Mixed Fluorocarbon/Hydrocarbon Surfactant Vesicles as Carriers of Metalloproteins: Scattering and Magnetic Resonance Experiments

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Spontaneously formed mixed vesicles were prepared with *n*-dodecylbetaine and the ammonium salt of perfluoropolyether carboxylate, and they were loaded with the water-soluble beef heart cytochrome *c*. The resulting dispersions were characterized with light and neutron scattering patterns, UV−vis spectra, and magnetic resonances (NMR and ESR). Proteins-containing vesicles showed scattering patterns that were interpreted in terms of a decrease of the membrane curvature with a resultant increase of the vesicle size that ranged from 83 to 113 nm, depending on the cytochrome *c* concentration in the dispersion. The interactions between surfaces of protein and of vesicle membrane, which could be responsible for the decreased curvature, did not alter either the optical properties of the heme unit of the protein (from UV−vis) or the dynamics of the vesicle membrane (from ^{1}H NMR of betaine and ESR of membrane-soluble doxylstearic acids). Cytochrome *c* was therefore suggested to be surrounded by a shell of water according to the so-called water-shell model. A comparison was made with the behavior of hydrophobic porphyrins introduced in the same vesicle system, as previously reported from the authors.

1. Introduction

Because of their structure, chemical composition, and controlled size, vesicles and liposomes are presently widely used in many applications involving carrier properties (drugs, vaccines, proteins, genes, immunoderivatives, etc).[1−8] The interest of liposomes for drug and biomolecule delivery in the body is continuously increasing.[9−11] It has been shown that the stability of ordered structures as molecular carriers is increased in a very advantageous way by using vesicles built up with mixed hydrocarbon and fluorocarbon surfactants.[12−17] Ristori et al.[18] have shown that a fluorinated surfactant, namely, perfluoropolyether (PFPE), when mixed in an appropriate molecular ratio with a hydrogenated surfactant, namely, *n*-dodecylbetaine, spontaneously gives rise to stable vesicles with a very low dispersitivity degree. Most of the physical properties of these aggregates, including scattering (light and neutron scattering) and magnetic resonance spectra (NMR and ESR), have been characterized thoroughly.[19] From the ESR data obtained by using small and large nitroxides, different domains in the vesicles exist, which are betaine-rich and PFPE-rich, respectively. These assembled structures have also been characterized as possible carriers for porphyrin-derivative molecules bearing long hydrocarbon and fluorocarbon chains, thus mimicking heme proteins. Scattering, optical, and magnetic resonance spectroscopies have been used in these cases also.[20,21]

In this work we report on the results obtained with the same techniques on the betaine/PFPE vesicles loaded with a very familiar and well-known water-soluble protein, that is, cytochrome *c* (Cyt-c). Cyt-c, together with other cytochromes, belongs to the multiprotein complex known as cytochrome oxidase, that is, the last structure of the respiratory chain, and catalyzes the electron transfer from Cyt-c−Fe(II) to molecular oxygen, which is the electron final acceptor:

$$4\text{Cyt-c}-\text{Fe(II)} + 4\text{H}^+ + \text{O}_2 \rightarrow 4\text{Cyt-c}-\text{Fe(III)} + 2\text{H}_2\text{O}$$

In eucaryotes, the cytochrome oxidase contains up to 13 polypeptide chains and it is known that three of them are coded by the mitochondrial genoma.

We used beef heart Cyt-c, which shows a single peptide chain bearing 104 residues and a covalently linked heme group. The 3D structure has been reported by Takano et al.[22] It appears as a quasi-spherical protein with a mean radius of 1.7 nm. This protein has also been used in the past as an example of protein insertion in nanometer scale systems such as reverse micelles (RM).[23,24] RM dispersions are indeed able to allow the solubilization of drugs and proteins in organic solvents without denaturing or loss of activity.[25,26] Electrostatic forces are supposed to dominate the protein solubilization process.[27−31] These conclusions have been reached by several authors including Kawakawi and Duncan,[32] who studied the insertion of α- and β-lactoalbumine in AOT/isooctane microemulsions, and Paradkar and Dordick.[33] In particular, Pileni et al.[23] have shown that, when inserted in AOT reverse micelles, Cyt-c interacts mostly electrostatically with the negative surface of the RM and no appreciable change in the AOT droplet radius is detected.

In this paper we found that the protein−vesicle interactions were not strong enough to modify the physicochemical properties of the water-soluble Cyt-c and the most relevant effect was a significant increase of the mean size of the carrier vesicles. The possibility that the number of interacting proteins was too low to induce an appreciable effect in the UV−vis and in the ESR data is also taken into account.

2. Experimental Section

Materials. The vesicles used in this work were spontaneously formed vesicles containing *n*-dodecylbetaine (**1**) as the hydrogenated surfactant, and the ammonium salt of a perfluo-

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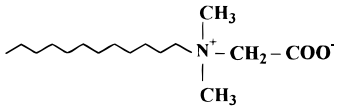
ropolyether (PFPE, **2**) as the fluorinated surfactant:



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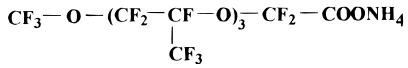

2

Synthesis, purification, and physical properties of **1** are reported in ref 18. The physical and chemical properties of surfactant **2** are described in previously published papers from this laboratory.[19−21]

Solutions of both surfactants were prepared in Milli-Q water containing 0.1 mol/L NH_4Cl. In this solution the molecular interactions among aggregates are strongly decreased. Vesicles were prepared on the basis of the phase diagram of the water/PFPE/betaine system.[18]

Horse heart cytochrome *c* (powder, from Sigma Chemicals, MW = 12 327, purity of 98%) was used as obtained. A stock solution of this protein (10^{-3} mol/L in 0.1 mol/L NH_4Cl water solution) was diluted as needed for the experiments.

Nitroxides used in the ESR experiments were purchased from Sigma Chemicals, München, Germany, and used without further treatment.

Insertion of proteins into the vesicle was accomplished by following two different procedures: (a) vesicles were formed in the presence of the protein by adding to the calculated amount of the Cyt-c solution the corresponding amount of betaine solution, and finally, after the solution was gently stirred for a few minutes, PFPE was added in such an amount to reach the desired protein/betaine/PFPE molecular ratios; (b) the calculated amount of Cyt-c solution was added to the vesicle system prepared as usual.

Instrumentation. Light scattering experiments were carried out on a standard setup as described in refs 18−21, whose light source was an argon laser (λ = 488 nm). In the static experiment the angular distribution was measured at θ ranging from 10° to 150°. As reported in previous investigations on pure vesicles and in porphyrin-containing vesicles,[18−21] all static spectra were well reproduced as the sum of the vesicle form factor plus a contribution due to isotropic scatterers, according to

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

where $I(q)$ is the benzene-normalized intensity, q is the scattering vector

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

R is the vesicle radius, and A and B are adjustable parameters.

Small angle neutron scattering (SANS) runs were made on the D22 line at the Institute Laue-Langevin, Grenoble, France. Solutions in D_2O were used for contrast reasons. More details on data handling and treatment are found in refs 18−21.

Electronic spectra in the UV and visible regions were registered with the aid of the UV−vis Lambda 5 Perkin-Elmer spectrometer.

Continuous-wave ESR spectra were run with the Bruker 200D spectrometer operating in the X band. Data acquisition and handling were done with the help of the Stelar EPR software.

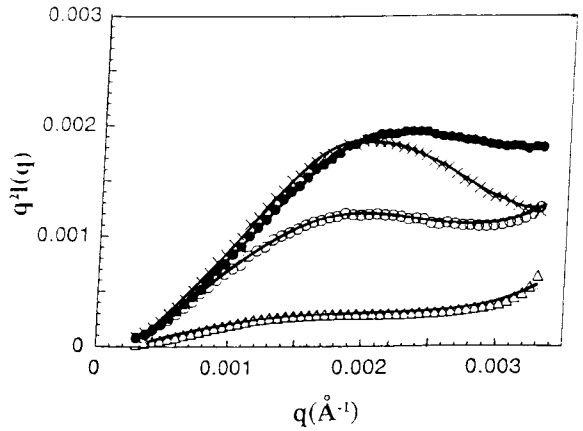

Figure 1. Static light scattering patterns of the betaine/PFPE vesicles dispersions (total surfactant concentration, 0.5%, x_{bet} = 0.75) in the absence (×) and in the presence of different concentrations of cytochrome *c*: (△) 5×10^{-4} mol/L; (○) 5×10^{-5} mol/L; (●) 5×10^{-6} mol/L.

Temperature was controlled (accuracy of ±1 °C) with the Bruker ST100-700 accessory. The procedure of spectral simulation was that reported by Schneider and Freed[34] for microscopically ordered and macroscopically disordered (MOMD) fluid phases as used in several cases of nitroxides inserted into organized systems, including mixed fluorocarbon/hydrocarbon vesicles.

^{1}H NMR 200 MHz spectra were recorded with a Bruker MSL-200 spectrometer by courtesy of the European NMR Large Scale Facility, directed by Professor I. Bertini. Tetramethylsilane (TMS) was used as an external standard. D_2O dispersions of the samples were put in 5 mm external diameter quartz tubes.

3. Results and Discussion

Figure 1 shows the light scattering angular distribution curves of mixed vesicle dispersions containing 0.5% w/w total surfactant concentration at molar fraction x_{bet} = 0.75, and different concentrations of Cyt-c as compared with the same system without added Cyt-c. The observed decrease of intensity after protein addition is to be attributed to the absorption of Cyt-c at λ = 488 nm (cf. the visible spectrum in Figure 3). Full lines are the best fit obtained with the following equation:

$$q^2I(q) = A'[1 - \cos(2qR)] + Bq^2 \qquad (3)$$

which is a modified form of eq 1. The following data were calculated:

	R (nm)	B/A'
pure vesicles	84	1.3×10^5
vesicles + 5×10^{-6} mol/L Cyt-c	82	2.2×10^5
vesicles + 5×10^{-5} mol/L Cyt-c	96	2.7×10^5
vesicles + 2×10^{-4} mol/L Cyt-c	113	4.8×10^5

As it happens for substituted porphyrins and fluorinated porphyrins,[20,21] the mean vesicle radius increased remarkably when a significant concentration of Cyt-c was introduced into the mixed vesicles. The contribution from isotropic objects, as calculated from the B/A' ratio, also increased. Because the uncertainty in the R calculation in these systems was ±3 nm, the presence of 5×10^{-6} mol/L Cyt-c did not change the vesicle size. The reason for the decrease of the bilayer curvature in the Cyt-c-containing vesicle was to be attributed to the interaction of the positive charges on the protein's surface with the negative polar heads of the fluorocarbon surfactants that

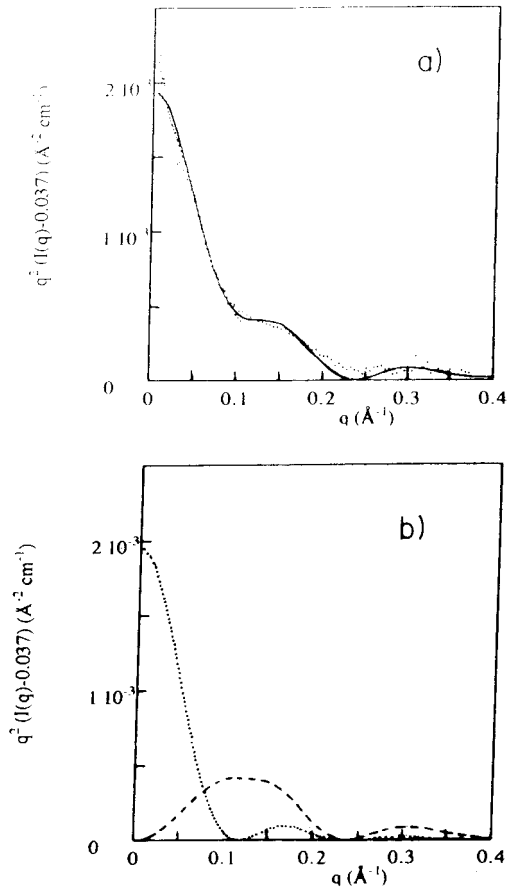

Figure 2. (a) Experimental and calculated (full line) small angle neutron scattering pattern of vesicles containing 2×10^{-4} mol/L of Cyt-c. The procedure followed for the fitting shown in part (a) from curves reported in part (b) is detailed in the text. In all curves an incoherent contribution of 0.037 cm^{-1} has been subtracted from the total intensity.

constitute a large fraction of the vesicle's membrane. The monodispersity of the vesicles was maintained also in the presence of the protein as evidenced by the occurrence of minima in the $q^2I(q) = f(q)$. The same curves were obtained in D_2O and with the two different procedures for vesicle preparation.

It must be noted that the Cyt-c concentrations reported in the above table refer to the total Cyt-c concentration, and only a part of it resided in the vesicle water pool. Although we are not able to determine which was the exact distribution of protein molecules between the inner and outer volumes of vesicles, from an approximate calculation that took into account the measured radii, the concentration of both surfactants, and the polar head areas (0.5 nm^2 per molecule for both surfactants), we evaluated the number of vesicles in a 10^{-4} m^3 volume, which ranged from $(2.3-2.5) \times 10^{15}$ (for vesicles with $r = 82-84$ nm) to $(1.2-1.4) \times 10^{15}$ (for vesicles with $r = 110-115$ nm), with an "outside" water volume to "inside" water volume ratio of 10 to 20. The mean numbers of Cyt-c molecules in the 82−84 nm vesicles and in the 110−115 nm vesicles (from the data of the above table) were 100−150 and 6000−7000, respectively. It clearly appears from these values, that in the water pool of the vesicles there were enough protein molecules that might affect the structure of the vesicle. It is reasonable to assume that proteins residing in the outer volume largely behaved as though they were in the absence of vesicles.

Figure 2a shows the experimental points and calculated SANS pattern (full line) of the same vesicle system shown in Figure

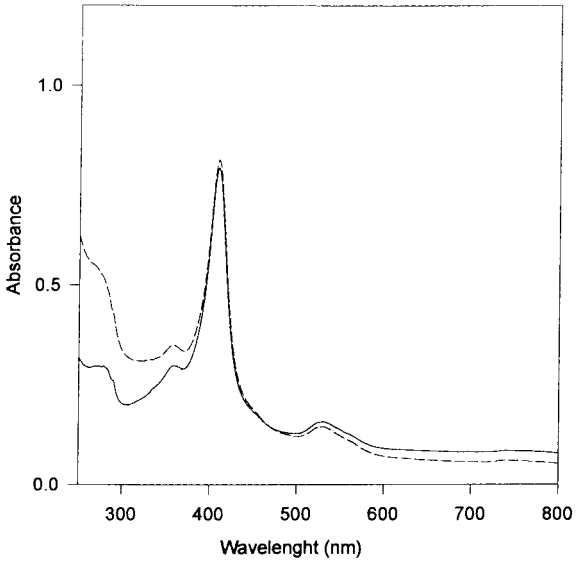

Figure 3. UV−vis spectrum at 298 K of 0.1 NH_4Cl mol/L water solution containing 5×10^{-6} mol/L Cyt-c (full line) and of the same Cyt-c concentration in the mixed vesicle dispersion (dashed line).

1 containing 2×10^{-4} mol/L Cyt-c. The overall shape of the $q^2I(q)$ versus q curve was significantly different from those observed in pure vesicles and in porphyrin-containing vesicles.[18,20,21] The present pattern could not be fitted by using the form factor of the bilayers only. This finding suggested that interactions of the vesicle-forming surfactants with the water-soluble protein might be strongly different from those reported for highly hydrophobic molecules such as porphyrins. A satisfactory fit was obtained by using a linear combination of the two contributions shown in the Figure 2b, that is, (i) the form factor of a bilayer (dotted line in the Figure 2b)

$$q^2I(q) = a[\sin(qd/2)]^2[qd/2]^{-2} \quad (4)$$

where d is the bilayer thickness, and (ii) the form factor of spherical objects with radius r (dashed line in the Figure 2b)

$$q^2I(q) = bq^2[\sin(qr) - qR\cos(qr)]^2[qr]^{3/2} \quad (5)$$

The best-fit values of the adjustable parameters were

$$a = 1.9 \times 10^{-3} \quad d = 5.3 \text{ nm}$$

$$b = 8.4 \times 10^{-3} \quad r = 1.9 \text{ nm}$$

The value $r = 1.9$ nm calculated from the best fit was well in line with the values expected on the basis of a roughly spherical shape of Cyt-c and was in agreement with values reported in the literature.[22] This finding agreed with the fact that a large fraction of protein maintained its overall globular structure after interaction with vesicles.

Figure 3 shows the optical spectra of 5×10^{-6} mol/L of the oxidized form Cyt-c−Fe(III) in an aqueous solution containing 0.1 mol/L NH_4Cl (full line) and in the 0.1mol/L NH_4Cl water dispersion of mixed fluorocarbon/hydrocarbon surfactants (dashed line). The considerations given above on the ratios between Cyt-c molecules in free water and in vesicle water suggest that electronic spectra are dominated by the spectrum of protein molecules in water outside the vesicles. However, the finding that the spectra in the two different systems were practically superimposed in the visible range meant that vesicle surface effects, if any existed, did not change the electronic structure of the prostetic group. In all cases they showed the Soret

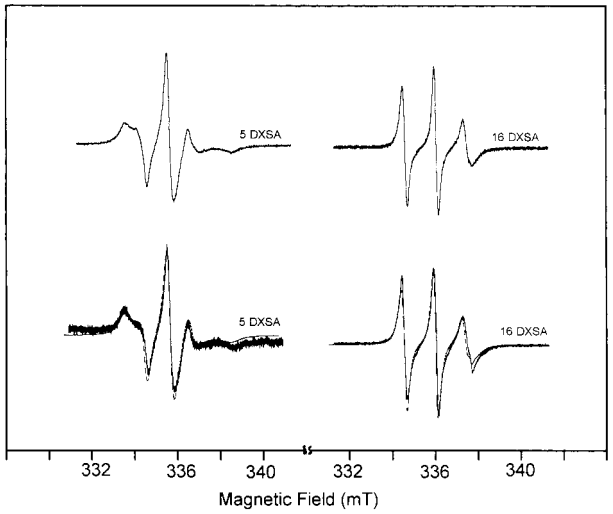

Figure 4. (Top) ESR spectra at 295 K of 5-DXSA (left) and 16-DXSA (right) in pure mixed betaine/PFPE (total surfactant concentration, 0.5%; $x_{bet} = 0.75$). (Bottom) Experimental (full lines) and simulated (dashed lines) ESR spectra of the same vesicle dispersions containing 5×10^{-5} mol/L Cyt-c.

transition at 409 nm and the complex Q bands at 530 nm. For the attribution of the above peaks, reference may be made to previous literature on porphyrins in general and on cytochrome in particular.[35] The increased absorption at $\lambda < 350$ nm was due to light scattering at these wavelengths. This is also observed when substituted porphyrins are introduced into the vesicle double layer.

Neither nuclear magnetic resonance nor electron spin resonance spectra gave results different from results of free vesicles. Figure 4 shows the 295 K ESR spectra in the field region 330−340 mT obtained from 5-doxylstearic acid (DXSA) (right) and 16-doxylstearic acid (DXSA) (left) introduced in pure vesicles (top) and in vesicles containing 5×10^{-5} mol/L Cyt-c (bottom). As is usually reported, the 5-DXSA radical gives details on the dynamics near the water/vesicle interface, whereas 16-DXSA reflects the dynamics of more internal regions of the vesicle membrane. The spectra of 5-DXSA and 16-DXSA, independently of the absence or the presence of the protein, were reproduced with the same structural and dynamic parameters, which were, respectively,[19]

5-DXSA:

$$g_{xx} = 2.0083(\pm.0003) \quad g_{yy} = 2.0070(\pm.0003) \quad g_{zz} = 2.0029(\pm 0.003)$$

$$A_{xx} = 0.53(\pm 0.02)\ \text{mT} \quad A_{yy} = 0.47(\pm 0.02)\ \text{mT} \quad A_{zz} = 3.44(\pm 0.03)\ \text{mT}$$

$$\langle \tau_c \rangle = 2(\pm 0.2) \times 10^{-9}\ \text{s} \quad S_{33} = 0.40(\pm 0.03)$$

16-DXSA (same errors as in 5-DXSA):

$$g_{xx} = 2.0083 \quad g_{yy} = 2.007 \quad g_{zz} = 2.0029$$

$$A_{xx} = 0.52\ \text{mT} \quad A_{yy} = 0.46\ \text{mT} \quad A_{zz} = 3.43\ \text{mT}$$

$$\langle \tau_c \rangle = 4.5 \times 10^{-10}\ \text{s} \quad S_{33} = 0.1$$

The observed lack of dependence of the magnetic and dynamic parameters on the presence of Cyt-c was due to the localization of the protein far enough from the surface of the membrane. The order parameters $S_{33} = 0.4$ and 0.1 in 5-DXSA and 16-DXSA, respectively, were indicative of relevant order near the water/membrane interface and of a diffuse disorder toward the center of the membrane itself. This molecular arrangement was, however, not affected by the occurrence of Cyt-c inside the vesicles. Timmins et al.,[24] in their ESR study of the motion of spin-labeled α-chymotrypsin, myoglobin, and cytochrome *c* enclosed within AOT/isooctane reverse micelles (RM), found that the protein solubilization leads to an increase of the RM size according to the water-shell model in which the protein is surrounded by a shell of water within the RM itself.[36,37] The same mechanism, together with electrostatic interactions, might have a considerable part in the size increase observed in the vesicles reported here. More details on this point could be obtained by using spin-labeled Cyt-c.

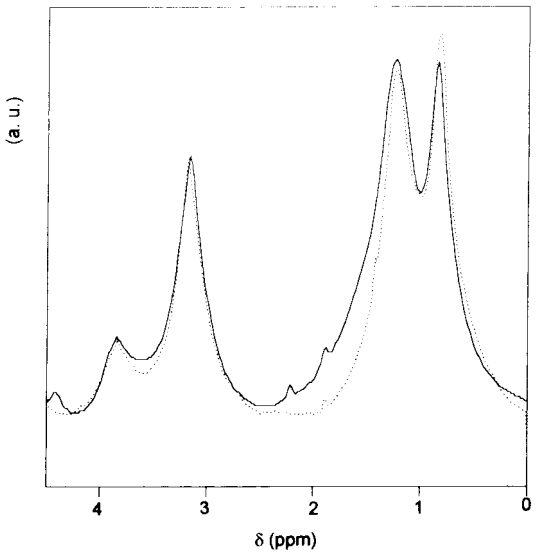

Figure 5. Comparison between ^{1}H NMR 200 MHz spectra at 295 K of pure betaine/PFPE vesicles (dotted line) and the same vesicles containing 5×10^{-5} mol/L Cyt-c (full line). See ref 19 for details of the pure vesicle spectrum.

The same conclusion arose from the NMR spectra. Figure 5 compares the ^{1}H NMR 200 MHz spectrum of pure vesicles, which is due to the proton nuclei of betaine molecules in a partially ordered system, with the spectrum of the same vesicle dispersion containing 5×10^{-5} mol/L Cyt-c. No large spectral changes appeared, showing that the interaction between the protein and the vesicle-forming surfactant molecule was very limited. Together with UV−vis and ESR spectra, NMR results also proved that the Cyt-c molecules that had penetrated the vesicle membrane were localized in the water pool without direct interaction with betaine or PFPE molecules. From this point of view, the water-soluble protein Cyt-c behaved in a different manner with respect to the hydrophobic porphyrins previously studied in the authors' laboratory.[20,21] The lack of narrow peaks due to betaine micelles in the spectrum of Figure 5 seemed to be anomalous if the large isotropic contribution to scattering data observed in the presence of Cyt-c (Figures 1 and 2) is taken into account. However, they might be masked under the larger bands of the vesicle system.

4. Conclusion

Various direct (light and neutron scattering, UV−vis and NMR) and indirect (ESR) spectroscopic techniques on aqueous dispersion of mixed vesicles formed by a fluorosurfactant (PFPE) and a hydrocarbon surfactant (betaine) and containing

a water-soluble protein (Cyt-c) allowed us to suggest that the very simple structural model of mixed vesicles, which was suggested as carriers of biomimetic molecules such as porphyrins, holds for a water-soluble biological model such as Cyt-c.

In a very restricted range of total surfactant concentration and betaine/PFPE molar ratio, monolamellar mixed vesicles formed spontaneously and their radius ranged from 70 to 90 nm, depending on the system's composition. These vesicles are able to accommodate water-soluble proteins such as Cyt-c, whose presence induces a smaller curvature of the vesicle membrane with a resultant increase of the mean radius that depends on the protein content. No perturbation of the dynamics, stability, and shape of the vesicle system is recorded. As in the case of hydrophobic molecules such as porphyrins, these systems proved therefore to be promising in the search for stable carriers of hydrophilic large biomolecules.

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