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New Photoactive Compounds To Probe Cholic Acid and Cholesterol inside Mixed Micelles

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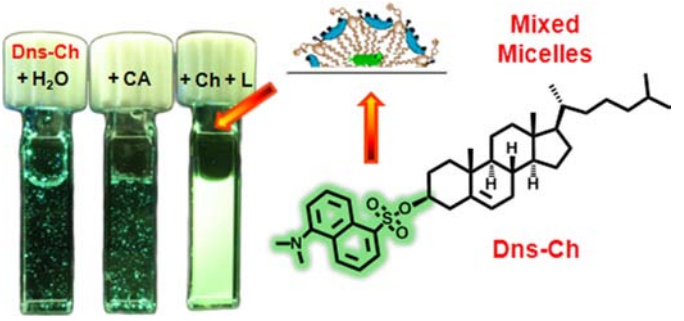
ABSTRACT



New photoactive dyads have been synthesized by derivatization of cholic acid (CA) or cholesterol (Ch). These compounds have proven to be efficient tools to monitor incorporation of CA and Ch to mixed micelles (MM) and to probe the microenvironment experienced inside these entities. The outstanding capability of MM to solubilize Ch has been demonstrated.

Bile salts (BS), phospholipids (*e.g.*, lecithin (L)), and cholesterol (Ch) are the major components of bile.[1,2] It is believed that Ch and L are cosecreted by hepatocytes as vesicles, which are then partially solubilized by BS,[3−6] forming mixed micelles (MM).[1,2,7,8]

Both species, which exist in a dynamic equilibrium,[9,10] play an important role in the digestion and in the intestinal absorption of dietary lipids, as well as in the formation of Ch gallstones[10] and in the maintenance of the appropriate Ch levels in humans.

Under fasting conditions vesicles formation prevails; however, in the presence of exogenous lipids (from food, medication, etc.) the total BS concentration increases, which results in the predominance of MM and therefore in a higher solubilization capability.[1]

Despite the considerable effort devoted to understand the association properties of BS,[11,12] the precise mechanisms of lipid vesicle solubilization by surfactants and some interesting properties of MM deserve further attention.

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These issues have been addressed using different techniques including calorimetry,[13] dynamic light scattering (DLS),[14,15] transmission electron microscopy (TEM),[16] small-angle X-ray scattering,[17] ultracentrifugation,[9] or NMR;[18] however, direct and sensitive methodologies to provide reliable information on the architecture of MM are still needed.

In this context, photophysical properties are very sensitive to the environment, and thus excited states can be used as probes for microheterogeneous systems, provided that an appropriate photoactive chromophore is available.[19−27] Specifically, we have recently reported on the enhancement of the fluorescence quantum yield of the dansyl (Dns) chromophore covalently linked to cholic acid (CA), together with an increase of the singlet lifetime. This has allowed us to build up the speciation diagrams of a variety of BS.[12] Moreover, quenching of the singlet and triplet excited states of naproxen (NPX) derivatives by using salts that mainly remain in water has revealed the distribution of the chromophore between the bulk solution and the lipophilic environments provided by CA aggregates.[28] With this background, the present work nicely illustrates the use of new photoactive compounds (**1a**−**c** and **2a**−**c**, Figure 1) to probe incorporation of CA and Ch to MM. The unique properties of these new probes are associated with their close structural similarity to CA and Ch, which are among the main components of MM. Hence, all the probes are expected to occupy equivalent positions in the aggregates to the parent compounds, resulting in a nearly negligible influence on micellization.

Here, the singlet excited state of the Dns fluorophore and the triplet excited state of NPX have been used as reporters. These chromophores have been covalently attached to the 3-β-position of the CA and Ch skeleton, to give a series of dyads: Dns-CA (**1a**), (*R*)-NPX-CA (**1b**), (*S*)-NPX-CA (**1c**), Dns-Ch (**2a**), (*R*)-NPX-Ch (**2b**), and (*S*)-NPX-Ch (**2c**). They were prepared from CA or Ch and NPX or Dns, following standard procedures (see details in Supporting Information).

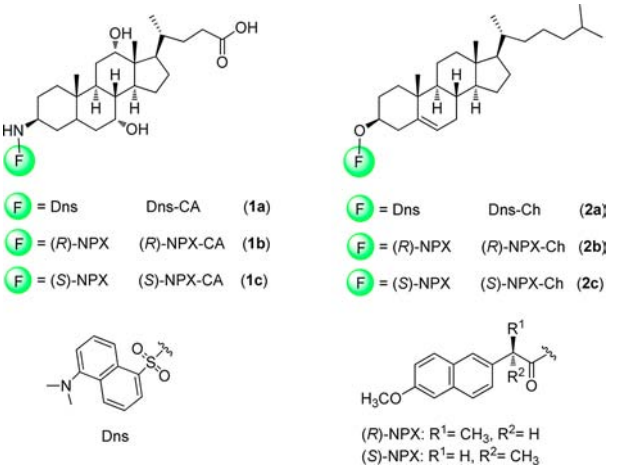

Figure 1. Chemical structures of dyads **1a**−**c** and **2a**−**c**.

The MM were prepared from L (13 mM), Ch (4 mM), and CA (28 mM) according to a described procedure.[29] To monitor incorporation inside MM, 0.7 or 5% of CA or Ch, respectively, was replaced by the corresponding dyad (**1a**−**c** or **2a**−**c**); thus, the concentration of the chromophore (2×10^{-4} M) was appropriate for the photophysical measurements without introducing significant changes in the structure of MM.

The obtained MM were characterized by DLS and by TEM; the combined results revealed the presence of real MM as a major component, together with a lower amount of vesicles (Figure 2A). The structure of MM with and without the photoactive units is represented as a cartoon in Figure 2B.

For a better characterization, the photoactive MM were submitted to photophysical studies. Thus, identical emission and excitation spectra (maxima at 510 and 358 nm, respectively) were obtained with either Dns-CA (**1a**) or Dns-Ch (**2a**) inside MM (Figure 3A). For comparison, analogous experiments were also performed in solution. Interestingly, in the case of **1a** a blue-shifted emission spectrum, accompanied by the corresponding red-shifted excitation spectrum, was observed in MM; in the case of **2a** identical emission and excitation maxima were recorded in the MM system and in CH_2Cl_2. This indicates that the nature of the environment experienced by the labeled dyads inside the MM is markedly lypophilic and similar to that provided by the nonpolar organic solvent. In aqueous media, the emission quantum yield of **1a** increased from 0.05 (0.2 M NaCl) to 0.12 (MM). In the case of **2a**, the corresponding values were < 0.01 (0.2 M NaCl) and

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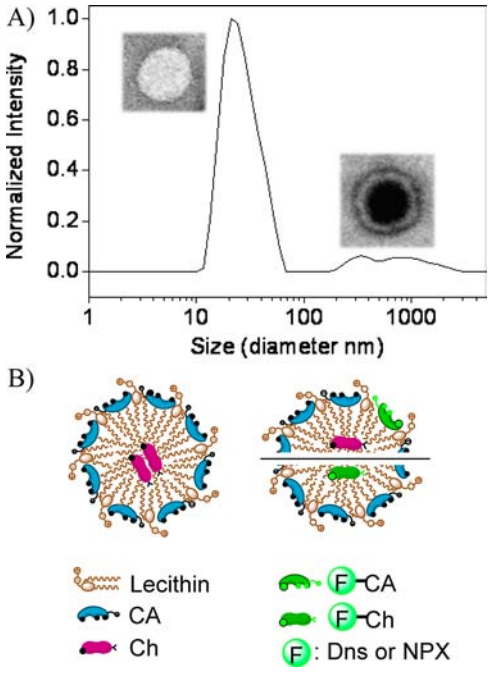

Figure 2. Bile salts/lecithin/cholesterol MM. (A) Size distribution curves for mixed micelles and vesicles in 0.2 M aqueous NaCl, obtained by DLS and TEM image. (B) Cartoon representation of MM.

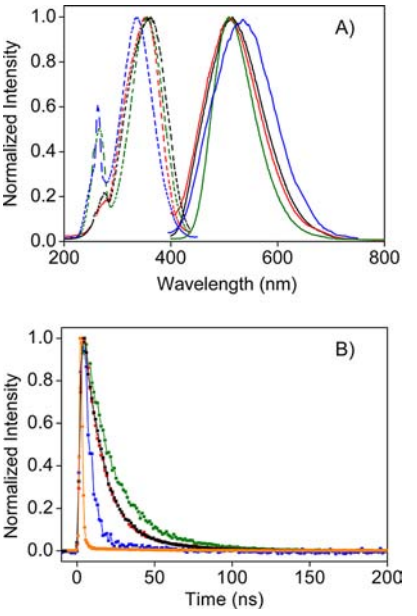

Figure 3. (A) Normalized emission (solid line) and excitation spectra (dashed line) of **1a** into MM in aqueous 0.2 M NaCl (red); **2a** into MM in aqueous 0.2 M NaCl (black); **1a** in aqueous 0.2 M NaCl (blue) and **2a** in CH_2Cl_2 (green). (B) Decay traces of **1a** and **2a** solutions under identical conditions; the color codes remain as above. The lamp response time is included (orange). Concentration of the samples were fixed at the value of 2×10^{-4} M, under an aerobic atmosphere.

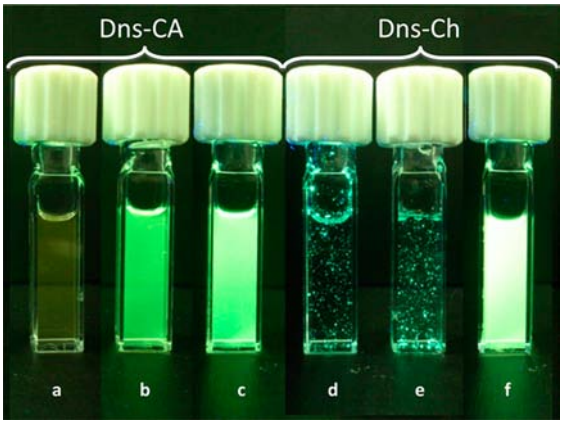

Figure 4. Photograph of real-color emission (λ_{exc} = 254 nm) of Dns-CA and Dns-Ch in aqueous solution (a, d), in the presence of 28 mM CA (b, e), and incorporated in MM (c, f). The concentration of the samples was fixed at 2×10^{-5} M.

0.12 (MM). Accordingly, when time-resolved emission was recorded in solution, a singlet lifetime of 4.8 ns was obtained for **1a** in an aqueous medium, while a value of 15 ns was obtained for **2a** in CH_2Cl_2. An intermediate lifetime of *ca.* 10 ns was measured upon incorporation of either **1a** or **2a** in MM, which indicates that both probes are integrated in the architecture created by the supramolecular entities.

An eye-catching demonstration that clearly proves how the labeled derivatives of CA and Ch are incorporated into the MM was achieved by photographing **1a** and **2a** under a UV lamp, in three different environments (Figure 4): aqueous solution (a, d), 28 mM CA aggregates (b, e), and MM (c, f). As the pictures illustrate, in the case of **1a** an efficient incorporation into both the supramolecular CA aggregates and MM was assessed by the observed fluorescence (Figure 4a–c). More interestingly, in agreement with the fact that the solubility of Ch in aqueous media is extremely limited, the pictures revealed that the fluorescent Ch derivative (**2a**) is hardly solubilized in water or in CA aggregates (Figure 4d and e, respectively). By contrast, a highly emissive solution was observed in the presence of MM (Figure 4f), in agreement with the paramount importance of these micelles in the solubilization of Ch in biological systems.[1]

A complementary approach to investigate incorporation of the CA- or Ch-derived probes was based on laser flash photolysis (LFP) experiments. When NPX was chosen as the labeling chromophore and the resulting dyads **1b,c** and **2b,c** were incorporated into MM and submitted to LFP, transient absorption spectra exhibiting a main band at 440 nm, characteristic of the NPX triplet–triplet absorption in solution, were recorded (see Figure 5A for **2c** as an example). The fact that the spectra of **2b** and **2c** could be measured at 2×10^{-4} M with a good signal-to-noise ratio shows again that solubilization of Ch in an aqueous

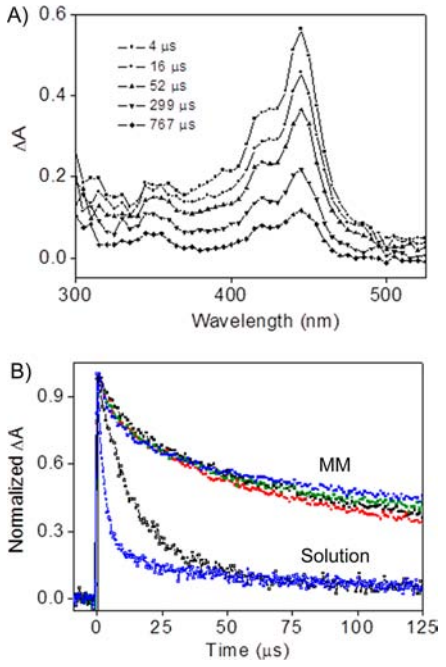

Figure 5. (A) Transient absorption spectra (λ_{exc} = 266 nm) obtained at different delay times after the laser pulse for **2b** within mixed micelles in aqueous 0.2 M NaCl, under nitrogen. (B) Decay traces monitored at 440 nm (λ_{exc} = 266 nm) for **1b** (red), **1c** (black), **2b** (green), and **2c** (blue) incorporated in MM and for comparison **1c** and **2c** in solution (aqueous 0.2 M NaCl or CH_2Cl_2, repectively). All measurements were made under anaerobic conditions, and the concentration of samples was fixed at 2×10^{-4} M.

medium can only be achieved in the presence of MM. Interestingly, a remarkable enhancement of the triplet lifetime (to hundreds of microseconds) was noticed by comparison with the values found for **1c** in an aqueous solution (*ca.* 18 μs) or for **2c** in CH_2Cl_2 (*ca.* 3.2 μs), without any significant stereodifferentiation (Figure 5B). This huge increase in the triplet lifetime can also be exploited as a sensitive parameter to monitor the photoactive probes inside the supramolecular entities provided by MM.

In summary, the incorporation of CA and Ch to the supramolecular architecture provided by MM and the capability of these entities to solubilize Ch have been demonstrated by means of DLS and TEM, combined with photophysical techniques. For this purpose, new tools have been developed by converting CA and Ch into photoactive reporters in which highly sensitive Dns or NPX chromophores are covalently linked to the steroidal skeleton. Replacement of less than 5% of the original components of the MM by any of the photoactive units results in an impressive enhancement of the fluorescence intensity (by 1 order of magnitude in the case of Dns-Ch) accompanied by a corresponding change in the singlet lifetime. Moreover, a remarkable enhancement of the triplet lifetime of NPX-marked CA and Ch has been found by transient absorption spectroscopy. Overall, this is a direct, sensitive, and straightforward methodology to probe incorporation of CA and Ch into MM, which can in principle be extended to other microenvironments experienced by BS or Ch in biological systems.

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Supporting Information Available. Experimental details, spectroscopic characterization of **1a**−**c** and **2a**−**c**, and additional figures. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.