble into tubules that constitute interesting structures for their potential applications in nanotechnology.^{11,13-15} Furthermore, self-assembly of amino acid derivatized BS has been widely investigated as a potential strategy for the preparation of nanostructured biomaterials with a wide range of applications in nanomedicine.¹⁶⁻¹⁸

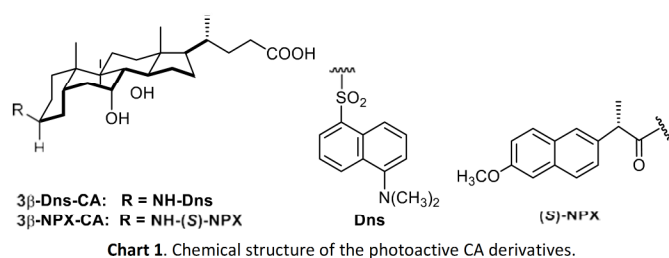
We have recently demonstrated that derivatization of CA by incorporating a dansyl (Dns) fluorophore,^{19,20} which exhibits a strongly medium-dependent photophysical behavior, affords valuable reporters to investigate the aggregation of BS.^{5,21} This is achieved by replacement of less than 1% of original BS and therefore does not affect the micelle-forming properties of the natural BS.

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Electronic Supplementary Information (ESI) available: Additional UV-vis, steady-state emission and time-resolved spectra and quenching experiments. See DOI: 10.1039/x0xx00000x

Herein, we want to report on the singular self-assembling properties of dansyl and naproxen derivatives of CA (3β -Dns-CA and 3β -NPX-CA, Chart 1). In the absence of non-labeled BS, these modified analogs aggregate in aqueous solution at concentrations 2-3 orders of magnitude lower. Characterization of the new photoactive aggregates has been based on steady-state and time-resolved emission spectroscopy, laser flash photolysis and confocal microscopy measurements. Furthermore hybrid 3β -Dns-CA: 3β -NPX-CA aggregates are formed at even lower concentration ranges.



Results and discussion

Photophysical characterization of self-assemblies based on 3β -Dns-CA

The concentration-dependent photophysical behavior of the two cholic acid derivatives was investigated at micromolar concentration range (25-140 μ M). The UV-Vis spectrum of 3β -Dns-CA showed the characteristic absorption band at around 330 nm (Figure ES1). Interestingly, the emission spectra of 3β -Dns-CA registered at increasing concentrations showed a remarkable non-linear enhancement in the emission intensity (Figures 1 and 2) accompanied by a hypsochromic shift of the maximum (Figure 2 top right) and up to three-fold increase in the average singlet lifetime (Figure 2 bottom left and Figure S2-S4). Control experiments using a cyclohexyl analog (Chx-Dns) at the same concentration range showed the expected small and linear increase in the relative emission intensity (Figure 2 bottom right and S2B) without significant changes in the position of the emission maximum or singlet lifetime. The inflection point in the behavior of 3β -Dns-CA was observed at around 50-70 μ M (Figure 2), pointing to the formation of unprecedented assemblies at such low concentrations. Moreover, the behavior of 3β -Dns-CA is in clear contrast with the aggregation response observed for other bile acids having two hydroxyl groups, such as deoxycholic or chenodeoxycholic acids, that start forming aggregates at millimolar concentration (typically 5 mM).⁶ Clearly, the facial amphiphilia of natural CA is altered upon incorporation of the hydrophobic dansyl chromophore on the β face of the skeleton. As a result, the usual tendency to aggregate upon interaction of the β faces is enhanced; therefore aggregation starts at concentrations two orders of magnitude lower.

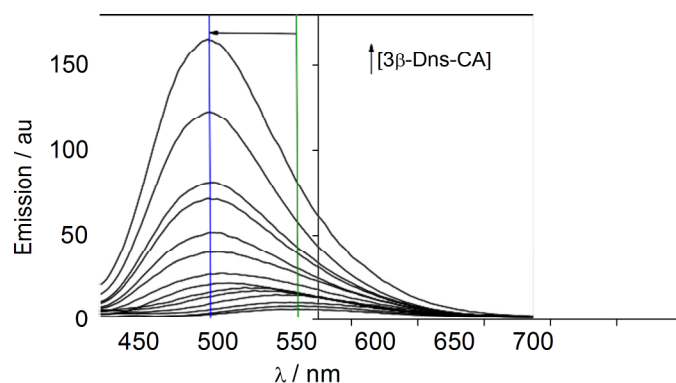


Fig. 1. Fluorescence spectra ($\lambda_{exc} = 390$ nm) of 3β -Dns-CA in aqueous solution upon increasing concentrations (25 – 140 μ M).

Photophysical characterization of self-assemblies based on 3β -NPX-CA

After having illustrated the unusual self-assembling properties of the diluted Dns-CA derivative, the study was extended to a naproxen-labelled CA (3β -NPX-CA, Chart 1). Although the NPX singlet excited state properties are not particularly sensitive to the hydrophobicity of the surroundings, its triplet lifetime is strongly dependent on the environment, as we have recently demonstrated upon incorporation into cholic acid aggregates or mixed micelles.^{20,22} Hence, the behavior of the triplet excited state of 3β -NPX-CA was investigated by laser flash photolysis (LFP) (Figure 3). As a matter of fact, longer lifetimes were found at higher concentrations up to two-fold, but more interestingly, when the relative triplet lifetime was plotted against concentration, a marked increase was observed at 40-70 μ M. This trend is in contrast to the behavior reported for the isolated NPX unit, which suffers triplet self-quenching.²³⁻²⁵ Control experiments performed at the same concentration range, using commercial (S)-naproxen evidenced indeed the expected decrease in the triplet lifetime (Figure 3 bottom). Therefore, the observed tendency in the triplet lifetime behavior found for 3β -NPX-CA vs concentration indicates formation of new 3β -NPX-CA assemblies at ca. 50 μ M. Again, the more hydrophobic β face of 3β -NPX-CA compared to the natural CA results in a tendency to aggregate at concentrations two orders of magnitude lower.

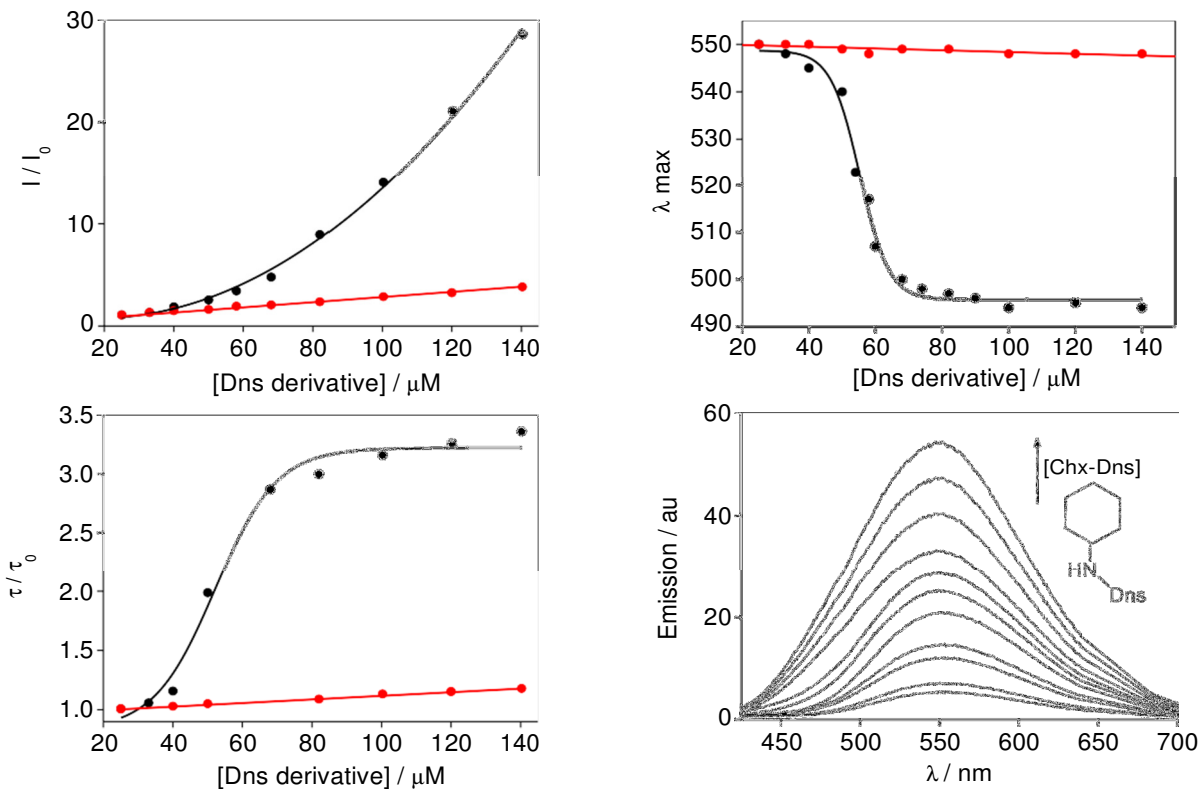


Fig. 2. Singlet excited state behavior of 3 β -Dns-CA (black) or Chx-Dns (red) in aqueous solution versus concentration ($\lambda_{\text{exc}} = 390$ nm): Top left) changes in the relative emission intensities recorded at the corresponding maximum (I/I_0); top right) changes in the position of the emission maximum (λ_{max}); bottom left) changes in the relative singlet lifetime (τ/τ_0); bottom right) fluorescence spectra ($\lambda_{\text{exc}} = 390$ nm) of Chx-Dns in aqueous solution upon increasing concentrations (25 – 140 μM).

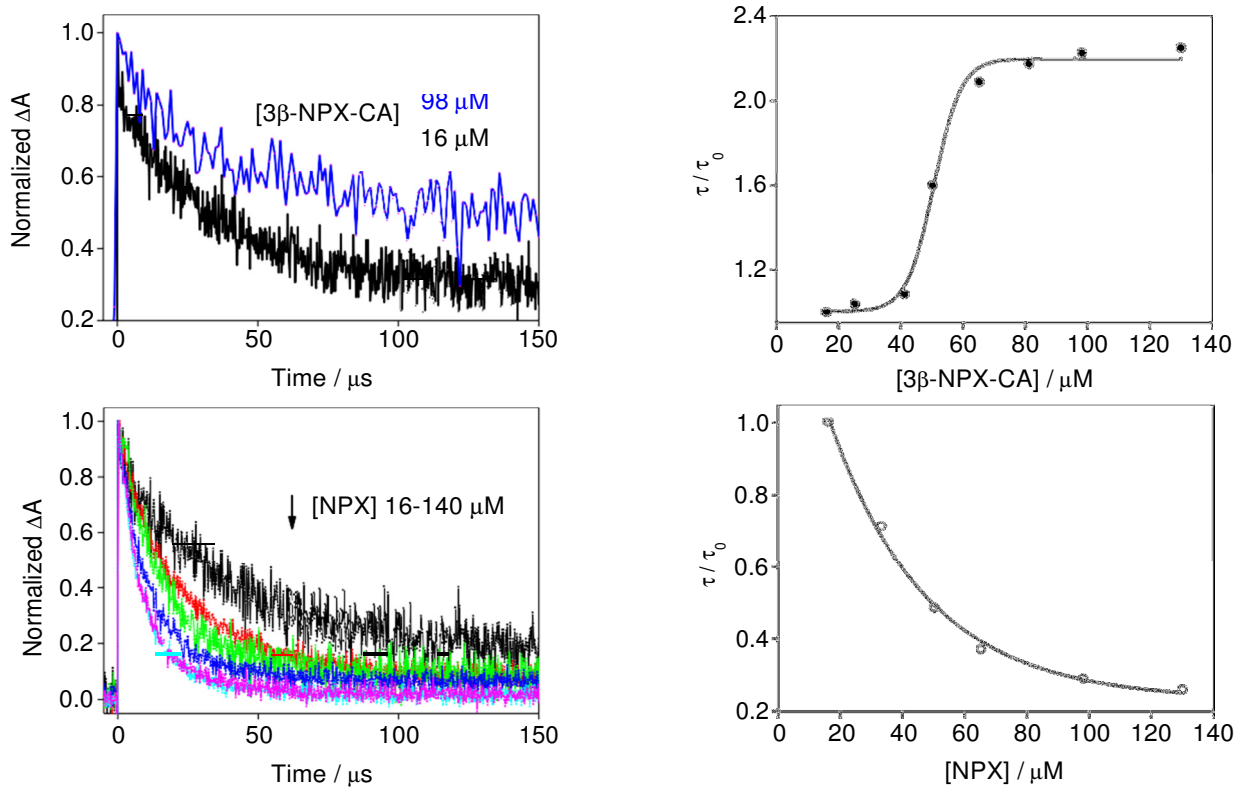


Fig. 3. Left) Traces corresponding to $^3\text{NPX}^*$ monitored at 440 nm upon laser flash photolysis excitation ($\lambda_{\text{exc}} = 266$ nm) and right) changes in the relative triplet lifetime (τ/τ_0), in deaerated 0.2 M aqueous NaCl at different concentrations. Top) 3 β -NPX-CA; Bottom) (S)-naproxen.

Fluorescence quenching of self-assemblies based on 3 β -DnsCA or 3 β -NPX-CA

A further piece of evidence supporting formation of new 3 β -Dns-CA or 3 β -NPX-CA assemblies at 40-70 μ M concentration was obtained from quenching experiments using iodide (Figures 4 and S5).^{22,26} Rate constant values (k_q) were determined for 3 β -Dns-CA and 3 β -NPX-CA at two different concentrations: 16 μ M (still in solution) and 100 μ M (forming self-assemblies). For 3 β -Dns-CA k_q decreased from $7.5 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$ to $1.5 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$; analogously, for 3 β -NPX-CA, k_q decreased from $3.1 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$ to $4.5 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$. The less efficient quenching observed in the self-assemblies compared to solution agrees well with formation of the new supramolecular entities.

and their behavior compared to that of the pure photoactive derivatives at the same concentrations (Figure 5 and S6-7). Experiments conducted with pure 3 β -Dns-CA or 3 β -NPX-CA shown in Figure 5 top and middle revealed a linear increase in the emission upon increasing concentrations in the studied range (up to 30 μ M). By contrast, when 1:1 mixtures were subjected to emission experiments, a different behavior was noticed (Figure 5 top). In fact, a lower increase in the emission was observed for 3 β -NPX-CA, while a huge increase was experienced in the case of 3 β -Dns-CA with concentration in the same range (up to 60 μ M total). This fact revealed a partial intermolecular energy transfer from NPX to Dns consistent with their reported singlet energies (355 and 280 kJ mol^{-1} , respectively).²⁷

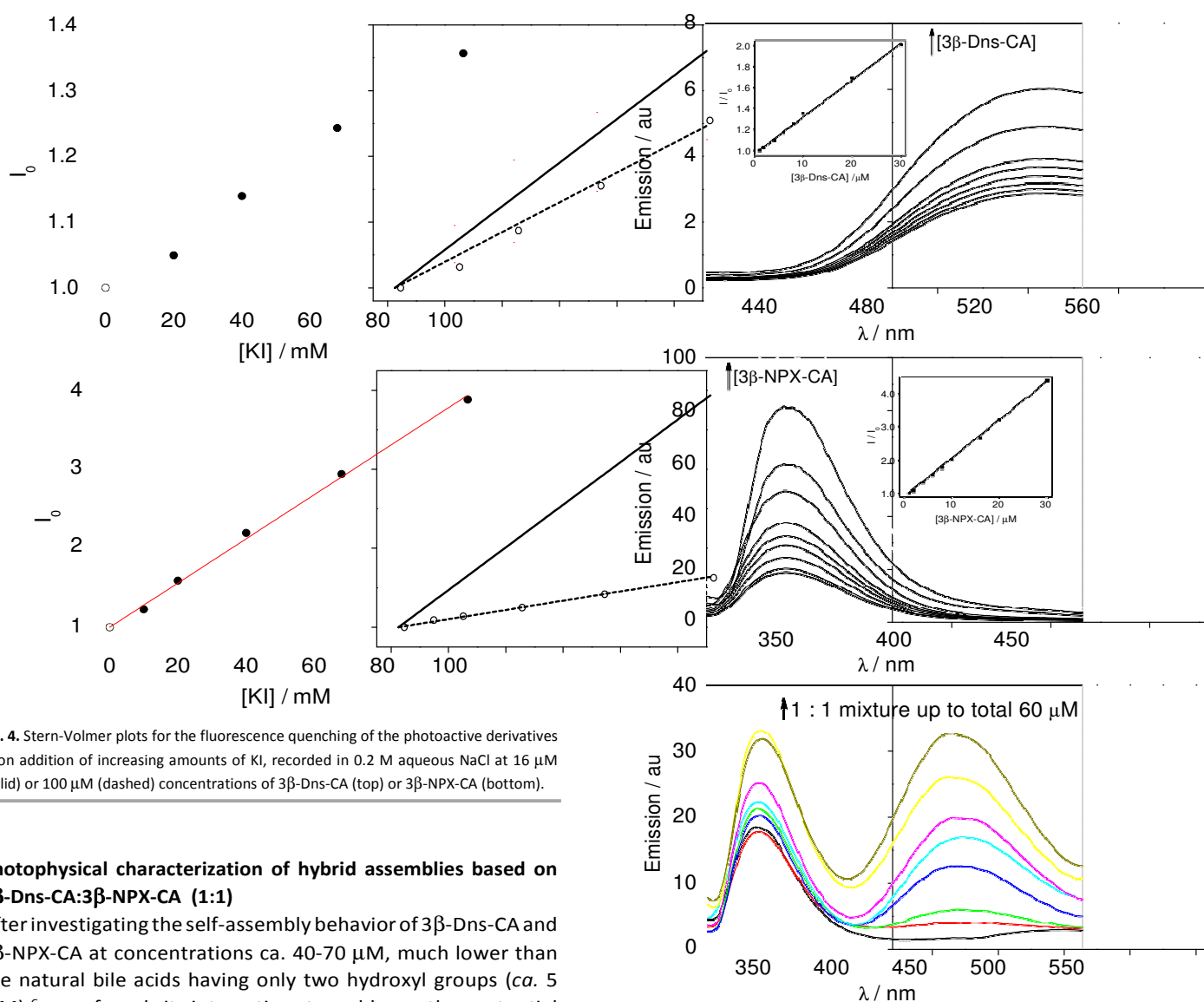


Fig. 4. Stern-Volmer plots for the fluorescence quenching of the photoactive derivatives upon addition of increasing amounts of KI, recorded in 0.2 M aqueous NaCl at 16 μ M (solid) or 100 μ M (dashed) concentrations of 3 β -Dns-CA (top) or 3 β -NPX-CA (bottom).

Photophysical characterization of hybrid assemblies based on 3 β -Dns-CA:3 β -NPX-CA (1:1)

After investigating the self-assembly behavior of 3 β -Dns-CA and 3 β -NPX-CA at concentrations ca. 40-70 μ M, much lower than the natural bile acids having only two hydroxyl groups (ca. 5 mM),⁶ we found it interesting to address the potential formation of hybrid assemblies. For this purpose, 1:1 mixtures of 3 β -Dns-CA:3 β -NPX-CA (2-60 μ M, total concentration) were subjected to steady-state fluorescence experiments (Figure 5),

Fig. 5. Emission behavior of 3 β -Dns-CA (1-30 μ M) (top) and 3 β -NPX-CA (1-30 μ M) (middle) in aqueous solutions. Insets: Relative emission intensity vs concentration recorded at 540 nm (top) or 350 nm (middle); Bottom) Fluorescence emission behavior ($\lambda_{\text{exc}} = 290 \text{ nm}$) of 1:1 mixtures of 3 β -Dns-CA: 3 β -NPX-CA (2-60 μ M, total concentration).

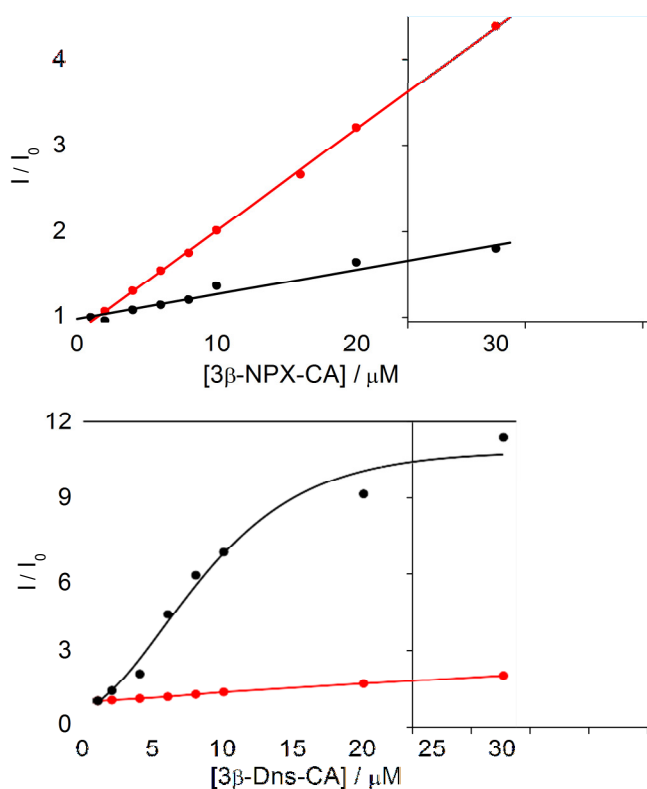


Fig. 6. Relative emission intensity ($\lambda_{\text{exc}} = 290 \text{ nm}$) versus concentration due to: top) 3β -NPX-CA (recorded at 350 nm); bottom) 3β -Dns-CA (recorded at the corresponding maximum) in pure (red) or hybrid (black) assemblies.

More interesting resulted to plot the relative emission of the two chromophores in the pure versus hybrid assemblies (Figure 6 top and bottom) upon excitation at 290 nm. Hence, in Figure 6 top, the relative intensity vs NPX concentration (recorded at 350 nm) in the case of pure 3β -NPX-CA (red line) was compared to hybrid assemblies (black line). Correspondingly, in agreement with an efficient intermolecular energy transfer, the behavior of the Dns chromophore in hybrid assemblies (black line, Figure 6 bottom), showed a huge non-linear emission increase with concentration in contrast with the linear behavior observed for the pure 3β -Dns-CA (red trace) with a clear change in trend at ca. 5-10 μM , pointing to the formation of hybrid assemblies at very low concentrations.

This fact could be explained considering that the tendency to aggregate in this case is not only due to the more hydrophobic β faces but also and more importantly, to the different nature of the aromatic rings. As naproxen (electron rich) and dansyl (electron poor) have a complementary charge distribution, their interaction is favoured, and results in aggregation at concentrations 3 orders of magnitude lower.

Images of the pure 3β -Dns-CA and 3β -Dns-CA: 3β -NPX-CA (1:1) assemblies at different concentrations

Emission photographs, taken under UV lamp irradiation (Figure 7), clearly illustrated the emissive properties of pure and hybrid entities. In fact, pure 3β -Dns-CA assemblies were highly emissive at concentrations as low as 90 μM , compared to the Chx-Dns control at the same concentration. Even more

interesting was the result that hybrid assemblies were extremely fluorescent at only 32 μM (total concentration). To the best of our knowledge, this is the first time that photoactive hybrid assemblies based on BS derivatives are described.

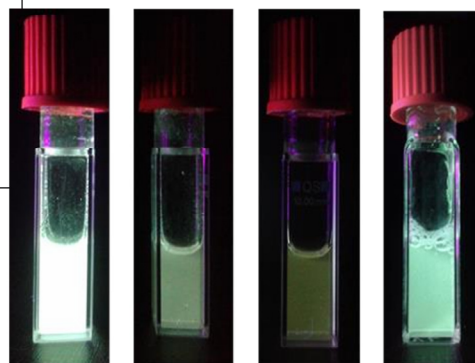


Fig. 7. Photograph of emission ($\lambda_{\text{exc}} = 254 \text{ nm}$). From left to right: 3β -Dns-CA (90 μM), Chx-Dns (90 μM), 3β -Dns-CA (16 μM) and 1:1 3β -Dns-CA / 3β -NPX-CA (16 μM , Dns).

Confocal microscopy of 3β -Dns-CA assemblies

The morphology of these entities was investigated by means of confocal microscopy upon excitation at 405 nm. At 90 μM , 3β -Dns-CA showed disperse assemblies with sizes ranging from 1 to 10 μm . The emission spectra of these assemblies confirmed that they correspond to Dns chromophore with maximum at ca. 500 nm (Figure 8).

Conclusions

The singular self-assembling properties of photoactive dansyl and naproxen derivatives of cholic acid (3β -Dns-CA and 3β -NPX-CA) have been demonstrated on the basis of their optical properties. Formation of the unprecedented supramolecular entities at 50-70 μM is revealed by the changes in the emission maximum and relative singlet lifetime in the case of 3β -Dns-CA and by the increase in triplet lifetime for 3β -NPX-CA, in contrast to the triplet self-quenching reported for the isolated NPX unit. Quenching of the emission by iodide, with lower k_q values upon aggregation in both cases, also supports formation of the new entities. Even more, the presence of extremely fluorescent hybrid assemblies has been demonstrated for 1:1 mixtures of 3β -Dns-CA: 3β -NPX-CA at 5-10 μM , based on the emission corresponding to 3β -Dns-CA upon NPX excitation and subsequent intermolecular energy transfer. Confocal microscopy experiments for 3β -Dns-CA show disperse assemblies with sizes in the μm range.

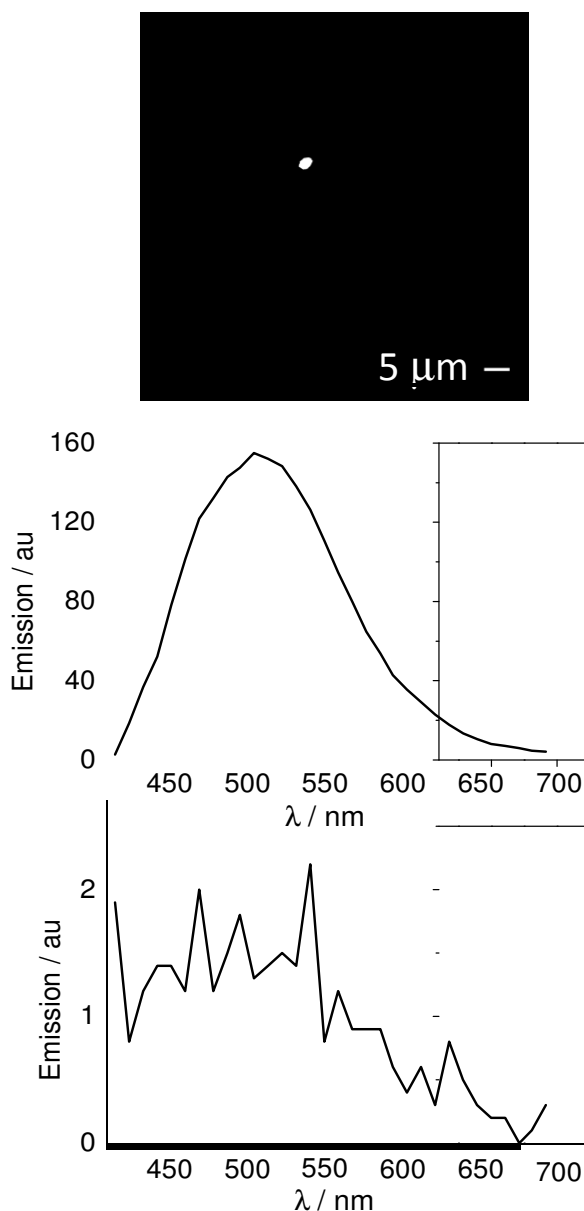


Fig. 8. Top) Image obtained from the confocal microscope ($\lambda_{\text{exc}} = 405$ nm) of 3β -Dns-CA at $90 \mu\text{M}$; middle) emission spectrum ($\lambda_{\text{exc}} = 405$ nm) taken inside the bright assembly (3β -Dns-CA at $90 \mu\text{M}$); bottom) background emission spectrum obtained in the dark region.

Acknowledgements

Financial support from the Spanish Government (Grants SEV-2012-0267 and CTQ2012-38754-C03-03) and the Generalitat Valenciana (Prometeo Program) is gratefully acknowledged.

Experimental section

Chemicals. NaCl, dansyl chloride, (*S*)-naproxen, cyclohexylamine (Chx-NH_2), dimethylsulfoxide and KI were purchased from Sigma–Aldrich; Milli-Q® water was used for sample preparation. The photoactive cholic acid derivatives 3β -Dns-CA and 3β -NPX-CA were prepared as previously

reported.^{19,20} Cyclohexylamine-Dns (Chx-Dns) was synthesized by direct reaction between cyclohexylamine and dansyl chloride (80% yield): $^1\text{H NMR}$ (300 MHz, CDCl_3): δ 1.00–1.80 (m, 10H), 2.90 (s, 6H), 3.20 (m, 1H), 4.60 (br d, 1H), 7.20 (d, 1H), 7.50–7.65 (m, 2H), 8.20–8.35 (m, 2H), 8.58 (d, 1H).

Instrumentation

Absorption and Emission Equipment. UV spectra were recorded on a Cary 300 (Varian) spectrophotometer. Steady-state fluorescence experiments were carried out using a Photon Technology International (PTI, Germany) LPS-220B fluorometer, equipped with a monochromator in the range of 200–700 nm. Time-resolved fluorescence measurements were performed with a Time Master fluorescence lifetime spectrometer TM 2/2003 from PTI, using a hydrogen/nitrogen flash lamp as the excitation source. The kinetic traces were fitted by one monoexponential decay function, using a deconvolution procedure to separate them from the lamp pulse profile.

Laser Flash Photolysis. A pulsed Nd:YAG L52137 V LOTIS TII was used at the excitation wavelength of 266 nm. The single pulses were *ca.* 10 ns duration, and the energy was lower than 20 mJ/pulse. The laser flash photolysis system consisted of the pulsed laser, a 77250 Oriel monochromator and an oscilloscope DP04054 Tektronix. The output signal from the oscilloscope was transferred to a personal computer.

Confocal Microscope. A LSM 780 AxioObserver from Zeiss was used at the excitation wavelength of 405 nm. Emission measurements were taken in the region 411–696 nm every 8.9 nm. All the measurements were performed at room temperature and the obtained data were analyzed by means of a free software called Fiji.

Sample Preparation

New photoactive assemblies at different concentrations were freshly prepared by dissolving the specified amount of the photoactive derivative (from a stock solution in DMSO) in 0.2 M NaCl.

Photophysical Experiments

Emission measurements were performed in the region of 330–600 nm with excitation in the range of 200–340 nm under air-equilibrated conditions. The triplet lifetimes of 3β -NPX-CA or (*S*)-naproxen were determined in 0.2 M NaCl aqueous solution from the monoexponential fitting of the decay traces registered at 440 nm under N_2 . All photophysical measurements were performed at room temperature using $10 \times 10 \text{ mm}^2$ quartz cells of 4 mL capacity.

Quenching Experiments

The specified volumes of a freshly prepared KI solution (2 M) were added to aerated solutions of the photoactive derivatives at the indicated concentrations.

Confocal Microscope Images

The images were taken from freshly prepared 3β -Dns-CA assemblies at $90 \mu\text{M}$ concentration in 0.2 M NaCl.

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