Novel Heterotopic Colloids of Anionic Porphyrins Entangled in Cationic Amphiphilic Cyclodextrins: Spectroscopic Investigation and Intracellular Delivery

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Abstract: The entanglement process between water-soluble 5,10,15,20-tetrakis(4-sulfonatophenyl)-21*H*,23*H*-porphine and the amphiphilic cyclodextrin (CD) heptakis(2-ω-amino-*O*-oligo(ethylene oxide)-6-hexylthio)-β-CD and the occurrence of various species at different porphyrin:CD ratios were studied by a combination of UV/Vis absorption, fluorescence anisotropy, time-resolved fluorescence, resonance light scattering, and circular dichroism. The effect of the entanglement process on the mean vesicle diameter was investigated over a wide concentration range by quasielastic light-scattering techniques. The experimental results indicate that the presence of porphyrins in this colloidal system promotes some structural rearrangements, essentially driven by charge interaction, which are responsible for a sensitive change of vesicle dimensions. In the range of porphyrin:CD molar ratios between 1:10 and 1:50, the porphyrin is solubilized in monomeric form ($\tau_1 = 11.5$ ns) and photosensitizes the production of singlet oxygen ($^1O_2$). At the same molar ratio the ability of this amphiphilic cyclodextrin to transport porphyrins into tumor cells indicates specificity at the nuclear-compartment level. These findings may be of potential interest for the of development agents for photodynamic therapy of tumors.

Keywords: cyclodextrins · drug delivery · fluorescence spectroscopy · nanostructures porphyrinoids

Introduction

Over the past decade cyclodextrin (CD) colloids with lyotropic and thermotropic properties have been widely reported. In particular, macrocyclic amphiphiles of hydrophobically modified CDs can form a variety of supramolecular assemblies, including monolayers,[1] micelles,[2] and nanoparticles of pharmaceutical importance.[3] Amphiphilic CDs have been admixed with phospholipid monolayers[4] and liposomes.[5] Recently, some of us prepared micellar aggregates[6] and vesicles[6a,7] entirely composed of amphiphilic cyclodextrins. Their structural properties in water are due to the balance between hydrophobic tails, such as thioalkyl chains, and the hydrophilic components, such as ethylene glycol oligomers. The presence of ethylene glycol chains, in particular, increases the colloidal stability of the nanoparticles while potentially decreasing their adverse immune response as "stealth" liposomes.[8] The presence of a complex hydrophilic head group, on the other hand, constitutes an important difference to most of the investigated double-tailed lipids, such as phospholipids. In this respect the corresponding supramolecular aggregates are very sensitive to the geometrical properties of the individual molecule, such as sur-

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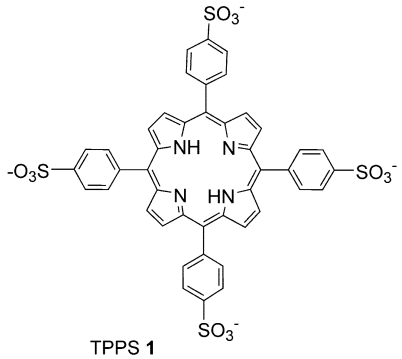
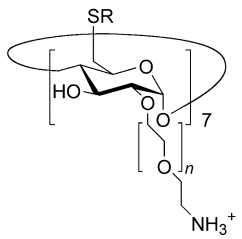
face area or head group hindrance.[9]Cationic vesicles were prepared at physiological pH by transforming OH terminal groups into NH_2 groups in ethylene glycol conjugates.[10] Recently, anionic vesicles of sulfate-modified cyclodextrins were also reported.[11] The ability of anionic and cationic CD vesicles to act as hosts towards guest molecules opens new perspectives in supramolecular chemistry. Our interest is focused on the properties of photochemical biovectors in which a photoactive component is assembled together with an organized receptor (cyclodextrin aggregate), which at the same time can act as an organizing system.[12] As photoactive component we used a porphyrin which photosensitizes the production of singlet oxygen ($^1O_2$), whose manifold oxidative effects are the basis of photodynamic therapy of tumors (PDT).[13] Spectral features (fluorescence, UV/Vis absorption) of a sensitizer are prerequisites for photodynamic action, and its binding properties towards carrier vectors that transport the sensitizer to tumor tissue are crucial for the ultimate photodynamic efficiency, since they may result in changes in physicochemical, photophysical, and photochemical properties.[14] Cyclodextrinss have been widely studied as host molecules for complexing porphyrin derivatives,[15] and many spectroscopic investigations on charged porphyrins interacting with neutral and charged micelles have been reported,[16] as well as equilibrium[17] and kinetic studies.[18]

Here we report on a spectroscopic study on the heterotopic colloidal system formed by cationic heptakis-(2-ω-amino-*O*-oligo(ethylene oxide)-6-hexylthio)-β-CD (SC6CDNH2; **2**), (synthesized from the precursor heptakis(2-ω-iodo-*O*-oligo(ethylene oxide)-6-hexylthio)-β-CD,[6,10] and water-soluble 5,10,15,20-tetrakis(4-sulfonatophenyl)-21*H*,23*H*-porphine (TPPS; **1**). Preliminary results on the interaction of TPPS and other, water-insoluble porphyrins with cationic and neutral nanoaggregates of CDs were reported.[19] The combination of UV/Vis absorption spectroscopy and quasielastic light scattering (QELS) at different relative molar concentration of porphyrins and CDs, as well as steady-state and time-resolved fluorescence measurements, has given insights into the structure of these heteroaggregated species and have revealed the entanglement process.



TPPS **1**



R = C_6H_{13} *n* = 1-2 SC6NH2 **2**

The localization patterns of photosensitizers and the correlation between these patterns and the primary targets of PDT are widely influenced by the physicochemical properties of the drug. In particular, the degree of hydrophobicity[20] often determines the extent of interaction of the photosensitizer molecules with serum proteins, as well as their partitioning among specific sites or domains of cell organelles.[21] In our case, CDs would constitute a suitable hydrophobic environment for specific intracellular delivery and portioning of the investigated porphyrin in cell compartments. The potential application in cancer therapy is due to the special photodynamic properties of porphyrins, combined with the carrier behavior of the above-mentioned nanoaggregates. Therefore, it is important to gain further information on this topic in order to optimize the development of second-generation photosensitizers having improved selectivity for tumor targeting and phototherapeutic efficacy.

Results and Discussion

Spectroscopic investigation of the entanglement process: TPPS usually aggregates under acidic conditions and in the presence of templating species to form various type of aggregates (H- and J-type).[22] Under neutral conditions this porphyrin is mainly present as a monomer, and dimerization in the presence of crown ethers has been reported.[23] Quasielastic light scattering (QELS) measurements (vide infra) showed that SC6CDNH2 forms nanoaggregates whose sizes range from 120 to 1000 nm under our experimental conditions and at concentrations between 1×10^{-6} and 6×10^{-4} M. The effect of the nanoparticles was investigated by titrating TPPS with increasing concentrations of SC6CDNH2 and monitoring the changes in the UV/Vis absorption, fluorescence emission, and resonance light scattering (RLS) spectra. The UV/Vis electronic spectra of the system TPPS/SC6CDNH2 are reported in Figure 1, in comparison with a neat sample of TPPS**.**

The UV/Vis spectrum of the starting TPPS porphyrin shows a Soret band at 414 nm (Figure 1, trace a). At a 1:1 molar ratio this spectral feature is hypochromically affected with respect to the free porphyrin. At 1:2 molar ratio, the Soret band becomes broader, and two components centered at 414 and 421 nm, respectively, are evident.

With increasing concentration of SC6CDNH2, the Soret band at 421 nm, assigned to the new species, becomes more intense and reaches a maximum at 1:50 molar ratio. A red shift in the Soret band was reported for this porphyrin, solubilized as monomer in the micellar phase of neutral TX-100.[16a] At higher CD concentrations (1:80, 1:100 molar ratio) the intensity of the Soret band shows an apparent decrease in intensity (spectra not shown) due to the dominant scattering effect of an excess of suspended particles.

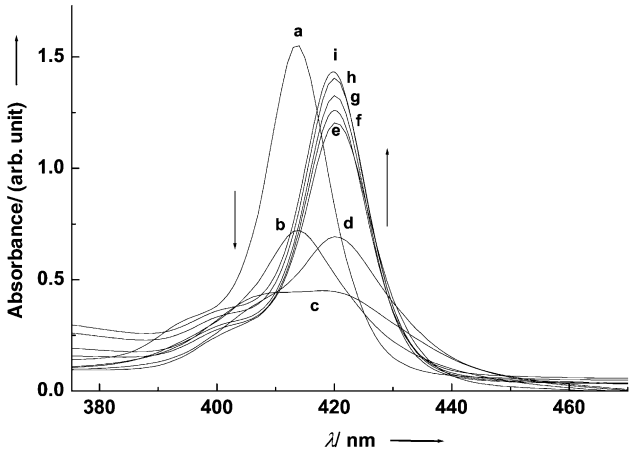


Figure 1. UV/Vis titration of TPPS (3 μM) in phosphate buffer (10 mM, pH 7) with SC6CDNH2. Absorption spectra in the Soret region of TPPS (trace a) and at 1:1, 1:2, 1:5, 1:10, 1:20, 1:30, 1:40 and 1:50 molar ratios TPPS/SC6CDNH2 (traces b to i, respectively).

Circular dichroism (c.d.) spectra of the heterotopic aggregate (see Supporting Information) show a very weak signal centered at 230 nm in the carbohydrate UV region relevant to self-assembled cyclodextrins. The lack of an induced Cotton effect in the Soret region of the porphyrin rules out inclusion of the chromophore in the interior or proximity of the CD cavity.[15c,g]

Fluorescence spectra of the system TPPS/SC6CDNH2, together with their anisotropies, are reported in Figure 2A. The emission spectrum of the starting TPPS, obtained by excitation at $\lambda_{ex}=520$ nm, shows maxima at 642 nm and 706 nm.[22c] At 1:1 molar ratio, the most intense fluorescence band is still centered at about 642–645 nm, and it shows very low anisotropy. At 1:2 molar ratio, the maxima of the emission spectra move to 654 and 710 nm, whereas at higher CD concentration the fluorescence spectra show maxima at 650 and at 716 nm and a larger anisotropy. To relate the steady state anisotropy to the molecular motion, information on the lifetime is needed. Perrin's formula[24] $r=r_0/[1+(\tau/\tau_R)]$ furnishes this relationship, where r is the steady-state anisotropy, τ is the fluorescence lifetime of the fluorophore, τ_R is its the rotational correlation time, and r_0 is the anisotropy of the immobilized fluorophore (e.g., in a highly viscous medium). Fluorescence lifetimes were obtained by time-resolved fluorescence measurements (Figure 2B and Table 1).

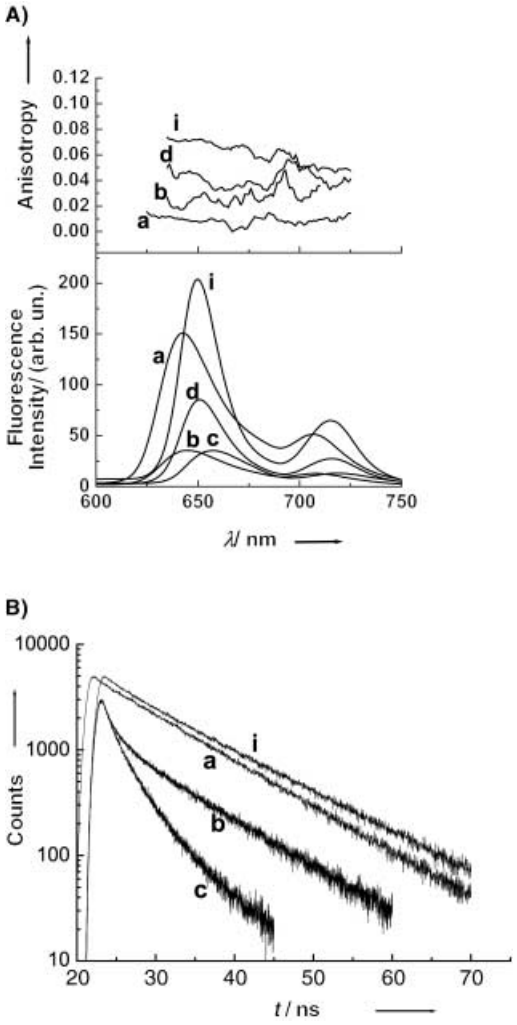


Figure 2. A) Fluorescence emission ($\lambda_{ex}=520$ nm) and relative anisotropy of TPPS (3 μM) in phosphate buffer (10 mM, pH 7, trace a) and in the presence of SC6CDNH2 at 1:1, 1:2, 1:5, 1:50 molar ratios (traces b, c, d, and i, respectively). B) Fluorescence emission decay traces ($\lambda_{ex}=374$ nm) of TPPS (3 μM) in phosphate buffer (10 mM, pH 7, trace a) and in the presence of SC6CDNH2 at 1:1, 1:2, and 1:50 molar ratios (traces b, c, and i, respectively).

As described by the Perrin formula, the molecular reorientational freedom is reflected in the values of the steady-state anisotropy and the lifetime. Inspection of Figure 2B and Table 1 shows that in buffered solution in the absence

Table 1. Fluorescence lifetime parameters for aqueous solutions of TPPS porphyrin (alone) and at different TPPS/SC6CDNH2 molar ratios.[a]

	τ_1 [ns]	τ_2 [ns]	τ_3 [ns]	τ_1A_1 [%]	τ_2A_2 [%]	τ_3A_3 [%]
TPPS[b]	9.7 (100%)			100		
1:1[c]	9.7 (74%)	2.0 (17%)	0.5 (9%)	95	4	1
1:2[d]	8.8 (20%)	3.0 (60%)	0.8 (20%)	47	48	4
1:2[c]	9.6 (22%)	2.8 (58%)	0.7 (20%)	54	42	4
1:5[e]	11.5 (84%)	4.6 (13%)	0.9 (3%)	94	6	0.3
1:10[e]	11.6 (88%)	5.3 (10%)	1.0 (2%)	95	5	0.2
1:50[e]	11.6 (89%)	5.0 (9%)	0.8 (2%)	96	4	0.1

[a] Fluorescence decay parameters were measured in 10 mM phosphate buffer pH 7, 298 K, and $\lambda_{ex}=374$ nm. $\tau\cdot A$ is the relative contribution to the total fluorescence emission (χ^2 is between 0.98 and 1.02). The concentration of TPPS alone in solution and in aggregated systems is 3 μM. [b] $\lambda_{em}=642$ nm. [c] $\lambda_{em}=645$ nm. [d] $\lambda_{em}=654$ nm. [e] $\lambda_{em}=650$ nm.

of CD (Figure 2B, trace a), the steady-state anisotropy and fluorescence lifetime are 0.01 and 9.7 ns, respectively. At high CD concentration, the anisotropy increases to 0.07, which, together with the increased lifetime ($\tau = 11.5$ ns), indicates that the rotational correlation time τ_R increases and is higher than that obtained in the absence of CD. This suggests that porphyrin molecules are embedded in the CD aggregate.

At low CD concentration the use of the Perrin formula does not allow definite conclusions about the rotational correlation time. However, some considerations can be proposed. At the molar ratio 1:1, although the decay is not exponential (Figure 2B, trace b), the fluorescence intensity corresponds almost totally to the free molecule lifetime (9.7 ± 0.2 ns), and the anisotropy has a slightly higher value of 0.02. This indicates that most of the porphyrin molecules remain free and solvated in solution. At the molar ratio 1:2 the picture becomes more complex: the fluorescence decay is strongly nonexponential (Figure 2B, trace c), consisting essentially of two characteristic lifetimes ($\tau_1 = 8.8$–9.6 ns, $\tau_2 = 3.0$–2.8 ns, depending on the emission wavelength), which suggests that the porphyrins are distributed between the solvent and the colloidal phase.

However, in these samples short-lived fluorescent species are also present (Table 1), probably attributable to porphyrins entangled in different hydrophobic compartments, or to minor porphyrin oligomers due to a small degree of self-aggregation, a hypothesis supported by decreased intensity of fluorescence spectra (Figure 2A, traces b and c).

In contrast, at higher CD concentrations (1:5, 1:10, and 1:50) more than 90% of the total fluorescence emission is due to species with lifetimes of 11.5–11.6 ns, and the anisotropy increases up to 0.07. In these cases the Perrin equation suggests that TPPS is probably in a monomeric form, solubilized in SC6CDNH2 and less free to rotate. Indeed, a very similar lifetime value (11.8 ns) was reported by Maiti et al. for TPPS solubilized in Triton X-100 micelles.[16a] These authors assigned such a lifetime to the monomer of TPPS stabilized in the micellar phase of the surfactant. The presence of short-lived fluorescent species, whose contribution is very low (5–6%), also at higher CD concentrations (see Table 1) can be ascribed to porphyrins interacting in more hydrophobic environments than the solvent. At the cited ratios, RLS spectra do not show any intense resonant signal (data not shown), and this is a clear indication that the porphyrins do not extensively self-aggregate in water and in the colloidal phase. This is strictly in line with previous findings reported in literature.[16a] Further investigations on long- and short-lived species in the CD colloidal system are underway in our laboratory in comparison with properties of excited TPPS in aqueous solutions.[22m] In comparison with aggregates of insoluble porphyrins and neutral vesicles of CDs,[19] the fluorescence anisotropies of the system TPPS/SC6CDNH2 are lower and indicative of a larger hydrophilicity of the cationic colloid.

Optical microscope images registered in fluorescence mode show that the red emission of free TPPS in solution (Figure 3, top) becomes highly localized on interaction with the CD vesicles (Figure 3, bottom).

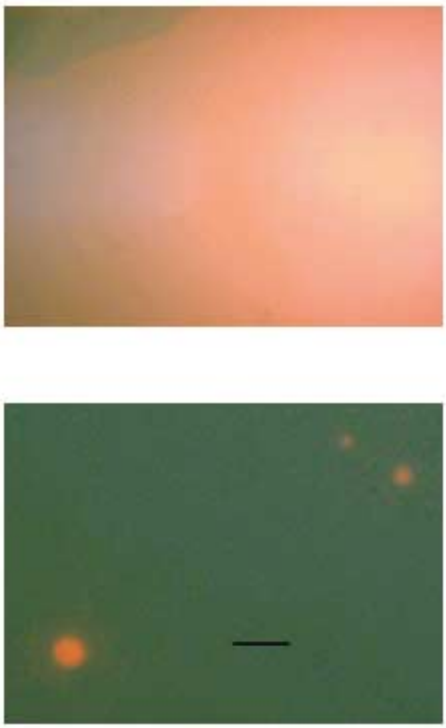


Figure 3. Fluorescence optical microscopy on solutions of TPPS alone (3 μM, top) and in the presence of SC6CDNH2 (1:50 ratio, bottom). The bar measures 5 μm.

Study on structural parameters in the colloidal heteroaggregate: The influence of the entanglement process on the mean vesicle diameter was investigated by QELS techniques. In a QELS experiment the measured intensity–time correlation function $g^{(2)}(t)$ is related to the electric field correlation function $g^{(1)}(t)$ by the Siegert relation [Eq. (1)],[25a–c]

$$g^{(2)}(t) = B(1 + f|g^{(1)}(t)|^2) \quad (1)$$

where B is the baseline and f is a spatial coherence factor. In dilute solutions of monodisperse particles $g^{(1)}(t) = \exp(-k^2 D t)$, where D is the translational diffusion coefficient. From the diffusion coefficient D, the mean hydrodynamic radius R_H of diffusing particles can be calculated by using the Stokes–Einstein equation [Eq. (2)],

$$D = \frac{k_B T}{6\pi\eta R_H} \quad (2)$$

where k_B is the Boltzmann constant, T is the absolute temperature, and η is the viscosity of the solvent. In general, $g^{(1)}(t)$ can be expressed as the Laplace transform of a continuous distribution $G(\Gamma)$ of decay times (relaxation rates Γ).[25a–c] The effective diffusion coefficient $D(k) = [\Gamma(k)/k^2]$ can be obtained by standard second-order cumulative analysis of the autocorrelation functions.[25d,e]

Figure 4 shows the variation of the hydrodynamic radius R_H of CD vesicles with CD concentration. The error bars are related to the polydispersity of the generated aggregates, as evidenced by the observation of a continuous distribution of decay times in the intensity correlation functions. In the

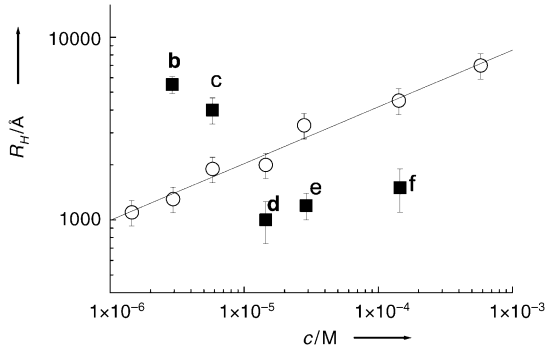


Figure 4. Hydrodynamic radii of aggregates vs SC6CDNH2 concentration in absence of TPPS (○) and in the presence of TPPS (3 μM, ■) at different porphyrin:CD molar ratios. Points b to f refer to 1:1, 1:2, 1:5, 1:10, 1:50 TPPS/SC6CDNH2 ratios, respectively.

absence of porphyrins, the vesicle dimensions increase as a function of the concentration (empty circles). Moreover, a sensitive increase in the mean vesicle hydrodynamic radius R_H is detected after addition of porphyrin at high values of the porphyrin:CD molar ratio. More specifically, it is possible that the charge balance between cationic CDs and anionic porphyrins drives reorganization and growth of the system by incorporating charged anionic species which would induce fusion of the cationic CD aggregate (see Scheme 1).[25f]

Singlet oxygen generation: To test the potential use of these nanoaggregates as novel second-generation photosensitizers, we carried out typical experiments on in vitro $^1O_2$ formation. Figure 5 shows the rate of bleaching of RNO (absorbance maximum at $\lambda = 440$ nm) as a function of irradiation time for samples of TPPS and for aggregated TPPS/SC6CDNH2 at a molar ratio of 1:50. The data ($\delta OD_{440\,nm}$) measured for TPPS in aqueous solution were used as a secondary standard with a quantum yield of 0.6,[26] (the ratio of the rates of bleaching is equal to the ratio of quantum yields). The experimental results show that the investigated aggregate is still an efficient $^1O_2$ generator (quantum yield: 0.2 ± 0.02).

Interestingly, after irradiating the aggregates for 5 min, apart from RNO bleaching, the Soret band centered at 421 nm shifted to 414 nm. This finding suggests that SC6CDNH2 (in particular the thioalkyl groups) could be oxidized and degraded by $^1O_2$, with consequent release of free TPPS in solution The photostability of SC6CDNH2/TPPS is under investigation in order to compare the toxicity of the nanoaggregate towards cell membranes with those of other drug/SC6CDNH2 complexes.[27]

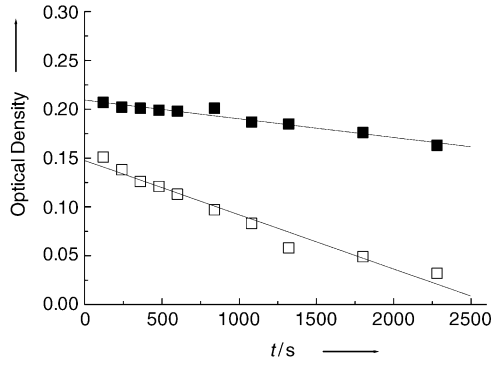


Figure 5. RNO bleaching data in the presence of TPPS without (□) and in the presence of SC6CDNH2 (■) at 1:50 molar ratio.

Intracellular delivery: To verify the ability of SC6CDNH2 to transport porphyrin into the internal compartment of target tumorogenic cells, HEP-2 cell lines were treated with the CD/porphyrin nanoaggregates at different molar ratios, and compared with treatment by TPPS alone. Optical microscope images registered in fluorescence mode (Figure 6) show the results 5 h after exposure of the cells to treatments.

In the absence of CD vesicles, TPPS binds mainly to the cell membrane, as evidenced by the diffuse red fluorescence emission from the single cells (Figure 6, top). A completely different pattern is seen in the presence of CD vesicles. The brightest fluorescence was observed when samples were treated with the aggregate at molar ratios greater than 1:10 (Figure 6, bottom). We estimated that, under these condi-

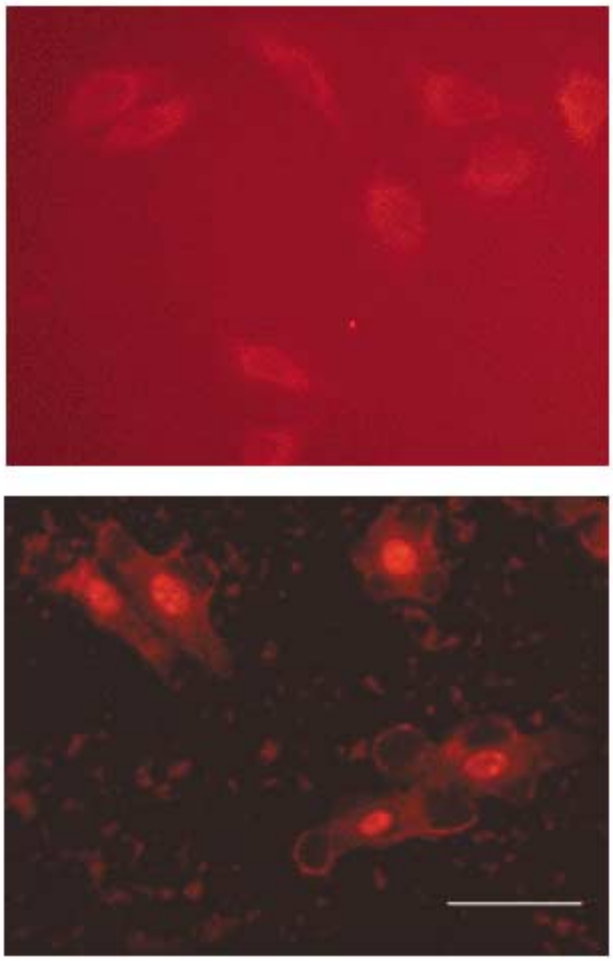


Figure 6. HEP-2 cell lines treated with TPPS alone (top) and with TPPS/SC6CDNH2 nanoaggregates at 1:50 molar ratio (bottom). The bar measures 10 μm.

tions, approximately 90% of the cells were positive for fluorescence emission, that is, porphyrin incorporation.

A diffuse, readily detectable level of intracellular fluorescence intensity in porphyrin-positive cells indicates that cyclodextrin has the ability to deliver porphyrins into the cells, presumably also into the nuclear compartment. It has been reported that either porphyrin inner core protonation or other noncovalent interactions can induce complexation between anionic porphyrins and DNA.[28] Furthermore, literature data show a strong binding between TPPS and histones, positively charged nucleoproteins which play a key role in the compaction of chromatin in the nucleus.[29] Therefore, our findings point to the possibility that TPPS binds to the chromatin of the target cells. Further experiments will verify this possibility and clarify the significance of our observation.

The trypan blue exclusion test showed that, in the absence of exposure to light, cells remain alive for at least 5 h, after which cell damage increased.

The ultimate goal of this experimental approach is to develop new strategies for cancer PDT. The above-described results, together with those already reported on photodynamic therapy of tumors with porphyrins,[30] indicate that cyclodextrin delivery of porphyrin associated with photodynamic activation should be seriously considered in future studies.

Conclusion

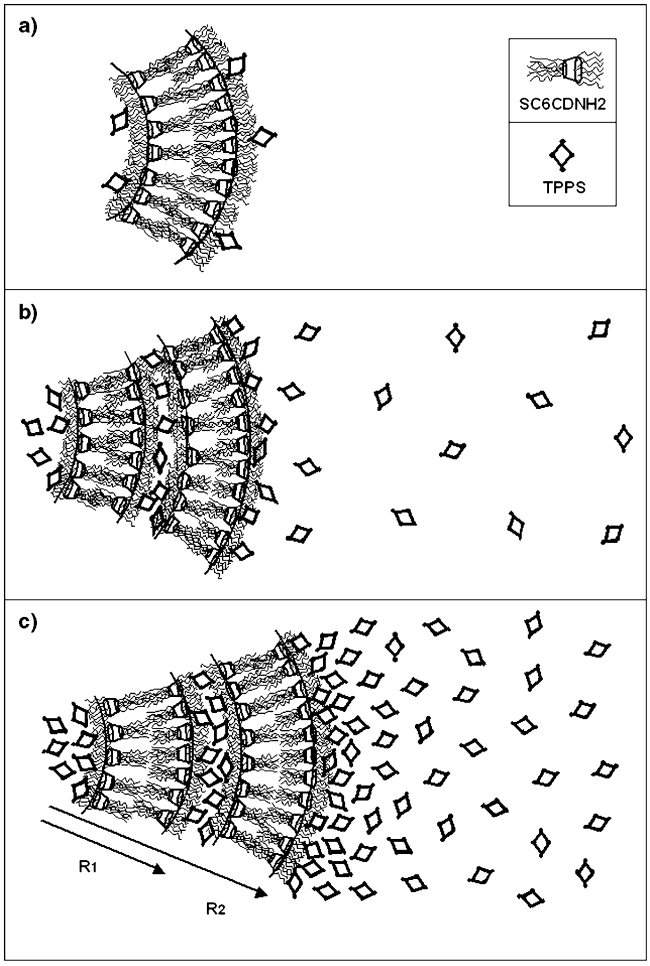
The above experimental findings suggest a scenario in which different species are present at different ratios. Hypothetical models of the heterotopic aggregates are shown in Scheme 1.

At higher CD concentration, porphyrins can interact statistically with more nanoparticles, and most of them are probably entangled in the amphiphilic phase. At these ratios (Scheme 1a), monomeric TPPS is solubilized in the colloidal phase and rotates less freely, as shown by the larger fluorescence anisotropy. Overnight dialysis experiments on solutions at 1:50 molar ratio and electronic and emission spectra of the purified solutions show only a small decrease in TPPS bands, and this is further confirmation for an entanglement process.

At low CD concentration (i.e., at molar ratio 1:2, Scheme 1b), the anionic porphyrin interacts electrostatically with cationic amphiphilic CDs, and saturation of the vesicle surface charge occurs. Actually, only porphyrins which neutralize the charges on the surface of the particles have a changed environment, rotate slowly, and consequently contribute to a larger anisotropy, while most of them are still free in the solvent (Soret band at 414 nm). However, in the self-assembled CDs a small number of porphyrin molecules are hindered by CD chains and are probably "caged" as supramolecular dimers or trimers of TPPS (short-lived fluorescent species).

Strong charge effects at the highest concentration of porphyrins (molar ratio 1:1, Scheme 1c) cause at least an increase in the size of the aggregates (from R_1 to R_2).

Therefore, negatively charged porphyrins cause the progressive neutralization of the vesicle positive charges at the surface, generating a sort of "onionlike" aggregate in which porphyrins surround the original colloidal cyclodextrin aggregate.



Scheme 1. Hypothetical models for the heterotopic aggregates. a) [TPPS/SC6CDNH2] = 1:50, b) [TPPS/SC6CDNH2] = 1:2, c) [TPPS/SC6CDNH2] = 1:1. See text for details.

Porphyrin/CD nanoaggregates are able to photosensitize singlet oxygen production, albeit with lower efficiency than the unbound TPPS porphyrin. Furthermore, the CD vesicles seem to be an efficient system for delivering TPPS into cells. Our experiments in vitro in the absence of irradiation clearly demonstrate a distribution of porphyrin in the cytoplasmic compartment and mostly inside the nucleus, and suggest a tendency to interact with chromatin. These properties seem promising for the design of novel second-generation photosensitizers for PDT, in which the observed decreased photosensitizing efficiency can lead to a reduction of invasiveness without affecting the PDT activity. Further investigations on this issue and the development of more specific carriers for different tumor cells are currently in progress.

Experimental Section

Materials: β-Cyclodextrin (Wacker) was crystallized from distilled water and dried under vacuum (0.1 mmHg, 80°C) for 4 h. Heptakis(2-ω-amino-

O-oligo(ethylene oxide)-6-hexylthio)-β-CD was synthesized according to general procedures.[6,10] 5,10,15,20-Tetrakis(4-sulfonatophenyl)-21*H*,23*H*-porphine tetrasodium salt was purchased from Aldrich Chemical Co. The solvents used were purified and dried by standard techniques. All other reagents were of the highest commercial grade available and were used as received or were purified by distillation or recrystallization when necessary.

Sample preparation: Preparation of cyclodextrin vesicles, encapsulation of porphyrins, dialysis, and fluorescence polarization measurements were carried out by using conventional experimental procedures for liposomes.[31] All solutions were prepared in pure microfiltered water (Angelini). An aqueous solution of TPPS (0.3 mM) was added to different aqueous colloidal solutions containing increasing concentrations of SC6CDNH2. The samples were adjusted in volume with phosphate buffer (10 mM) at pH 7 and equilibrated overnight. The investigated colloidal systems were studied at 1:1, 1:2, 1:5, 1:10, 1:20, 1:30, 1:40 and 1:50 porphyrin:CD molar ratios ([TPPS] = 3 μM).

Measurements: The formation of TPPS/SC6CDNH2 aggregates was studied by UV/Vis spectroscopy, fluorescence, circular dichroism, RLS, and QELS techniques and by measuring the electrophoretic mobility. To check the reproducibility, all spectroscopic titrations were run at least five times. Images were obtained by using an optical microscope (Zeiss Axiovert S100 with Carv) working in transmission and fluorescence modes with Plan Neofluar objectives (63×/N.A. 1.25, 10×/N.A. 0.3, and 50 ×/N.A. 0.50).

UV/Vis absorption spectra were recorded on a Hewlett Packard mod. HP 8453 diode-array spectrophotometer and a Varian UV-Vis-NIR Cary 500 spectrophotometer.

Steady-state fluorescence and RLS experiments were performed on a Jasco model FP-750 spectrofluorimeter by using a synchronous scan protocol for the RLS technique.[32] Fluorescence and RLS spectra are not corrected for the absorbance of the samples. Depolarized fluorescence spectra were measured by using Equation (3), [24]

$$r = (I_{VV}I_{HH} - I_{VH}I_{HV})/(I_{VV}I_{HH} + 2I_{VH}I_{HV}) \quad (3)$$

where r is the anisotropy and I_{VV}, I_{HH}, I_{VH}, and I_{HV} are the fluorescence intensities registered with different polarizer orientations (V = vertical, H = horizontal). The depolarized spectra of the CDs vesicles without porphyrin were also subtracted from the measured spectra.

Time-resolved fluorescence measurements were made on an IBH5000U apparatus with a NanoLED UV centered at 370 nm as excitation source. The experimental data were obtained by time-correlated single-photon counting (TCSPC), and a deconvolution procedure gave a time resolution of about 200 ps. The goodness of fit was assessed by using the plots of weighted residuals, reduced χ^2 values. Circular dichroism spectra were recorded on a Jasco mod. J-600 spectropolarimeter in a quartz cell with path length $d = 0.1$ cm. The ellipticity values $\Delta\varepsilon$ were calculated by using the concentration of SC6CDNH2 at the investigated porphyrin:CD ratios and by considering $d = 0.1$ cm.

QELS measurements were carried out on a computerized homemade goniometer with the 532 nm line of a doubled Nd:YAG laser as excitation source. A Malvern 4700 correlator was used to collect the intensity autocorrelation function in the time domain ranging from 10 μs to 1 s. The temperature in light-scattering experiments was fixed at $T = 295 \pm 0.01$ K. The investigated range of scattering angles was $20 \leq \theta \leq 150°$, which corresponds to exchanged wavevector values in the range of $5.5 \leq k \leq 30.5$ μm^{-1} ($k = [(4\pi n)/\lambda]\sin(\theta/2)$, where n is the refractive index of the sample and λ the vacuum-incident wavelength). Measurements at different angles showed a linear dependence of the relaxation rate on the square of the scattering wavevector and thus indicated the diffusive nature of the detected relaxations.[25a-c]

Singlet-oxygen assay: The amount of $^1O_2$ produced was determined by a standard method based on the bleaching reaction of *p*-nitroso-*N*,*N*′dimethylaniline (RNO).[33,26] The tested sensitizers (nanoaggregate SC6CDNH2/TPPS, or TPPS alone as reference) were dissolved in phosphate-buffered solutions (50 mM, pH 7.4) with imidazole (10 mM) and RNO (50 μM), so that the optical density at the excitation wavelength did not exceed the value 0.1, to avoid shielding effects. In a typical experiment, 500 μL of reaction mixture was poured into a quartz cuvette (Hellma) with a path length $d = 0.1$ cm, and exposed to a homogeneously distributed 100 mW green Nd:YAG laser source ($\lambda = 532$ nm) for different periods of time (from 0 to 40 min). The decrease in absorbance at $\lambda = 440$ nm due to RNO in the samples was registered 1 min after completion of irradiation. The obtained data were normalized by considering $d = 0.1$ cm, in order to compare the changes in optical density (δOD) with literature data.

Cell culture preparation: HEP-2 cells were obtained from American Type Culture Collection and propagated at 1:6 ratio by using Dulbecco's modification of Eagle's Minimal Essential Medium supplemented with new born calf serum (10%). The cells were seeded in a four-well slide and incubated overnight at 37°C in the presence of CO_2 (5%). After incubation the cells were treated for 5 h with TPPS alone and with TPPS/SC6CDNH2 aggregate dissolved in a phosphate-buffered solution (10 mM, pH 7) at molar ratios of 1:1, 1:2, 1:5, 1:10, 1:20, and 1:40 ([TPPS] = 3 μM). Five hours after treatment, nonadherent cells were removed by gentle washing, and the slides were mounted in 90% glycerol and phosphate buffered solution. Unfixed, viable cells were analyzed by fluorescence microscopy.

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