Journal of Inclusion Phenomena and Macrocyclic Chemistry **44:** 127–132, 2002.

Entangled Porphyrins in Cyclodextrin Vesicles

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(Received: 7 May 2002; in final form: 1 October 2002)

Key words: aggregation, amphiphilic cyclodextrins, fluorescence anisotropy, nuclear magnetic resonance, porphyrins, resonance light scattering

Abstract

The entanglement process between porphyrins and some amphiphilic cyclodextrins and the occurrence of different species have been proved by the combination of UV-Vis absorption, fluorescence anisotropy, resonance light scattering and ^{1}H NMR spectroscopy.

Introduction

Porphyrins are requisite substances in most living systems. A wide range of porphyrin derivatives were prepared in order to investigate the porphyrin core behaviour in oxygen affinity, emoprotein activation, electron transfer, and photosynthetic processes. In addition porphyrins photosensitise production of singlet oxygen ($^1O_2$) whose multiform oxidative effects are the basis for photodynamic therapy (PDT) of tumours [1]. Spectral features (fluorescence, UV-Vis absorption) of a sensitizer are prerequisites for photodynamic action. Also their binding to carrier vectors, transporting the sensitizer to tumour tissue, is crucial for the final photodynamic efficiency, since it can result in changes of physico-chemical, photophysical and photochemical properties [2–4]. Cyclodextrins have been widely studied as host molecules for complexing porphyrin derivatives [5–10]. Furthermore, spectroscopic investigations of both cationic and anionic porphyrins interacting with cationic, neutral and anionic micelles have been reported [11–17] as well as equilibrium [18] and kinetic studies [19]. Micellar aggregates [20, 21] and vesicles [20, 22] of amphiphilic cyclodextrins were prepared. Their dynamical properties in water are due to the balance between hydrophobic tails, such as thioalkyl chains, and the hydrophilic heads, such as ethylene glycol oligomers, which increase the colloidal stability of these nanoparticles while potentially decreasing their adverse immune response as "stealth" liposomes [23]. Here we report on the porphyrin organization in nanostructures formed by some amphiphilic cyclodextrins. The potential application in cancer therapy is due to the peculiar photodynamical properties of porphyrins combined with the carrier behaviour of the above mentioned nanoaggregates. The system has been studied by using neutral heptakis (2-O-oligo(ethylene oxide)-6-hexadecylthio-)-β-CD **2** [20–22] and cationic heptakis(2-ω-amino-O-oligo(ethylene oxide)-6-hexylthio)-β-CD **3** (synthesized from precursor heptakis(2-ω-bromo-O-oligo(ethylene oxide)-6-hexylthio) **1** [20, 24] and different porphyrins either insoluble (5,10,15,20-tetrakis(4-hydroxyphenyl)-21H,23H-porphyrin (H_2TPOH, **4**) and 5,10,15,20-tetrakis(4-pyridyl)-21H,23H-porphyrin (H_2TPyP, **5**) or water soluble (5,10,15,20-tetrakis(4-sulfonatophenyl)-21H,23H-porphyrin (H_2TPPS_4, **6**). Figure 1 reports the formulas relative to the investigated systems. The combination of ^{1}H-NMR and UV-vis absorption spectra at different relative molar concentration of porphyrins and CDs as well as static fluorescence depolarisation experiments point out the entanglement process.

Materials and methods

β-Cyclodextrin (Wacker) was crystallised from distilled water and dried under vacuum (0.1 mmHg, 80 °C) for 4 hours. Heptakis (2-O-oligo(ethylene oxide)-6-hexadecylthio)-β-CD and heptakis(2-ω-amino-O-oligo (ethylene oxide)-6-hexylthio)-β-CD were synthesized in our laboratory according to general procedures [20, 24]. 5,10,15,20-tetrakis(4-hydroxyphenyl)-21H,23H-porphine, 5,10,15,20-tetrakis(4-pyridyl)-21H,23H-porphine and 5,10,15,20-tetrakis(4-sulfonatophenyl)-21H,23H-porphine were purchased from Aldrich. The solvents used were purified and dried by standard techniques. All the other reagents were of the highest commercial grade available and were used as received or were purified by distillation

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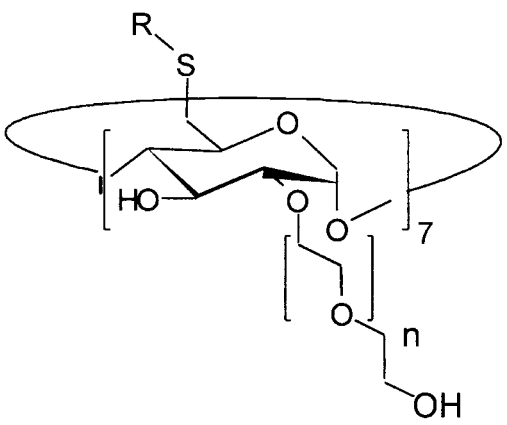
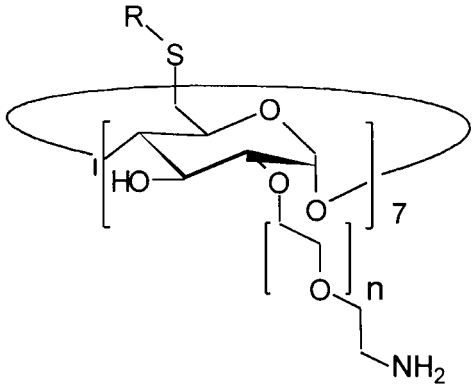
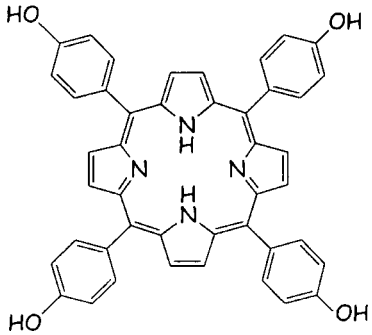


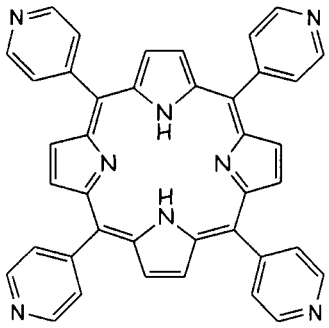
R= $C_{16}H_{33}$ **2** n_{ave}= 1



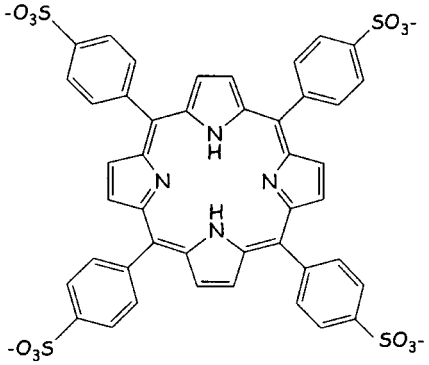
R= C_6H_{13} **3** n_{ave}= 2



H_2TPOH **4**



H_2TPyP **5**



H_2TPPS$_4$ **6**

Figure 1. Amphiphilic cyclodextrins and porphyrins used.

or recrystallization when necessary. Preparation of cyclodextrin vesicles, encapsulation of porphyrins, dialysis, and fluorescence polarization measurements were carried out by using conventional experimental procedures used for liposomes [25].

UV-Vis absorption spectra were obtained by using a HP 8453 diode array spectrophotometer. Fluorescence and resonance light scattering (RLS) experiments were performed on a Jasco model FP-750 spectrofluorimeter, using a synchronous scan protocol for RLS technique. ^{1}H-NMR spectra were recorded on a Bruker AMX-R 300 spectrometer operating at 300.13 MHz. Chemical shifts (δ) are reported in ppm downfield from Me_4Si.

Porphyrin-cyclodextrin interaction

Stock solutions of cyclodextrin **2** and **3** in CH_2Cl_2 were prepared by weighting the proper amount of the compounds. Fixed amounts of a stock solution of **4** in CH_2Cl_2/MeOH (80:20) and of **5** in CH_2Cl_2, respectively, were distributed in a series of vials containing different amount of **2** (in CH_2Cl_2) and the solvent was evaporated in the open air. Phosphate buffer (pH 7) was added and the resulting turbid solutions were sonicated for 3–4 h and equilibrated overnight. An aqueous solution of **6** (0.3 mM) was added to different aqueous colloidal solutions containing increased concentration of CD **3**. The samples were adjusted in volume and equilibrated overnight. All of the investigated systems were studied at 1:1, 1:2, 1:5, 1:10, 1:50 and 1:100 porphyrin/CD ratios ([**4**] = 5.2 μM, [**5**] = 3.1 μM, [**6**] = 2.9 μM).

The formation of aggregated systems (**2/4**, **2/5**, **3/6**) was studied by UV-Vis spectroscopy, fluorescence and RLS techniques. In UV-Vis spectra, the scattering contribution was subtracted from extinction by using the corresponding RLS spectrum, according to a recently reported procedure [26]. The fluorescence spectra are not corrected for the absorbance of the samples. Depolarisation fluorescence spectra were elaborated by using the following equation [27]:

$$r = (I_{VV} \cdot I_{HH} - I_{VH} \cdot I_{HV})/(I_{VV} \cdot I_{HH} + 2I_{VH} \cdot I_{HV})$$

where r is the anisotropy and I_{VV}, I_{VH}, I_{VH} and I_{HV} are respectively the fluorescence intensities registered with different polarizers orientations (being V = vertical and H = horizontal). The depolarised spectra of the CDs vesicles without porphyrin were also subtracted. NMR samples were

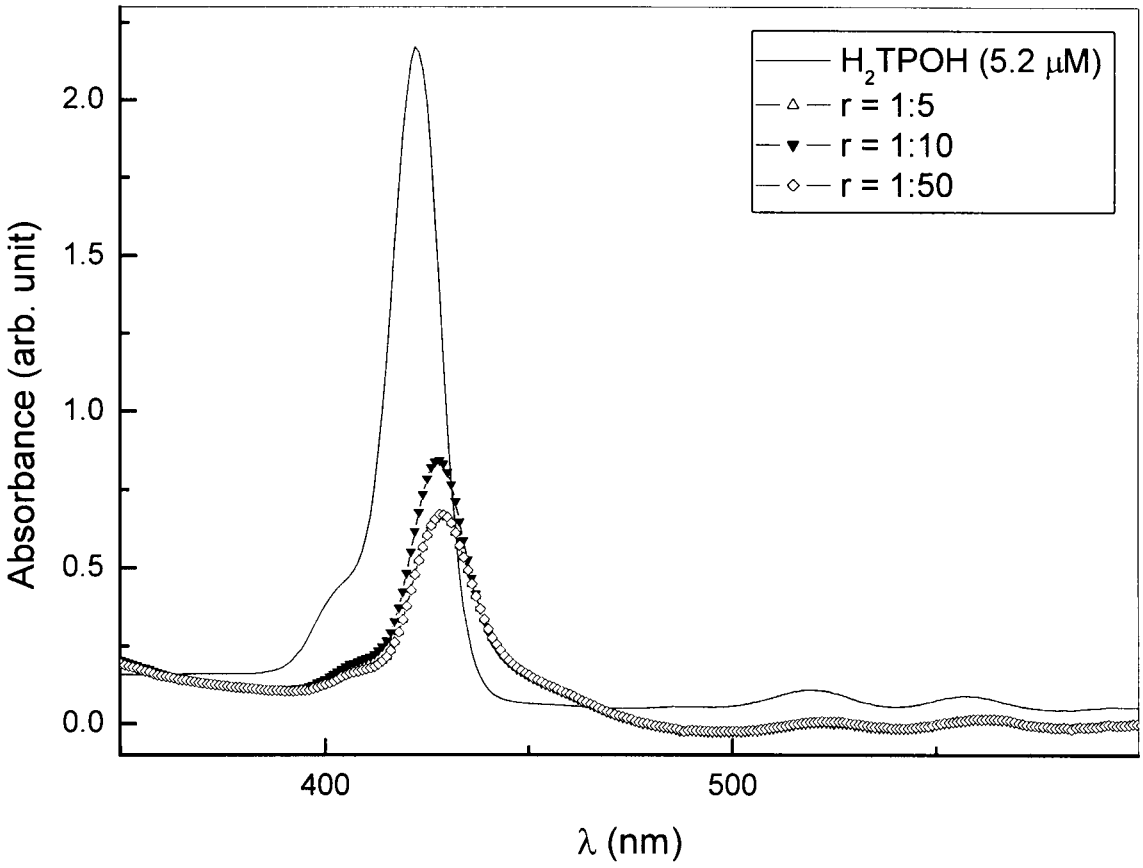


Figure 2. Extinction corrected by scattering[26] of **4** in CH_2Cl_2/MeOH (no CD), and in presence of CD **2** and CD **3** (*r* is porphyrin/CD molar concentrations ratio in buffer pH = 7).

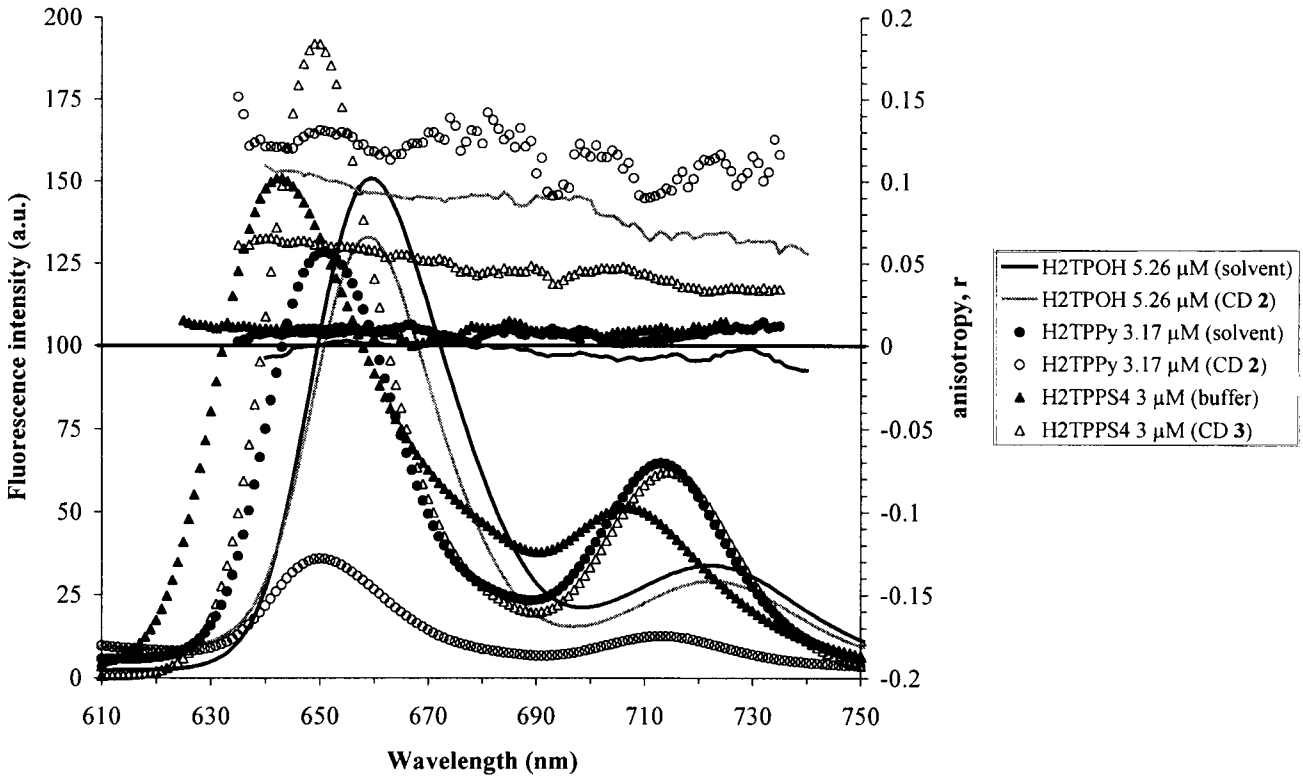


Figure 3. Fluorescence emission and relative anisotropy of all porphyrins (**6** in buffer, **5** in CH_2Cl_2, **4** in CH_2Cl_2/MeOH) and in presence of CD **2** and CD **3** (porphyrin/CD molar concentrations ratio is 1/50) in buffer (pH = 7).

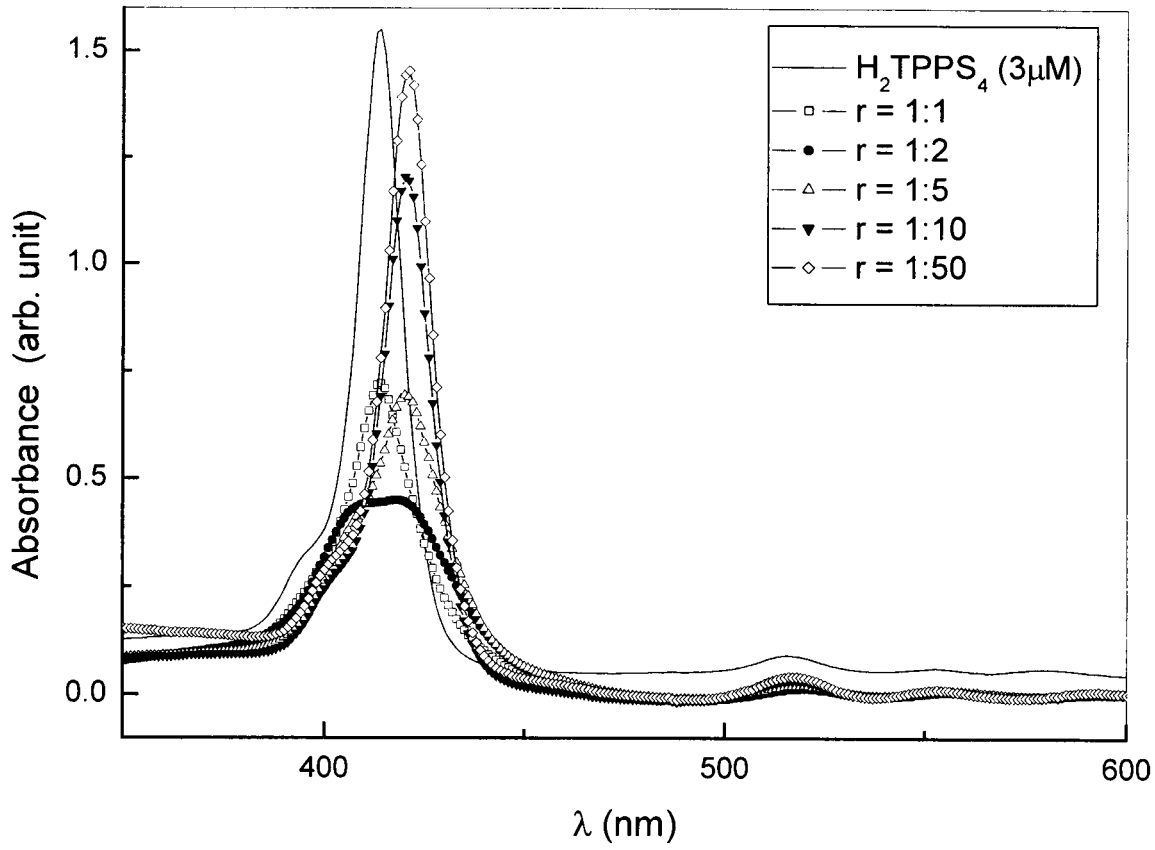


Figure 4. Titrations in buffer (pH = 7) of **6** (3 μM) with increasing amounts of CD **3** (r is the porphyrin/CD molar concentrations ratio).

obtained by dissolving porphyrin **6** (1 mM) in DMSO-d_6 and by adding increasing amounts of CD **3** (porphyrin/CD ratios are 1:1 and 1:2).

Results and discussion

CD **2** forms vesicles at very low concentration (about 0.1 mg/ml) [22]. An investigation of the vesicles effect has been carried out by titrating a fixed quantity of porphyrins **4** and **5** with increasing concentration of CD **2** and monitoring the changes in the UV-Vis absorption, fluorescence emission and RLS spectra. The UV-Vis electronic spectra of **2/4** system are reported in Figure 2, and compared with a neat sample of **4**. This latter exhibits a Soret Band centred at 422 nm ($\epsilon = 2.47 \times 10^5$ M^{-1} cm^{-1}) in CH_2Cl_2/MeOH. On adding CD **2** (according to the conventional procedure used for liposomes [25]), UV-Vis spectra of the new system **2/4** show a Soret band centred at $\lambda = 428$ nm ($\Delta\lambda = +6$ nm), suggesting that the porphyrin core is influenced by the cyclodextrin environment. Besides, by subtracting the scattering contribution from extinction, the Soret band is hypochromically affected with respect to the free porphyrin at different [**4**]/[**2**] relative ratios (1:5, 1:10 and 1:50). The corrected spectra evidence similar intensities (traces with r = 1:5 and r = 1:50 are overlapped) of the Soret band at all investigated ratios except for 1:1 and 1:2. In these latter samples, the UV-Vis spectra show small bumps in the Soret region, suggesting a very low solubility of **4** in CD **2**. Again a bathochromic shift ($\Delta\lambda = +5$ nm) and hypochromic effect were evidenced in UV-Vis spectra (not reported) for **5** in CD **2** vesicles with respect to **5** in CH_2Cl_2 (Soret band at 417 nm). Furthermore, the system **2/5** shows a Soret Band at 422 nm which becomes more intense by increasing the concentration of CD **2**. Fluorescence spectra of H_2TPOH/CD (**2/4**) and H_2TPyP/CD (**2/5**) are depolarised suggesting that porphyrins **4** and **5** are deeply entangled in the vesicles (Figure 3).

Dynamic light scattering measurements evidenced that CD **3** forms nanoaggregates whose sizes range from 120 nm to 1000 nm under our experimental conditions and at concentrations between 1×10^{-6} and 6×10^{-4} M. A fixed quantity of porphyrin **6** was titrated by UV-Vis technique with increasing amount of CD **3** (Figure 3). UV-Vis spectrum of **3/6** system (Figure 4), in 1:1 relative ratio, shows a Soret band centred at 414 nm which is ipochromically affected with respect to the free porphyrin. A broadening of the Soret band is evident at 1:2 relative ratio and, it is probably due to the contribution of two component centred at 414 and 421 nm, respectively. On increasing concentration of CD **6**, the Soret band at 421 nm of the new species becomes more intense. At 1:50 relative ratio we found a maximum of absorbance, while for higher CD concentration the intensity of the Soret Band decreases due to the scattering effect of excess of particles. These spectroscopic observations suggest the presence of different species at above cited ratio. At low concentration of CD **3**, the anionic porphyrin interacts electrostatically with smaller cationic particles. Actually, only the porphyrins which neutralize the charges on the surface

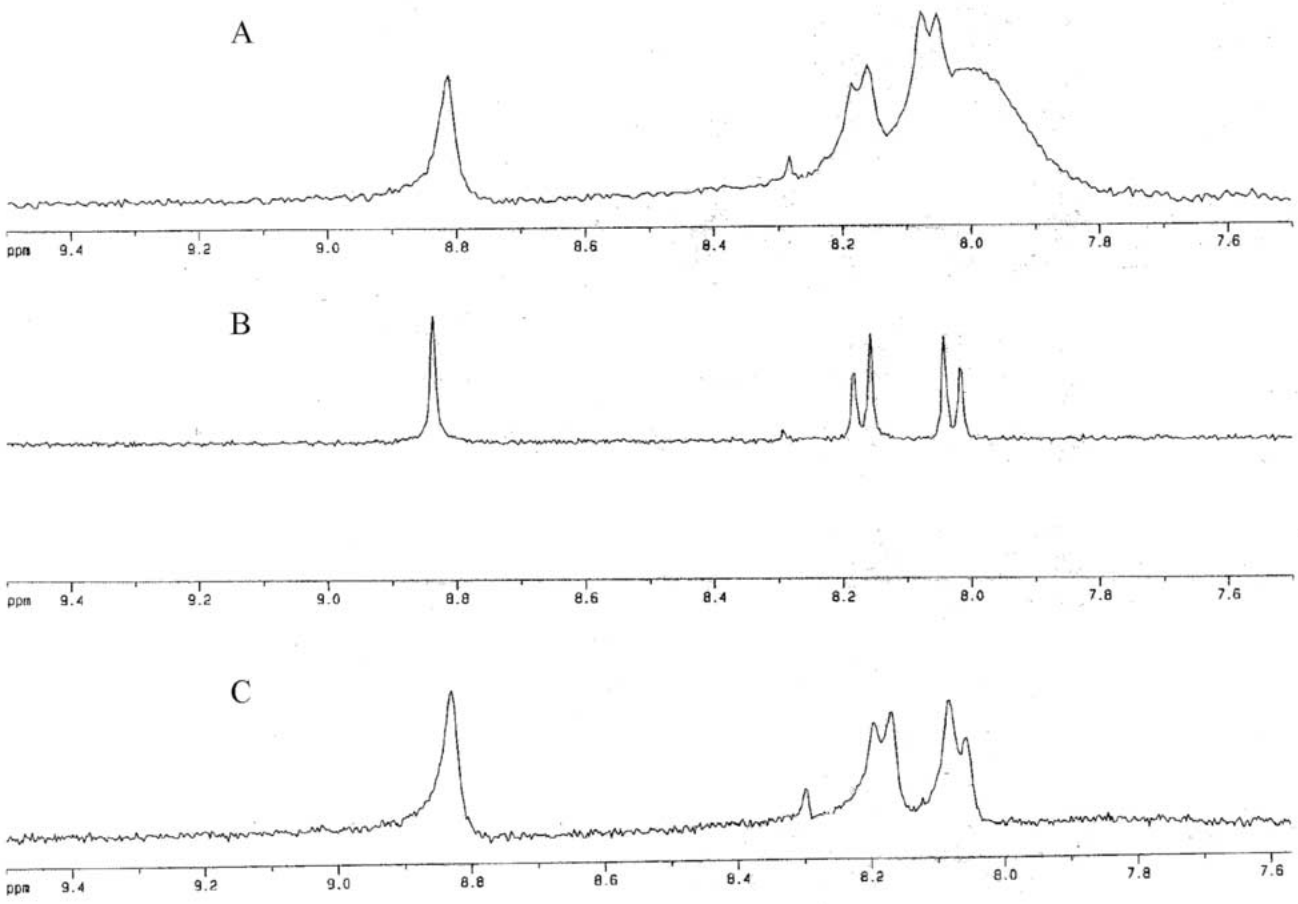


Figure 5. Aromatic region of the ^{1}H-NMR (DMSO-d_6) spectra of porphyrin **5** (1 mM). (A) In the presence of CD **4** (2 mM); (B) no CD; (C) in the presence of CD **6** (1 mM).

of the particles have an affected environment, while most of them are still free in the solvent (Soret band at 414 nm). We presume that steric hindrance can inhibit a full interaction of porphyrins on the smaller particles (120 nm diameter). At higher concentration, the porphyrins are completely encapsulated in bigger nanoparticles, in which their core strongly interacts with a more hydrophobic compartment than water, as confirmed by overnight dialysis experiments. In comparison with **2/4** and **2/5** systems, the fluorescence anisotropy value of **3/6** system is lower pointing to a larger hydrophilicity of the catanionic system. ^{1}H-NMR (Figure 5) of the heteroaggregated **3/6** shows wider signals in the porphyrin aromatic region with respect to those of the free porphyrin, suggesting the occurrence of interactions even in DMSO-d_6.

Conclusion

In summary, the entanglement process, as evidenced by UV-Vis spectroscopy and fluorescence anisotropy, increases remarkably the porphyrin solubility in water, in the case of water insoluble species (**4**, **5**) and promotes a transfer of free molecules from solvent into the vesicles, in the case of the water soluble porphyrin **6**. Interestingly, the heteroaggregate (CD **3**/porphyrin **6**) is formed in DMSO (at high concentrations, NMR experiments) and in H_2O (at low concentrations, UV-Vis absorption experiments) where different species probably exist depending on the relative concentrations. The structure, morphologies and relative sizes are currently under investigation through dynamic and elastic light scattering techniques.

Acknowledgements

The authors thank Dr N. Micali, Dr N. Angelini, Dr D. Lombardo, Dr V. Villari (IPCF-CNR, Messina) for helpful discussion. MURST and CNR, Enterprise Ireland, South African Druggist provided financial support.

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