Fluorinated/Hydrogenated Mixed Vesicles as Carrier of Model Biomolecules: A Spectroscopic Study

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Received: March 31, 1997; In Final Form: July 2, 1997[⊗]

Light scattering (LS), small-angle neutron scattering (SANS), electron spin resonance (ESR), and electronic spectroscopy in the UV−vis region were used to characterize hydrogenated/fluorinated mixed vesicles to be used as carriers for molecules of biological and pharmaceutical interest. *n*-Dodecylbetaine and the ammonium salt of a perfluoropolyether carboxylate are known to spontaneously give mixed stable vesicles. Because of its heme-like structure, an octakis(octylthio)tetraazaporphyrin, under the form of the free base and of its Cu(II) metal complex, was utilized as a model cargo to penetrate into the vesicles. The porphyrins appreciably aggregated, as indicated by their electronic spectra, without disturbing the bilayer dynamics and polarity as revealed by ESR data using nitroxide probes located in different regions of the vesicle's membrane. Light scattering data showed, conversely, a significant contraction of the vesicle's radius, which is in agreement with the increased curvature expected from the insertion of polyazamacrocycles in the bilayer membrane.

1. Introduction

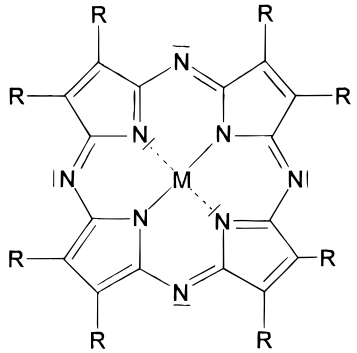
One of the most interesting properties of vesicles made of natural lipids is their ability to trap, either in the limiting membrane or into the inner core, molecules that are then carried in a very specific way.[1−4] The idea of *drug carrier* was born for vesicles in the early 70s with the pioneering studies reviewed by Gregoriadis.[5,6] Since then, liposomes have found applications in the transport and delivery of enzymes, antimicrobic and antiblastic drugs, as immunoliposomes, and for gene therapy. This is mainly due to their biocompatibility, biodegradability, and lack of antigenic activity.[6]

Porphyrins, as well as synthetic phthalocyanines, belong to a class of compounds characterized by a 16-member aromatic ring that can chelate a metal ion and act as an active center in bioprocesses. These molecular skeletons occur in many enzymes and proteins, including heme proteins, such as hemoglobin and cytochromes. It is therefore of relevance to find stable systems able to load and carry porphyrins and derivatives in biological fluids. Recently, Schenning et al.[7] have incorporated porphyrins in positively and negatively charged vesicles by using the ethanol procedure described by van Esch et al.[8] These systems have been characterized mainly by spectroscopic methods.

In the past few years many fluorinated amphiphilics have been synthesized, which show high biocompatibility and high surface activity so that their emulsions are investigated as oxygen carriers, as contrast agents in MRI, and as drug carriers.[9−14] Preliminary evaluation of the biotolerance of fluorinated and perfluorinated surfactants indicates their very low hemolytic activity compared with that of their hydrogenated counterparts. This is particularly interesting for applications in the biomedical, pharmaceutical, and cosmetic fields.

Recently it has been shown, mainly by light scattering techniques, that mixtures of perfluorinated and hydrogenated surfactants, namely, the ammonium salt of a perfluoropolyether carboxylate (PFPE) and *n*-dodecylbetaine, spontaneously form stable vesicles of well-defined radius in a narrow range of proportions of PFPE and betaine.[15] Furthermore, spectroscopy data from hydrogenated and fluorinated ESR probes inserted into these mixed vesicles suggested that domains exist that are either betaine- or PFPE-rich.[15]

In this paper we report on the insertion of a substituted tetraazaporphyrin and of its copper derivative into the hydrophobic regions of vesicles made of fluorinated and hydrogenated surfactants. The structure of the molecules to be carried is given below:



2,3,7,8,12,13,17,18-octakis(octylthio)-5,10,15,20-tetraazaporphyrin

M = H_2: free base (TAP)

M = Cu: copper(II) derivative (Cu-TAP)

Because as many as eight long hydrophobic chains can be grafted in peripheral positions on the porphyrin skeleton, these molecules can be assumed to mimic to a certain extent the steric behavior of some natural compounds, such as the heme-containing proteins.

These porphyrin/vesicle complexes were characterized using direct methods, such as electronic (UV−vis) spectroscopy, static light scattering (LS), and small-angle neutron scattering (SANS),

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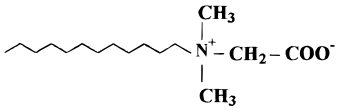
⊗ Abstract published in *Advance ACS Abstracts,* September 1, 1997.

S1089-5647(97)01128-0 CCC: $14.00

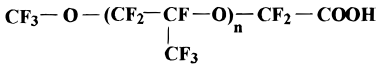
and by an indirect method, that is, electron spin resonance spectroscopy (ESR), which requires the addition of a spin label.

2. Experimental Section

Materials. The vesicles used in this work contained *n*-dodecylbetaine, as the hydrogenated surfactant, and the ammonium salt of a perfluoropolyether (PFPE) as the perfluorinated surfactant:



n-dodecylbetaine



perfluoropolyether carboxylate, ammonium salt (n = 3)

n-Dodecylbetaine (MW = 271, density = 0.93 kg/L) was synthesized and purified as reported in ref 17. Its critical micelle concentration (cmc) is 2×10^{-3} mol/L.[18] The PFPE ammonium salt ($n = 3$, MW = 681, density = 1.8 kg/L) was prepared by treating the acidic form of the surfactant obtained as an intermediate in the synthesis of the PFPE polymers by Ausimont SpA, Bollate, Milano.[19] The monodispersity of the acidic form is ≥95%, as determined by chromatographic analysis. The aggregation properties and the spectroscopic features of the assembled fluorinated systems are reviewed in refs 20 and 21.

The polar head area of the two surfactants is almost the same, i.e., 0.5 nm^2/molecule.[22,23]

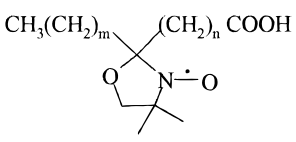
Solutions of both surfactants were prepared in Milli-Q water containing 0.1 mol/L NH_4Cl. In the ammonium chloride solution, molecular interactions among aggregates are strongly decreased.

Vesicles were prepared as described in refs 15 and 16.

The ring-substituted porphyrins, under the form of the free base and of its copper(II) derivative, were used as model compounds to be carried by PFPE/betaine vesicles. Solutions of the porphyrins in chloroform were used for the preparation of vesicle/porphyrin systems, since both TAP and Cu-TAP are very scarcely soluble in water.

The supramolecular structures (micelles, vesicles) containing TAP or Cu-TAP were prepared by deposition of the porphyrin as a thin film on the bottom of a vial by evaporation of the solvent and subsequent addition of the vesicle dispersion. The system was stirred at moderate temperature (308−313 K) for a week.

Nitroxide derivatives of stearic acid (Sigma Chemicals, München, Germany; used without further purification) were the spin probes for ESR characterization:



n + m = 15

m = 12	n = 3	5-DXSA
m = 5	n = 10	12-DXSA
m = 1	n = 14	16-DXSA

Samples containing spin probes for ESR runs were prepared by deposition of thin films of nitroxide on the bottom of a vial by solvent (ethanol) evaporation and subsequent recovery with dispersion of the vesicle after 12−14 h stirring.

No particular treatment was required for UV−vis spectra.

Instrumentation. Light scattering runs were carried out on a standard setup as described in ref 15. The light source was an argon laser (λ = 488 nm), and the angular distribution was measured at θ ranging from 10° to 150°. The scattering parameter q, given by

$$q = (4\pi n/\lambda)\sin(\theta/2)$$

(with $n = 1.33$, the refractive index of water) ranged from 3×10^{-4} to 3×10^{-3} Å^{-1}. The intensity $I(q)$ of the scattered light was scaled with respect to the benzene scattering intensity under the same conditions.

Neutron scattering data (SANS) were collected on the D22 line at the Institut Laue-Langevin (Grenoble, France). For contrast reasons, heavy water was used instead of H_2O.

The absence of any isotopic effect was ensured by carrying out LS measurements on H_2O and D_2O samples with the same molar ratio of all components. The neutron wavelength was 7 Å. Two different sample−detector distances (2 and 8 m) were used, and a total q range of 6×10^{-3} to 0.4 Å^{-1} was covered. Experimental data were corrected using standard methods, including absolute scaling by comparison with the scattering of pure H_2O.

Electronic spectra were registered with the UV−vis Lambda 5 Perkin-Elmer spectrometer interfaced to a PC.

ESR spectra were run with the Bruker 200D spectrometer operating at the X-band. The frequency used in all the reported spectra was 9.59 GHz. Data acquisition and treatment were carried out with the STELAR EPR software. Temperature was controlled with the Bruker ST 100-700 apparatus. ESR spectra and the order parameters S_{33} were computed by using the procedure given by Freed and co-workers[24] as used in several cases of doxylnitroxides inserted into organized systems.

3. Results

The phase diagram of the PFPE/betaine system in 0.1 M NH_4Cl has recently been determined by light scattering experiments.[15] Monodispersed vesicles occur at low total surfactant concentrations, together with polydispersed aggregates of unknown nature and mixed micelles. The mean vesicle radius ranges from 130 to 70 nm, depending on the betaine mole fraction, x_{bet}, and on the total surfactant concentration, with decreasing values obtained at decreasing total surfactant content. The relative ratios of different aggregates in the dispersion also change with the total surfactant concentration and with x_{bet}.

We choose to work with selected and well-characterized vesicle dispersions. Most of the results reported in this work were obtained on vesicle dispersions at total surfactant concentration 0.5% and x_{bet} in the range 0.75−0.79, which contained monodispersed unilamellar vesicles, together with a small fraction of mixed micelles.[15] Other compositions were used when needed to complete the characterization of these systems.

No appreciable difference was observed in the static and dynamic LS patterns when *n*-DXSA paramagnetic probes were added to the vesicle systems. The fit of the experimental data was obtained with the same dynamic and structural parameters as in the absence of probes.[16]

Figure 1 compares the static LS patterns of the pure vesicle dispersion (x_{bet} = 0.75; total surfactant concentration = 0.5%) with the pattern of the same vesicle dispersion containing 3 ×

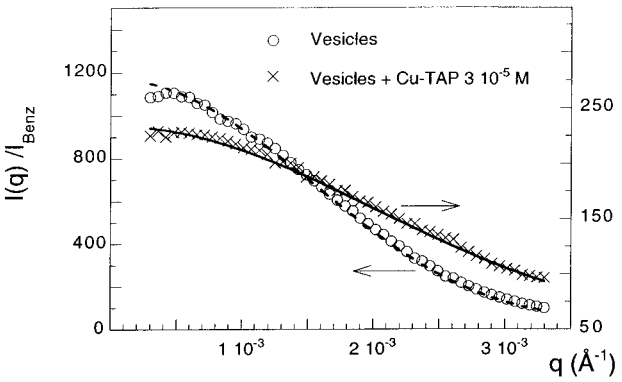


Figure 1. Static light scattering patterns of the betaine/PFPE vesicles (total surfactant concentration, 0.5%, $x_{bet} = 0.75$) in the absence and in the presence of Cu-tetraazaporphyrin. Full line was the best fit obtained from eq 1 (see text).

10^{-5} mol/L of copper tetraazaporphyrin (Cu-TAP) as determined by Cu atomic absorption. This pattern was fitted with the equation[25]

$$I(q)/I_{benz} = A[\sin(qR)/(qR)]^2 + B \qquad (1)$$

where A, B, and R are adjustable parameters. In particular R is the vesicle radius and B is an isotropic contribution to the scattering. Besides an appreciable increase of the isotropic component B, a radius of 65 ± 2 nm was calculated from the fitting for vesicles containing Cu-TAP porphyrin, compared with $R = 84 \pm 2$ nm for porphyrin-free vesicles. The decrease in radius was more apparent when the porphyrin concentration increased.

From the respective weights of the materials used and on the basis of the polar head area, of the vesicle radius, and of the porphyrin concentration, we calculated that for the sample containing 3×10^{-5} mol/L Cu-TAP the porphyrin/surfactant molecular ratio was about $^1/_{450}$. This means that each vesicle contained about 500 porphyrin molecules and that their presence led to a decrease in radius of 20−25%. A ∼5-fold increase in porphyrin concentration results in a loss of structure of the vesicle system. The LS patterns were then no longer represented by eq 1. This finding suggests that polydispersed, ill-defined aggregates were responsible for the scattering phenomena. The same results were observed with the free base and with its Cu derivative, i.e., the same decrease in vesicle radius was found for the same porphyrin content.

Figure 2 shows the SANS patterns of the porphyrin-free and porphyrin-containing vesicles after subtraction of the incoherent scattering contribution. The simulation was carried out with the form factor of a bilayer given by[26,27]

$$I(q) = A[\sin(q\delta/2)/(q\delta/2)]^2 \qquad (2)$$

where δ is the bilayer thickness. The fit gave $\delta = 27.0 \pm 0.5$ Å and $\delta = 27.4 \pm 0.5$ Å for the systems with and without porphyrin, respectively. The very small difference between the two values is probably within experimental errors. This proved that no marked changes took place in the bilayer mean thickness as a consequence of the insertion of the porphyrin. It is meaningful to observe that the above δ values were very close to that ($\delta = 27.8 \pm 0.5$ Å) evaluated by small-angle X-ray scattering in monophasic lamellar aqueous betaine/PFPE systems at much higher total surfactant content (10−60% w/w) for the same PFPE/betaine molar ratio.[28] On the other hand, the lamellar thickness of pure PFPE smectics in water is slightly but significantly smaller ($\delta = 24 \pm 0.5$ Å).[29]

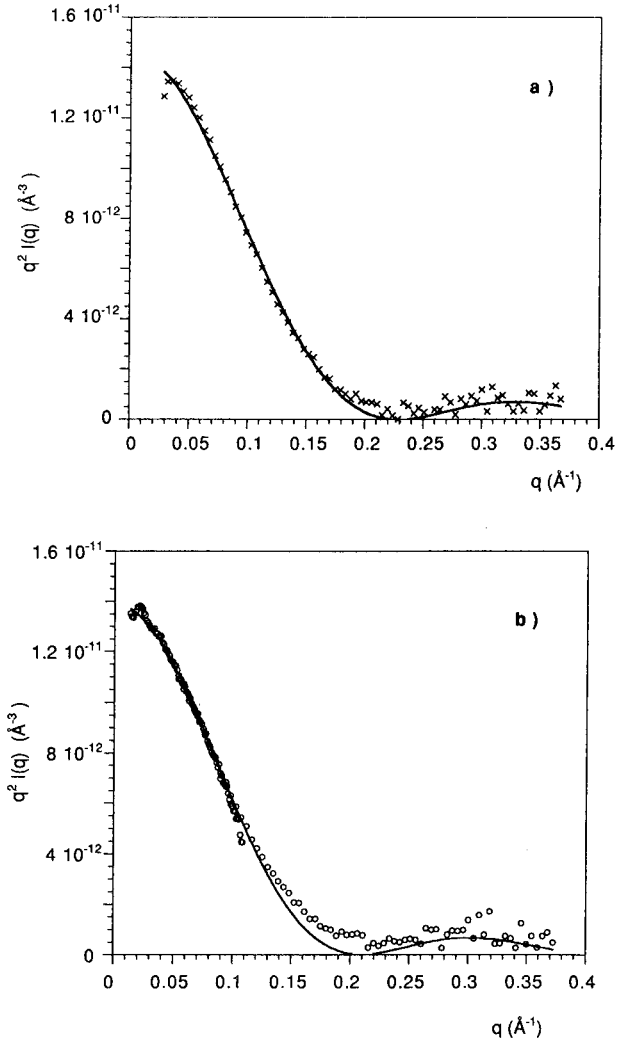


Figure 2. Small-angle neutron scattering patterns of the porphyrin-free (a) and Cu-porphyrin-containing mixed betaine/PFPE vesicles. The incoherent scattering contribution was subtracted and the full line was computed with eq 2 (see text).

Because of broad electron delocalization, the optical spectra of the polyazamacrocycle give most of the information useful for the characterization of systems containing porphyrins, phthalocyanines, tetraazaannulenes, etc.[30] Substitution on the ring's periphery does not appreciably change the electronic spectrum unless the electron conjugation is affected.

Cu-TAP in $CHCl_3$ solution shows the Soret band at 348 nm and the typical Q-band at 673 nm with a shoulder at 613 nm (Figure 3).[31] Both Q absorptions are due to $\pi \rightarrow \pi^*$ transitions, with the higher wavelength band attributed to the purely electronic Q(0,0), or Q_α, band. The 613 nm transition is the vibronic Q(1,0), or Q_β, transition. The large absorption at 500 nm arises from an $n \rightarrow \pi^*$ transition in the electron scheme of the S atoms, as suggested by Schramm and Hoffman.[32]

When Cu-TAP was introduced at different concentrations in the micellar phases made of pure betaine, the visible spectra (Figure 4) showed a marked broadening of the absorptions with significant blue shifts of the $n \rightarrow \pi^*$ band. A red shift to 631 nm was observed for the 613 nm band. No particular changes were observed on the Soret band. No porphyrin uptake was detected by UV−vis spectra for the pure PFPE aggregates in water, which are known to be essentially lamellar structures.[29]

The main features of the spectra the Cu-TAP spectra in betaine/PFPE vesicles (Figure 5) were again the strong broadening of the Q transitions and the decrease of the Q_α/Q_β intensity

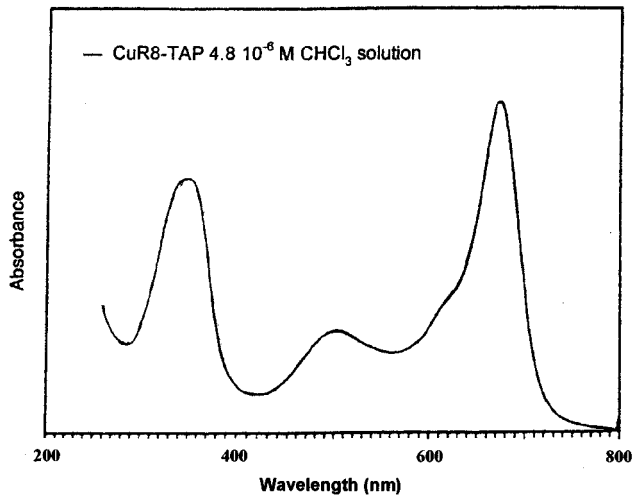


Figure 3. UV−vis spectrum at 298 K of the Cu(II) derivative of octakis(octylthio)tetraazaporphyrin in chloroform solution.

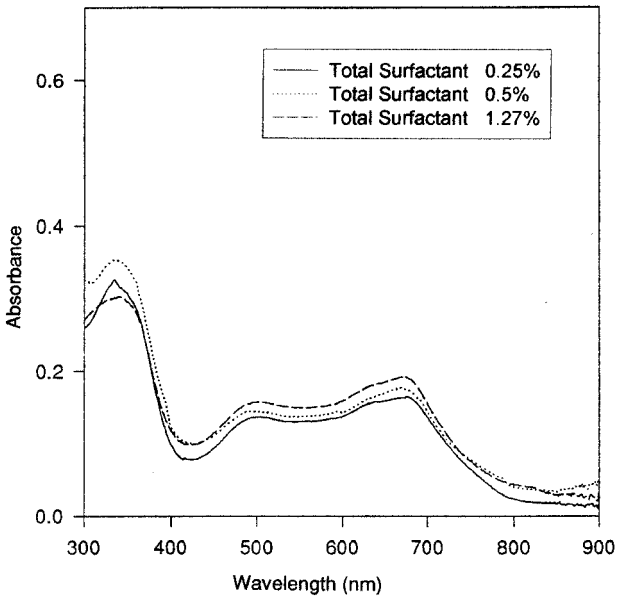


Figure 4. UV−vis spectra at 298 K of Cu-TAP in betaine micellar solutions at different betaine concentrations.

ratio. According to Snow and Jarvis,[33] the decrease of Q_α/Q_β indicates an increasing association. Thus, we suggest that the observed decrease of the Q_α/Q_β ratio in the spectra of porphyrins in vesicles, together with the band broadening observed when concentration increases, is due to strong molecular interactions leading to Cu-TAP association into dimers and/or multimers within the vesicles. These spectral features arise from exciton coupling among π-systems.[34−36] According to Kasha et al.[37] and to Hunter et al.,[38] strong exciton coupling is evidence of porphyrin association in dioctadecyldimethylammonium chloride (DODAC) and dihexadecyl phosphate (DHP) vesicles.[7,8]

The above considerations allow us to conclude that Cu-TAP penetrated the vesicle's double layer where it formed multimolecular assemblies with a behavior similar to that one shown by the same compound in the Langmuir−Blodgett film,[31] in particular showing strongly reduced mobility.

Further information on the status of the vesicles was obtained by ESR, as proved by the literature on vesicles, micelles, reversed micelles, and other supramolecular structures containing porphyrins and proteins.[7,8,21,39−46]

Figure 6 shows the ESR spectra of 5-DXSA inserted into pure PFPE/betaine vesicles and in the same vesicles containing

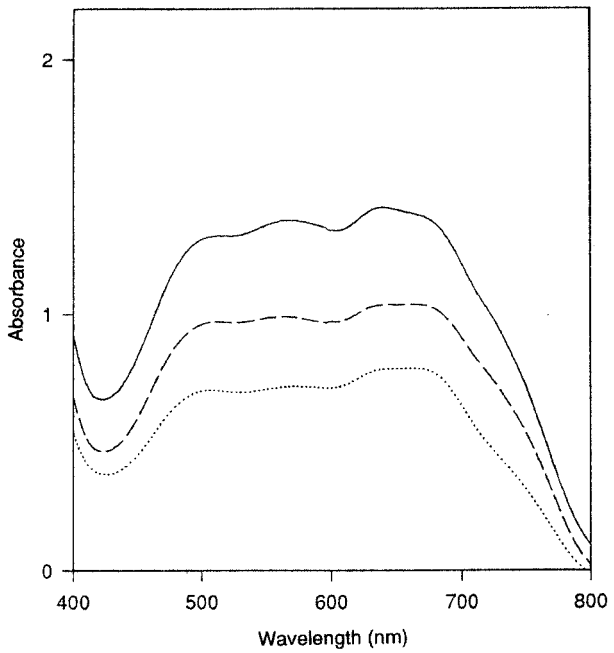


Figure 5. Visible spectra at 298 K of Cu-TAP in mixed betaine/PFPE vesicles at different total surfactant concentrations (tsc) and system compositions: full line, tsc = 1.27%, $x_{bet} = 0.79$; dashed line, tsc = 0.25, $x_{bet} = 0.75$; point line, tsc = 0.5%, $x_{bet} = 0.75$. The porphyrin concentration was 5×10^{-4} mol/L. The 0.5% and 1.27% solutions were diluted with the corresponding porphyrin-free solutions in order to have comparable absorbances.

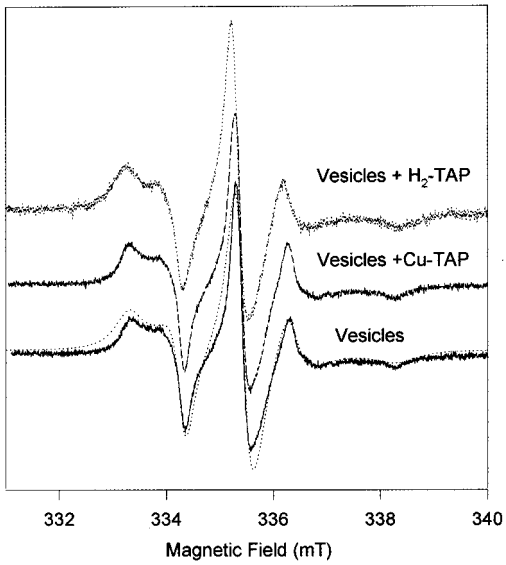


Figure 6. ESR spectra at 295 K of 5-DXSA in (from the bottom) (a) mixed betaine/PFPE vesicles without porphyrin (dotted line is the computed spectrum). Parts b and c (middle and top curves) show the same vesicles with Cu-TAP and H_2-TAP, respectively. Total surfactant concentration is 0.5%, and $x_{bet} = 0.75$.

TAP and Cu-TAP. The spectra were practically the same in the three cases. The presence of porphyrins did not alter significantly either the polarity around the >N−O group or its mean mobility. The analysis of the ESR spectra was done as for plain PFPE/betaine vesicles (dotted line superimposed on the bottom spectrum of Figure 6).[16] The spectra in Figure 6 gave a good fit when computed with $\langle \tau_c \rangle = (2 \pm 0.2) \times 10^{-9}$ s and order parameter $S_{33} = 0.4 \pm 0.1$.

The 12- and 16-DXSA also gave the same spectra in pure vesicles and in the presence of porphyrins, as in Figure 7 for 12-DXSA. In these spectra two different absorptions are identified, which are due to radicals in different motional

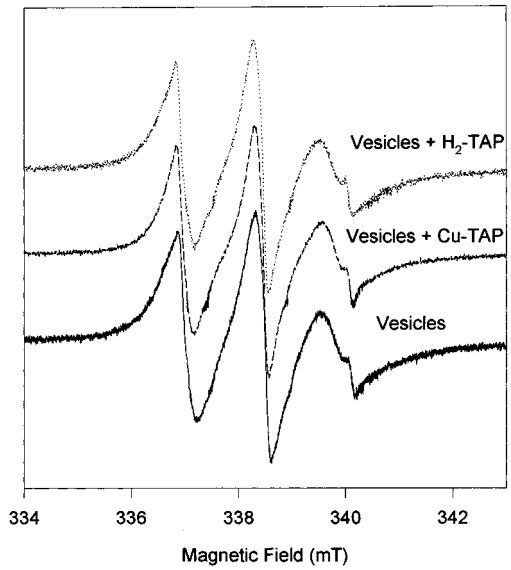


Figure 7. ESR spectra at 295 K of 12-DXSA in mixed betaine/PFPE vesicles, vesicles containing H_2-TAP, and vesicles containing Cu-TAP. Total surfactant concentration is 05%; $x_{bet} = 0.75$.

domains in slow exchange with respect to the ESR time scale, i.e, $\sim 10^{-9}$ s. The more intense one was assigned to radicals in slightly hindered motion, whereas the weaker one was the narrow three-line signal arising from the same radical in the pure water environment. It is noted that the ESR technique is the only one, among those used in this work, able to give information on the dynamics of the chains that form the bilayer. In particular, the DXSA probes allow us to describe the bilayer as being in a fluid state except for the region close to the polar heads, where, as indicated by 5-DXSA, motional restriction and local order are remarkable (cf. the relatively high value of S_{33} given above). This picture of the bilayer is consistent with the possibility of locally modifying the chain packing in order to accommodate large host molecules, such as the porphyrins.

The ESR technique gave clear information on the dynamics of the bilayer chains. The DXSA probes allowed us to describe the membrane as a fluid membrane, except for the region close to the surface, where 5-DXSA sensed a motional restriction and a local ordering. This picture of the vesicle membrane is consistent with a possible local modification of the chain packing so that large molecules as porphyrins may be hosted.

We never observed ESR signals from Cu-TAP. Cu-porphyrins usually give rise to well-resolved ESR patterns where the superhyperfine structure of the ^{14}N nuclei of the tetrapyrrole ring is often resolved. Cu-TAP has been characterized by its ESR spectrum[31,47] either in the form of magnetically diluted powder or in the form of Langmuir−Blodgett films.[31,48] Cu(II)-porphyrin ESR spectra have also been observed in the study of the molecular orientation of Cu-porphyrins into DODAC and DHP vesicles.[7] In this case, however, the vesicles were dried on a Mylar film and strips (up to 25) of these films were used for recording ESR spectra as a function of orientation. A well-resolved superhyperfine structure due to ^{14}N nuclei has been reported by Ishikawa and Kunitake[49] on anionic copper porphyrins in cationic bilayers, and this is a typical example of monomeric porphyrin.

The absence of observable ESR spectra from Cu(II) in our samples could therefore be largely due to the low Cu(II) final concentration, which was a very low concentration to be monitored with conventional ESR apparatus. Unfortunately, we could not increase this concentration because it led to large structural changes in the system under study. In addition, as assessed above, because TAP and Cu-TAP penetrated as molecular aggregates in the surfactant bilayer, Cu multimers with spin angular momenta higher than $^1/_2$ were expected, whose ESR signals are difficult to observe at room temperature.

4. Conclusions

The results reported in this paper on the properties of hydrogenated/fluorinated mixed vesicles show that these systems can be loaded with hydrophobic molecules, such as derivatives of porphyrins with long thiohydrocarbon chains in peripheral positions. These porphyrins can be considered as structurally analogous to some heme proteins. The betaine/PFPE vesicles are stable enough to accommodate many porphyrin molecules. The shape of the Soret and Q-bands in the electronic spectra of the tetrapyrrole ring suggests that porphyrins pile up in the betaine-rich region of the double layer as aggregated structures without appreciably modifying the structural order of the double layer itself or the mobility or the polarity of the region as sensed by paramagnetic probes. As for mixed micelles, whose presence has been indirectly detected by LS in most of the vesicle solutions investigated, we believe that they do not play a significant role in the process of porphyrin insertion within the vesicle bilayer. This is mainly based on the two following pieces of experimental evidence. (i) The number of micelles coexisting with vesicles is very low; they become directly detectable by QELS[15] (i.e., two separated relaxation times exist) and by ESR[16] only at betaine content much higher than those investigated in the present work. Preliminary neutron scattering data are in perfect agreement with these results. (ii) The ESR technique[16] shows that, at least on its time scale ($\sim 10^9$ s^{-1}), the two different types of aggregates behave as independent objects in solution.

The major effect of the porphyrin insertion is a radius contraction of 10−20 nm, the exact value depending on total surfactant concentration, betaine content, and porphyrin type. This increase of the external curvature is confirmed, for instance, by the fact that an upper limit exists for the amount of the porphyrin that can be loaded beyond which the system is no longer stable. The mean thickness of the bilayer does not appreciably change after the insertion of porphyrins. No further information on possible local changes could be given by the techniques used in this work.

Acknowledgment. Thanks are due to the Italian Ministero della Pubblica Istruzione (MPI-MURST) and Consiglio Nazionale delle Ricerche (CNR) for financial support. Financial help from Ausimont SpA, Bollate, Milano, Italy, is also acknowledged. The authors are in debt to Dr. J. Appell for her help in scattering measurements and for very useful discussions.

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