Interactions between Starburst Dendrimers and Mixed DMPC/DMPA-Na Vesicles Studied by the Spin Label and the Spin Probe Techniques, Supported by Transmission Electron Microscopy

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The interactions between polyamidoamine starburst dendrimers (SBDs) and vesicles consisting of dimyristoylphosphatidylcholine (DMPC) and its phosphatidylcholate salt (DMPA-Na) in various relative ratios (from 0% to 40% of DMPA-Na) were studied by electron paramagnetic resonance (EPR) and by transmission electron microscopy (TEM). The EPR spectra were computer-simulated to extract mobility and structural parameters of the probes and labels. The systems were analyzed as a function of the level of protonation of SBDs and generation (G = 2SBD and G = 6SBD). Both the hydrophobic 5doxylstearic spin probe (5DSA) and the positively charged nitroxide CAT16, a cationic surfactant with a C16 chain, inserted in the vesicles and monitored the formation of dendrimer−mixed vesicle (DMPC/DMPA-Na) complexes. 5DSA revealed a partial ordering of a double-layer-like structure occurring at the dendrimer surface. The spin labels at the vesicle/dendrimer interface modified the structural and mobility parameters upon complexation with the dendrimer. At high levels of protonation, dendrimers showed a larger interaction with the vesicles, especially for the mixed vesicles, compared to dendrimers at a lower level of protonation. In agreement with EPR results, TEM micrographs showed that the addition of DMPA-Na to DMPC modifies the vesicle shape from spherical to rodlike. The EPR analysis suggested that the vesicles wrap around the large dendrimers whereas the small dendrimer directly interacts with the vesicle surface. The results are very promising for a better understanding of the mechanism of interaction of dendrimers with cell membranes which may be an important feature in the role of SBDs as drug and gene carriers to target cells.

Introduction

Recently,[1,2] the electron paramagnetic resonance (EPR) technique has been employed to monitor the interactions occurring between dimyristoylphosphatidylcholine (DMPC, Scheme 1) vesicles and polyamidoamine starburst dendrimers (SBDs),[3] which belong to the fascinating family of dendritic macromolecules.[4] Such studies provide insight to the mechanism of delivery of drugs and genetic material to target cells by means of the SBDs.[5,6] Indeed, SBDs have been used in the pharmaceutical and biochemical fields as drugs and vehicles of biological materials,[5−8] as probes

for oligonucleotide arrays,[9−10] and as primers in polymerase chain reactions (PCR).[9] SBDs have been also coupled to antibodies for application in immunoassays.[6]

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Scheme 1. Structures of Dendrimers, Vesicles, and EPR Probes
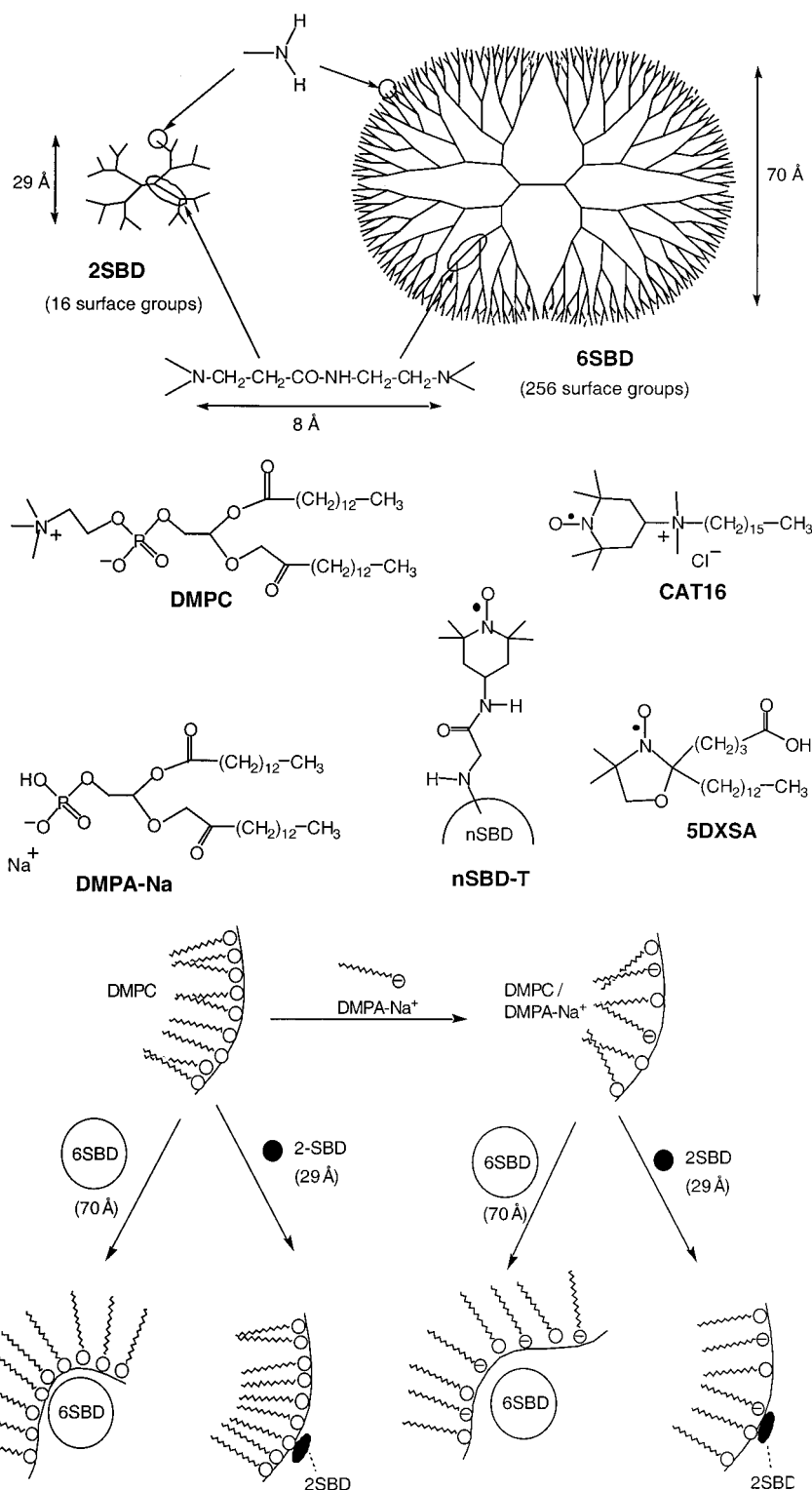
SBDs have been used as viral vectors and gene carriers to deliver DNA sequences in mammalian cells.[11] SBDs are able to mediate the delivery of both natural and synthetic DNA or RNA of various kinds and sizes.[11] In vitro and in vivo transfection experiments involving SBDs at different generations have been successfully carried out.[12-15] The first step in understanding the gene delivery and transfection processes is represented by the investigation of simple binary systems consisting of the dendrimers and each of the interacting biomolecules constituting the biosystem. For instance, the EPR technique has been demonstrated to be a useful tool to

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characterize the binary systems constituted by SBDs and DNA or polynucleotides.[16,17] The results demonstrated the formation of different supramolecular structures between DNA and dendrimers as a function of relative concentrations and dendrimer size.

One of the important issues in such delivery systems is the interaction of the dendrimer with the cell membrane. Since the phosphatidylcholine vesicles or liposomes[18,19] are widely used as model membranes,[20−22] information on dendrimer−vesicle interactions should be of importance and relevance in clarifying the structural details of delivery mechanisms. However, the use of pure DMPC (a zwitterionic phospholipid, Scheme 1) vesicles does not provide an appropriate mimic of cell membranes, which generally possess heterogeneity of the membrane composition. A better model may be achieved by using mixed vesicles, such as those produced by introducing a charged phospholipid to DMPC to produce mixed vesicles. In the present study, we used a mixture of DMPC and its phosphatidylcholate salt termed DMPA-Na (Scheme 1) to produce such mixed vesicles to serve as model membranes.

The use of EPR spin probes to investigate dendrimer−vesicle systems has proven to be very useful if the paramagnetic moiety is situated in the vicinity of the dendrimer/vesicle interface.[2] For this reason, we selected for the present study two spin probes, that is, 5DSA and CAT16 (Scheme 1), which are expected to be bound to the interface of the vesicle bilayer structure with the radical groups (nitroxides) close to the headgroups of the phospholipids. In particular, 5DSA, which is a strongly hydrophobic probe, is expected to bind with the doxyl group embedded in the lipid layer, but in the vicinity of the polar heads of the phospholipids. Consistent with this expectation, previous studies of DMPC vesicles containing 5DSA in the absence and in the presence of SBDs using the EPR and electron spin echo envelope modulation (ESEEM) techniques indicated that the mobility of the probe in the vesicles is partially quenched and has little accessibility to water molecules.[2] The addition of SBDs was also found to induce a partial ordering of the phospholipid layer due to the interactions between the dendrimer surface (amino) groups and the vesicle (phosphate) groups; at the same time, water molecules approach the NO site due to the compression of the water layer at the dendrimer/vesicle interface. In an analysis of the results from the mixed vesicles, we therefore refer to these results from pure DMPC vesicles, further discussed and integrated.

In contrast to the 5DSA probe, the CAT16 probe, a cation, is expected to bind with the nitroxide group directly inserted in the negatively charged headgroup layer of the vesicles. We did not present results from this probe in the previous study done with DMPC vesicles + SBD systems, because the variation of the EPR line shape from the absence to the presence of SBD was found to be almost negligible. But in this report, we will show that the line

shape variation for CAT16 embedded in the mixed vesicles is significant upon going from the absence to the presence of the dendrimers and, therefore, provides information on the structure of dendrimer−vesicle interactions. In this case, we will also use the pure DMPC vesicle system as a reference to evaluate these variations.

As a companion method to the spin probe method, a second EPR point of view of the dendrimer−vesicle interactions can be followed by covalently attaching a spin label to the dendrimer surface groups. In this regard, a previous study has shown that the variations in the spectral parameters of spin-labeled dendrimers serve as excellent monitors of the occurrence of electrostatic interactions between DMPC vesicles and SBDs. These interactions could be monitored as a function of the dendrimer generation (size), the level of protonation state of the dendrimer surface (pH), temperature, and other experimental conditions.[1] The results from this previous study provide a background for comparison with results obtained from the labeled dendrimers in the presence of the mixed vesicles.

In the present study, the SBD−mixed vesicle systems are analyzed under various experimental conditions: (1) variation of the level of protonation of the SBD surface, (2) variation of relative concentrations of DMPC and DMPA-Na composing the mixed vesicles, and (3) variation of temperature. The results are compared and integrated with one other to obtain an overall description of the dendrimer−vesicle systems. The micrographs of the vesicles obtained by transmission electron microscopy (TEM) also assisted greatly in interpreting the EPR results.

Experimental Section

Millipore doubly distilled water was used to prepare the aqueous solutions.

DMPC and DMPA-Na were purchased from Sigma and used as received. The vesicles were obtained using a procedure described by Reeves and Dowben:[23] hydration of the dried lipid film is accomplished by exposing the film to a stream of water-saturated nitrogen, followed by swelling in a sucrose solution without shaking. Spinning allows for the removal of floating multilamellar vesicles. Addition of a glucose solution and further spinning produce a pellet of vesicles, which was dissolved in water. Vesicles with different percentages (in moles) of DMPA-Na, from 0 to 40%, were prepared. The spectral differences between mixed vesicles and pure DMPC vesicles result gradually with the increase of the DMPA-Na content in the vesicles. The results from intermediate DMPA-Na content between 0 and 40% are not described for brevity's sake. Amounts larger than 40% of DMPA-Na in the mixed vesicles did not produce stable and reproducible vesicles.

A portion of a chloroform (Sigma) solution of 5DSA (Sigma, used as received), containing 2% (in moles) of DXSA out of the initial amount of DMPC (+ DMPA-Na), was dried and then left to equilibrate overnight in the vesicle solution in a nitrogen atmosphere. The same equilibration procedure was performed with CAT16 (Molecular Probes, used as received), previously dissolved in a water solution. This procedure favors the insertion of the probe chain in the lipid bilayer.[2] The DMPC−probe solutions, both in the absence and in the presence of the dendrimers, were used immediately after preparation.

Starburst dendrimers (Scheme 1) of generations 2 (2SBD, 16 surface amino groups) and 6 (6SBD, 256 surface amino groups), where the generation refers to the number of covalently attached amidoamine layers employed in this study, have been synthesized as described in previous papers.[3] 2SBD and 6SBD, representative of the "earlier" and "later" SBD systems, have been shown to demonstrate different supramolecular binding characteristics.[3] Careful purification of the dendrimers was accomplished by

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ultrafiltration. The purity of the dendrimers has been determined by ^{13}C NMR spectroscopy, by mass spectrometry, and by gel electrophoresis.[24] The labeling of the dendrimers (*n*SBD-T, Scheme 1) was accomplished by employing a modified method used by Pauly et al. for labeling of DNA-nucleosides[25] and was described previously.[16] The level of labeling was evaluated as 1 label for about 30 surface amino groups by quantitative EPR spectroscopy, employing a nitroxide standard and a flat cell fixed in the EPR cavity.

Water solutions of 2SBD-T and 6SBD-T were prepared at a concentration of 0.1 M in surface amino groups. Unless otherwise specified, the concentration of the SBD solutions is in the surface groups. The solutions were stored under nitrogen, to avoid oxidative degradation, and kept in the refrigerator when not in use.

Protonation of the surface amino groups was accomplished by adding controlled amounts of diluted HCl (0.05 M, Merck). The acid–base properties of the SBDs have been determined in a previous study.[26] Water solutions of *n*SBDs at a low level of protonation (termed *n*SBD) or at a high level of protonation (termed *n*SBD[+]) were then added to the DMPC–DMPA-Na vesicles (containing the probes, in case of unlabeled dendrimers) to obtain a final concentration of 0.05 M in surface amino groups. The natural pH of the dendrimer solution is about 8.5 due to the hydrolysis reaction performed by the amino surface groups. At this pH, previous studies[26] have demonstrated that the dendrimer surface is partially protonated and that complete protonation is accomplished at pH = 4.5. In this report, we are mainly interested in the variations in the EPR parameters relative to the nitroxide probe inside the vesicles or the labels attached to the dendrimers. Therefore, we shall consider that the interaction between dendrimers, both at low and high protonation, and vesicles takes place with no perturbation from the H^{+} and OH^{−} eventually present in solution from the hydrolysis reactions. Therefore, the specific pH of the solution is not taken into consideration.

The EPR spectra were recorded by means of a Bruker 200D or a EMX-Bruker spectrometer operating at X band (9.5 GHz), interfaced to a PC-IBM computer (Stelar software for 200D and Bruker software for EMX) for data acquisition and handling. The temperature was controlled with a Bruker ST 100/700 or a Bruker ST3000 variable-temperature assembly. For the spin probe experiments, the selected temperature was 303 K, whereas for the spin label experiments the selected temperature was 277 K (Results and Discussion).

Additional experiments were performed as a function of temperature and concentration: (1) Temperature increase up to 333 K (higher temperatures could be dangerous for the nitroxide signal surviving) gave rise to a progressive increase of the mobility of the radicals measured by the decrease of the correlation time for rotational motion, τ. The results from computation as a function of temperature (not shown) indicated a logarithmic decrease of τ as a function of the increase in $1/T$. Conversely, the decrease in temperature indicated a progressive immobilization of the radical environment. The analysis of the EPR spectra as a function of temperature supported the adequacy of the simulation model and the obtained magnetic and mobility parameters described in Results and Discussion but did not provide further information on the interactions between the dendrimers and the vesicles. Therefore, these additional experiments were not further discussed. (2) Variation in the relative concentrations of vesicles, probes, and dendrimers did not produce significant variations in the EPR results, with the exception of probe amounts above 2%, which led to spin–spin interactions among close probes embedded in the phospholipid bilayer. We expect a variation of the dendrimer–vesicle interactions by salt effect, for instance, by addition of different amounts of NaCl; this kind of study is in progress.

TEM experiments were carried out using the negative staining technique[1,27] employing 2% (wt/wt) uranyl acetate as the staining agent. The samples were stained for 2 h after adsorption onto a nickel net, covered by a Formvar (polyvinyl-formaldehyde) film. After washing out the excess unbound UO$_2$[2+], TEM measurements were obtained under a vacuum by means of PHILIPS-EM 201-TEM instrumentation.

The concentration of vesicles was about $10^{−10}$ M.

Results

Computation of the EPR Spectra. Computation of the spectral line shape has been carried out by means of the Schneider and Freed program and the NLSL program described by Budil et al.[28]

We assume that the components of the **g** tensor for the Zeeman coupling between the electron spin and the magnetic field are constant: for 5DSA, $g_{xx} = 2.0088$, $g_{yy} = 2.0061$, and $g_{zz} = 2.0027$, as used for the spectra of 5DSA in Langmuir–Blodgett films;[29] for labeled dendrimers, $g_{xx} = 2.009$, $g_{yy} = 2.006$, and $g_{zz} = 2.003$ as reported in ref 2 for SBD-T interacting with DMPC vesicles.

The A_{ii} values (components of the hyperfine tensor for the coupling between the electron and the nuclear spins) are modified in the computation according to a variation in environmental polarity; that is, A_{zz} decreases with the decrease in environmental polarity (A_{xx} and A_{yy} are assumed constant as 5 and 6 G, respectively).

The main parameters indicative of the vesicle–dendrimer interactions are (a) the correlation time for the rotational motion of the radical chain (τ) inserted in the vesicle, which monitors the local fluidity of the vesicle; (b) the diffusion tilt angle of the rotational axis with respect to the magnetic molecular direction. In this case, the true mobility parameter is the perpendicular component of the correlation time for motion, but for simplicity we assume the constant anisotropy value of $\tau_\perp/\tau_{//} = 10$ and calculated an averaged $\langle\tau\rangle = (\tau_\perp/\tau_{//})^{1/2}$; (c) the order parameter (S) which monitors the wobbling motion of the chains in the lipid bilayer, that is, an increase in the order parameter $(0 < S < 1)$ corresponds to an increased organization of the bilayer toward a packed structure characterized by the phospholipid chains parallel to one other, and a decrease in S corresponds to a looser packing of the bilayer where the chains oscillate and twist to and from one other; (d) the intrinsic line width (ΔH) which increases with the increase of spin–spin interactions due to the vicinity of the probes; (e) in some cases, the fitting needed the addition of two computed components. The different percentages of the two components were calculated by subtracting one component (or directly computed, or one experimental spectrum obtained in different experimental conditions) from the overall signal and computing the two components separately. Then, the two computed components were added to each other at the right relative percentage to reproduce the experimental signal.

We cannot exclude that different sets of parameters produce comparable good fitting between the experimental and the computed line shapes. However, the variation of each parameter produces a different modification of the line shape, and each set of parameters, producing a good fitting between computed and experimental line shapes, is the result of several computation attempts. The starting parameters to be modified are assumed on the basis of the physical expectation of the system. Moreover, for the

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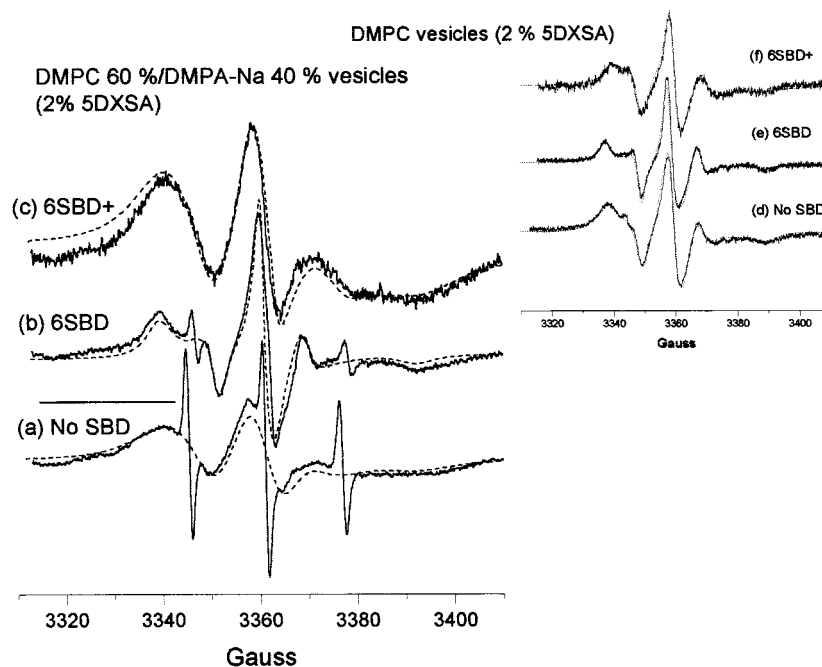(29) Bonosi, F.; Gabrielli, G.; Martini, G.; Ottaviani, M. F. *Langmuir* **1989**, *5*, 1037.

Figure 1. Full lines denote experimental EPR spectra at 303 K of (a) mixed DMPC/DMPA-Na (40%) vesicles containing 2% of 5DSA in the absence of 6SBD; (b) mixed DMPC/DMPA-Na (40%) vesicles containing 2% of 5DSA in the presence of 6SBD at low protonation of the surface and (c) at high protonation. The top inset shows the spectra of pure DMPC vesicles under the same experimental conditions (d−f). Dashed lines denote computation of the spectral components due to radicals inserted in the micelles. Concentrations are 0.05 M in surface groups for 6SBD and about 10^{-10} M for the vesicles.

purposes of the present study, we are interested in the spectral variations in a series of measurements on the same system in similar environmental conditions of the probes. Therefore, the variations in the parameters are significant of the environmental modifications of the probes.

All the parameters have an accuracy of 5% as obtained from the fitting procedure; that is, the fitting between the experimental and the computed spectra is lost when the parameters are increased or decreased by more than 5%.

Spin Probes Inserted in the Mixed Vesicles in the Absence and in the Presence of SBDs. *Results with the Spin Probe 5DSA.* Figure 1 shows the experimental (full lines) EPR spectra of mixed DMPC/DMPA-Na (60%/40%) vesicles containing 2% of 5DSA in the absence (spectrum a) and in the presence of 6SBD (0.05 M), both at a low level of protonation (pH = 8.5) (spectrum b) and at a high level of protonation (pH = 4.5) (spectrum c) at 303 K. In the inset (spectra d−f), the spectra of pure DMPC vesicles under the same experimental conditions are also reported.[1] The results from the small dendrimers (early generations),[3] when the probes inserted in the vesicles are investigated, differed only slightly from the results using the large dendrimers and are therefore not shown. The first main difference, which is evident by comparing the spectra from the pure (DMPC) (spectrum d) and the mixed (DMPC/DMPA-Na) vesicles (spectrum a), is the presence of a contribution from free probe radicals (a narrow three-line signal) in the case of the mixed vesicles. This contribution is absent for the pure DMPC vesicles. Also, the contribution of free probes diminishes in relative intensity upon addition of the SBD to the vesicles (and increases with the increase in DMPA-Na content, results not shown). The contribution of free probes totally disappears for the highly protonated dendrimers (spectrum c). The insertion of DMPA-Na in the vesicles increases the hydrophilicity and polarity of the vesicle surface. Since the 5DSA probe is strongly hydrophobic and it is added to the vesicles at the end of their

Table 1. Main Parameters Used for the Computation of the EPR Spectra of 5DSA Inserted in DMPC and DMPC−DMPA-Na Vesicles in the Absence and in the Presence of the Dendrimers

DMPA-Na (%)	dendrimer	A_{zz}[a] (G)	τ[a] ($\times 10^{-9}$ s)	S[b]	ΔH[c] (G)
0	no	34	6.0	0	1.2
0	6SBD	32	3.5	0.53	1.0
0	6SBD$^+$	35	1.5	0.38	1.5
40	no	34	4.5	0	4.0
40	6SBD	33	4.0	0.45	1.0
40	6SBD$^+$	35	3.5	0−0.2	4.0

[a] Hyperfine tensor component, accuracy 5% as obtained from calculation. [b] Correlation time for motion, accuracy 5% as obtained from calculation. [c] Line width, accuracy 5% as obtained from calculation.

preparation, a portion of 5DSA is unable to insert in the charged vesicle surface and is forced to remain in solution (water is less polar than the mixed vesicle surface). When the dendrimer is added, the polarity of the bulk solution increases, driving the free probes from the aqueous phase into the vesicles. This last effect is more significant for the highly protonated dendrimers. Interactions of the probes in solution with the dendrimers are not expected to occur due to the high polarity of the dendrimer surface.

The computed spectra are reported as dashed lines superimposed on the experimental spectra. For simplicity, only the component due to the radicals inserted in the vesicles is computed; therefore, the spectral contribution from free probes is not further discussed. Table 1 reports the parameters used for computation of the spectra in Figure 1 (also the parameters used for computing the spectra of pure DMPC vesicles are reported for comparison).

The mixed vesicles show an increase of τ (decrease in rotational mobility) and line width with respect to pure DMPC vesicles. The addition of dendrimers provokes the ordering of the bilayer (increase in the order parameter S) which is more substantial for pure DMPC vesicles with
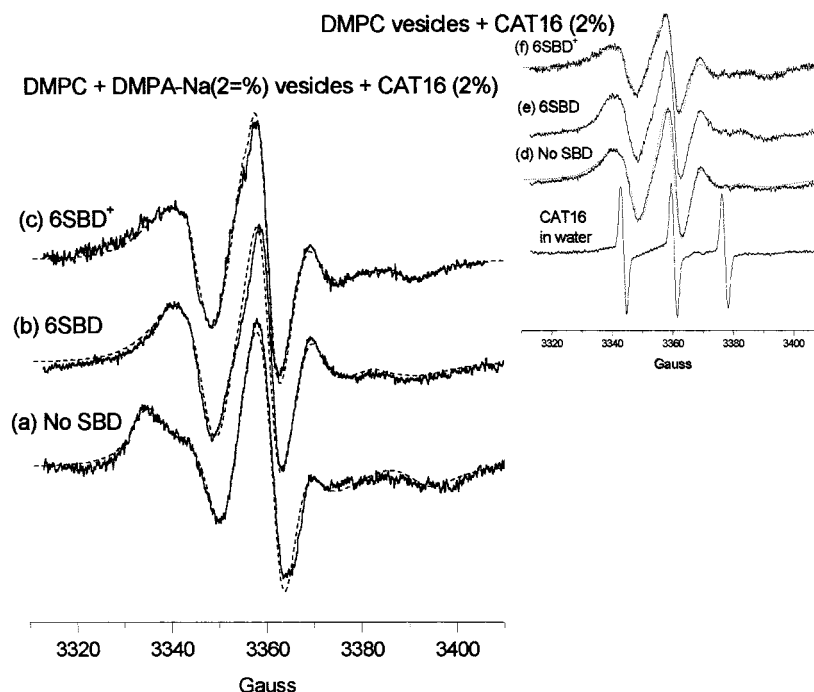
Figure 2. Experimental (full lines) and computed (dashed lines) EPR spectra at 303 K of (a) mixed DMPC/DMPA-Na (40%) vesicles containing 2% of CAT16 in the absence of 6SBD; (b) mixed DMPC/DMPA-Na (40%) vesicles containing 2% of CAT16 in the presence of 6SBD at low protonation of the surface and (c) at high protonation. The top inset shows the spectra of pure DMPC vesicles in the same experimental conditions (d−f) together with the spectrum of CAT16 in water solution. Concentrations are 0.05 M in surface groups for 6SBD and about 10^{-10} M for the vesicles.

respect to mixed vesicles. Contemporaneously, the line width decreases mainly for the probes in mixed vesicles.

The addition of 6SBD$^+$ to the mixed vesicles does not significantly modify the EPR line shape except for the complete disappearance of the contribution due to free radicals described above. The broadening of the signal (Figure 1c) prevents a correct computation of the line shape, and we could compute the spectrum assuming both a small S value (maximum S value = 0.2) and no ordering ($S = 0$). In both cases, the polarity slightly increases (A_{zz} = 35 G) and the rotational mobility also increases with respect to the mixed vesicles both in the absence and in the presence of 6SBD.

Results with CAT16. Figure 2 shows the experimental (full lines) EPR spectra of mixed DMPC/DMPA-Na (60%/40%) vesicles containing 2% of CAT16 in the absence (spectrum a) and in the presence of 6SBD (0.05 M), both at a low level of protonation (pH = 8.5) (spectrum b) and at a high level of protonation (pH = 4.5) (spectrum c) at 303 K. In the inset (spectra d−f), the spectra of pure DMPC vesicles under the same experimental conditions are reported. Also, in the inset the spectrum of CAT16 in water solution is reported for comparison. The dashed lines superimposed on the experimental spectra are the spectra computed as described above. The results from early generation dendrimers (2SBD) are not discussed since they do not differ significantly from those of later generation dendrimers (6SBD) as in the case of 5DSA as the spin probe. With respect to the computation of the spectra of 5DSA, in this case the A_{zz} parameter (= 35 G) does not change from one system to another, indicating almost the same environmental polarity for all the investigated systems. The hydration layer at the vesicle surface (which hydrates the nitroxide group) is therefore not modified by the interaction with the dendrimer. Furthermore, the ordering of the carbon chains is not monitored by the CAT16 groups, which are located at the external surface. The only parameter changing from one

Table 2. Correlation Times for Motion Used for the Computation of the EPR Spectra of CAT16 Inserted in DMPC and DMPC−DMPA-Na Vesicles in the Absence and in the Presence of the Dendrimers

DMPA-Na (%)	dendrimer	τ^a ($\times 10^{-9}$ s)
0	no	4.0
0	6SBD	4.0
0	6SBD$^+$	5.0
40	no	7.5
40	6SBD	4.0
40	6SBD$^+$	5.0

^{a} Accuracy 5% as obtained from calculation.

system to the other is the correlation time for motion, which is reported in Table 2 for all the systems with the exception of the free radical in water solution, whose spectrum is shown for comparison (τ is about 1×10^{-10} s).

For the pure DMPC vesicles, the spectral variation of CAT16 probes from the absence to the presence of the dendrimers is very minor; there is a small decrease in mobility (τ changes from 4×10^{-9} to 5×10^{-9} s) only when the highly protonated dendrimer is interacting. Conversely, the variation of τ is significant for the mixed vesicles from the absence to the presence of dendrimers.

The effect of the addition of the basic dendrimers to the vesicles cannot be simply reproduced by addition of a NaOH solution to provide the same pH level. This means that the results have to be correctly interpreted on the basis of dendrimer/vesicle interactions.

Labeled Dendrimers in the Absence and in the Presence of Mixed Vesicles. Figures 3 and 4 show the EPR experimental spectra (full lines) and the computed spectra (dashed lines) of labeled 2SBD-T and 6SBD-T (0.05 M for both *n*SBDs), respectively, both at a low level of protonation and at a high level of protonation of the dendrimer surface, in the presence of mixed vesicles (40% of DMPA-Na) at 277 K. The insets in the figures show the EPR experimental (full lines) and computed (dashed lines)
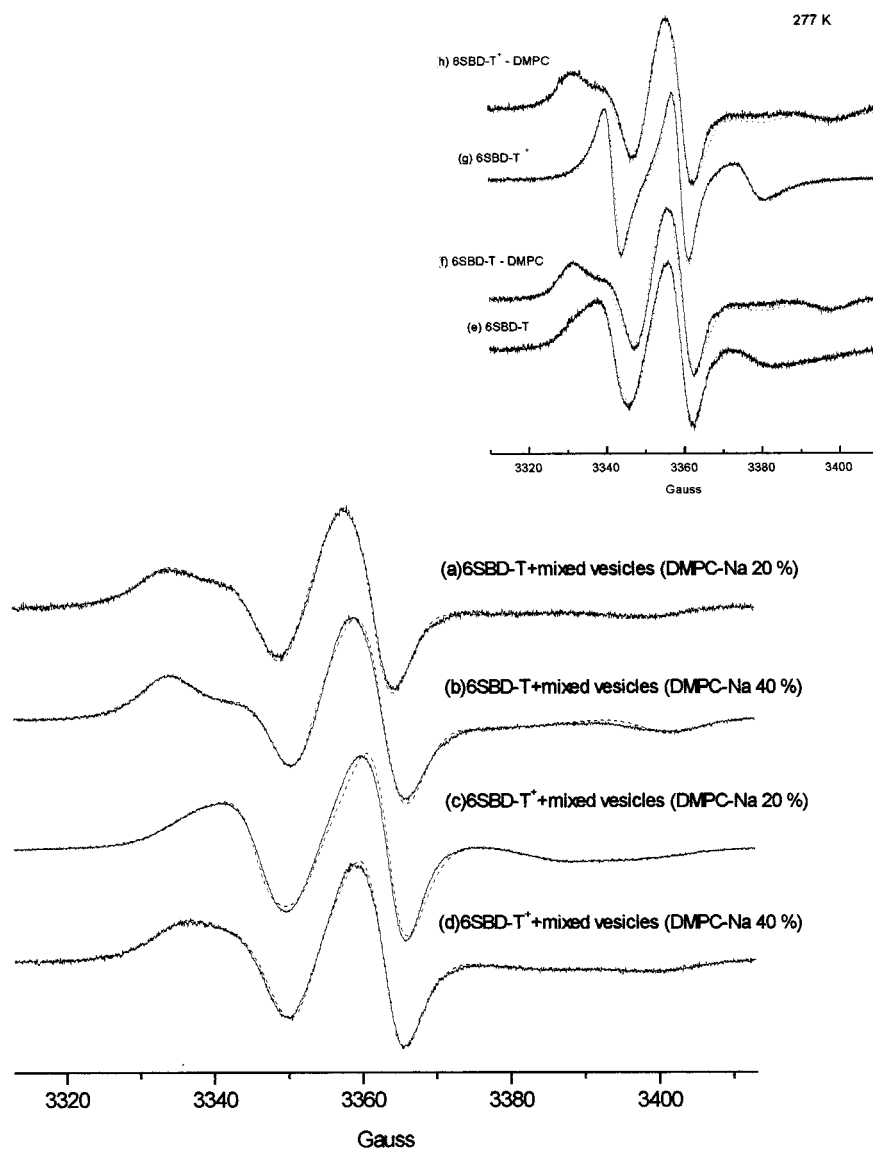
Figure 3. EPR experimental spectra (full lines) and computed spectra (dashed lines) at 277 K of (a) labeled 6SBD-T at low protonation of the dendrimer surface in the presence of mixed vesicles (20% of DMPA-Na), (b) labeled 6SBD-T at low protonation in the presence of mixed vesicles (40% of DMPA-Na), (c) labeled 6SBD-T at high protonation of the dendrimer surface in the presence of mixed vesicles (20% of DMPA-Na), and (d) labeled 6SBD-T at high protonation in the presence of mixed vesicles (40% of DMPA-Na). In the inset: EPR experimental spectra (full lines) and computed spectra (dashed lines) at 277 K of (e) labeled 6SBD-T at low protonation of the dendrimer surface, (f) labeled 6SBD-T at low protonation in the presence of DMPC vesicles, (g) labeled 6SBD-T at high protonation of the dendrimer surface, and (h) labeled 6SBD-T at high protonation in the presence of DMPC vesicles. Concentrations are 0.05 M in surface groups for 6SBD-T and about 10^{-10} M for the vesicles.

spectra of 2SBD-T and 6SBD-T, respectively, in the absence and in the presence of DMPC vesicles under the same experimental conditions as the mixed vesicle systems.[2] The temperature of 277 K was reached by initially decreasing the temperature below the freezing point of the free portion of dendrimers (not interacting with the vesicles) and then increasing the temperature while separating the noninteracting dendrimers, which therefore do not contribute to the overall EPR signal. This procedure has already been demonstrated to be useful in providing information concerning the dendrimer−vesicle interactions.[2] The variation in spectral line shape over temperature was analyzed and used to support the computation procedure and the magnetic and mobility parameters used for the computations. The principal variation with respect to the computation of the EPR spectra from the probes inserted in the vesicles is the tilting of the main rotation axis with respect to the magnetic axis (*x* axis). Table 3 reports this tilting together

with the direction of tilting (*z* or *y* axis). The table also shows the other relevant parameters obtained from spectral computation: the averaged correlation time for motion, $\langle \tau \rangle = (\tau_\perp / \tau_{//})^{1/2}$, the A_{zz} values, and the different percentages of the eventual two components constituting the spectra.

By addition of the vesicles to the dendrimers, an EPR component showing the main rotation axis between the *y*−*x* axis and faster rotational mobility decreased in intensity in favor of a component showing the main rotation axis between the *z*−*x* axis and slower rotational mobility. Also, the environmental polarity of the labels decreased from one component to the other (decrease in A_{zz}).

Lower A_{zz} decrease (decrease in environmental polarity) and higher rotational mobility (increase in τ) are shown by the labels interacting with mixed vesicles with respect to pure DMPC vesicles, with the main rotation axis in the *z*−*x* direction at 50−54°.
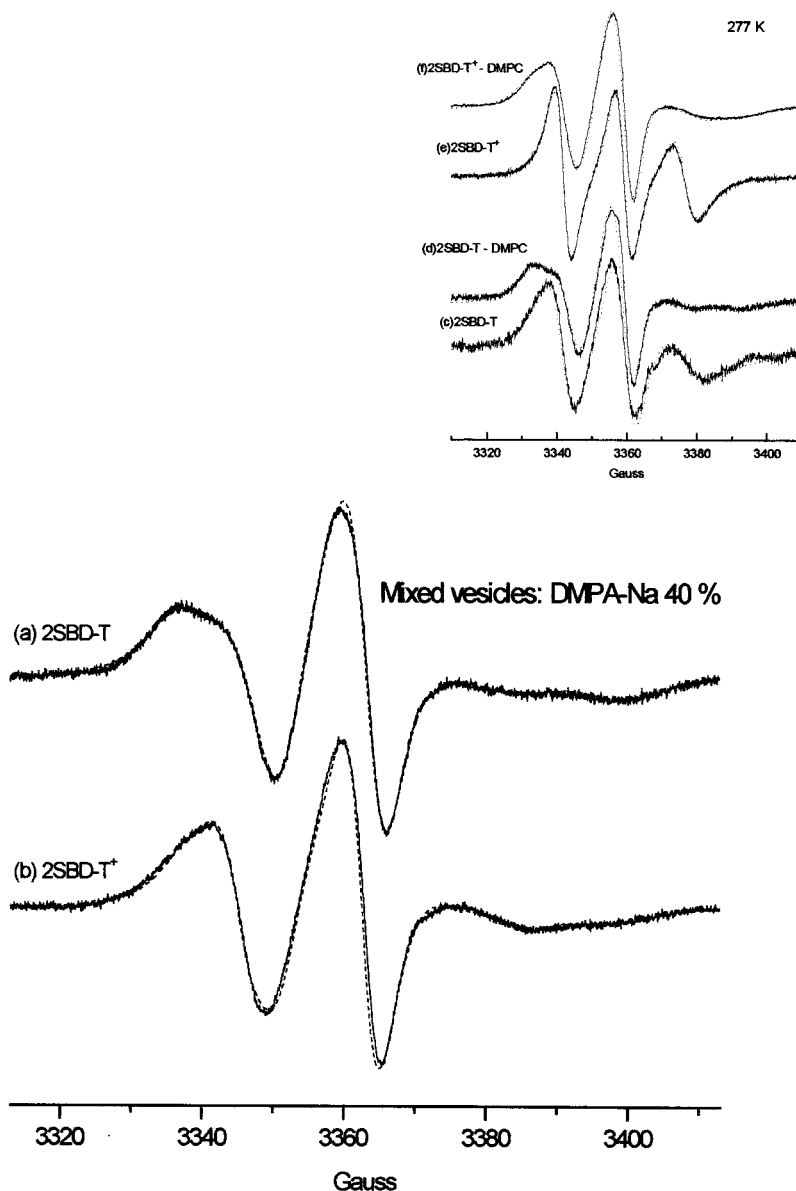
Figure 4. EPR experimental spectra (full lines) and computed spectra (dashed lines) at 277 K of (a) labeled 2SBD-T at low protonation of the dendrimer surface in the presence of mixed vesicles (40% of DMPA-Na) and (b) labeled 2SBD-T at high protonation of the dendrimer surface in the presence of mixed vesicles (40% of DMPA-Na). In the inset: EPR experimental spectra (full lines) and computed spectra (dashed lines) at 277 K of (c) labeled 2SBD-T at low protonation of the dendrimer surface, (d) labeled 2SBD-T at low protonation in the presence of DMPC vesicles, (e) labeled 2SBD-T at high protonation of the dendrimer surface, and (f) labeled 2SBD-T at high protonation in the presence of DMPC vesicles. Concentrations are 0.05 M in surface groups for 6SBD-T and about 10^{-10} M for the vesicles.

In the case of DMPC vesicles, we found a lower mobility for the labeled dendrimers interacting with the vesicles (more effective dendrimer–vesicle interactions), both for the absolute value of τ and for the variation of τ from the absence to the presence of the vesicles. For the mixed vesicles, the variation in mobility is smaller upon going from the absence to the presence of the vesicles. In addition, for the larger dendrimer there is a lower variation of $\langle\tau\rangle$. For example, the slow moving labels in 6SBD-T without the vesicles show $\langle\tau\rangle = 6 \times 10^{-9}$ s, whereas in the presence of the mixed vesicles (DMPA-Na 40%) $\langle\tau\rangle$ is 7.7×10^{-9} s. This corresponds to an increment of about 1.3 for the 6SBD-T, whereas for 2SBD-T the increment is 1.5. A similar simple calculation for all the systems was performed, and the results are summarized in Table 4.

Discussion

Analysis of the EPR results on the probes inserted in the vesicles leads to information on the variation of the vesicle structure both from the absence to the presence of the positively charged DMPA-Na in the DMPC vesicles and from the absence to the presence of the dendrimers in the vesicle solutions.

The increased mean hydrophilicity of the mixed vesicles compared to the pure vesicles forces the hydrophobic 5DSA probes to partially self-aggregate in regions far from the charged DMPA-Na phospholipids. However, the probes monitor a lower viscosity (lower τ) for the mixed vesicles compared to the pure DMPC vesicles. The higher hydration of the charged surface and the different packing of the phospholipids for the mixed vesicles are responsible for the increased mobility of the probes in the mixed vesicles as compared to pure DMPC vesicles.

The interaction of the dendrimer with the mixed vesicle provokes a redistribution of the probes: the charge neutralization plays an important role, where the negatively charged phospholipid groups are neutralized by the positively charged amino groups at the surface of the

Table 3. Main Parameters Extracted from Computation of the EPR Spectra of Labeled Dendrimers in the Absence and in the Presence of Vesicles at 277 K

system	$A_{zz}{}^a$ (G)	$\langle\tau\rangle^c$ ($\times 10^{-9}$ s)	axis − tilt angle^d	%^{e} signal
6SBD-T	39^b	2.3^b	Y − 50° ^{b}	50^b
	36^b	6.0^b	Z − 50° ^{b}	50^b
6SBD-T−DMPC	39^b	2.3^b	Y − 50° ^{b}	30^b
	36^b	15.0^b	Z − 50° ^{b}	70^b
6SBD-T−DMPC + DMPA-Na 20%	38	6.8	Z − 54°	100
6SBD-T−DMPC + DMPA-Na 40%	39	7.7	Z − 54°	100
6SBD-T$^+$	39^b	1.5^b	Y − 50° ^{b}	100^b
6SBD-T$^+$−DMPC	39^b	2.3^b	Y − 50° ^{b}	30^b
	36^b	17.0^b	Z − 50° ^{b}	70^b
6SBD-T$^+$−DMPC + DMPA-Na 20%	39	2.3	Y − 50°	50
	37	6.0	Z − 50°	50
6SBD-T$^+$−DMPC + DMPA-Na 40%	38	6.0	Z − 54°	100
2SBD-T	39^b	2.0^b	Y − 40° ^{b}	70^b
	36^b	4.0^b	Z − 50° ^{b}	30^b
2SBD-T−DMPC	39^b	2.0^b	Y − 40° ^{b}	85^b
	36^b	6.0^b	Z − 50° ^{b}	15^b
2SBD-T−DMPC + DMPA-Na 40%	38	6.0	Z − 54°	100
2SBD-T$^+$	39^b	0.9^b	Y − 50° ^{b}	100^b
2SBD-T$^+$−DMPC	39^b	2.3^b	Y − 50° ^{b}	35^b
	36^b	6.0^b	Z − 50° ^{b}	65^b
2SBD-T$^+$−DMPC + DMPA-Na 40%	39	2.3	Y − 40°	40
	36	6.0	Z − 50°	60

^{a} Hyperfine tensor component, accuracy 5% as obtained from calculation. ^{b} From ref 2. ^{c} Correlation time for motion, accuracy 5% as obtained from calculation. ^{d} Tilt angle of the main rotational axis of the nitroxide with respect to the perpendicular direction from the dendrimer surface. ^{e} Percentage of EPR signal intensity of individual component.

Table 4. Comparisons of Mobility Parameters

system	variation of $\langle\tau\rangle$ (slow component)
6SBD-T−DMPC	2.5
6SBD-T−DMPC DMPA-Na (40%)	1.3
6SBD-T$^+$−DMPC	11.3
6SBD-T$^+$−DMPC DMPA-Na (40%)	4
2SBD-T−DMPC	1.5
2SBD-T−DMPC DMPA-Na (40%)	1.5
2SBD-T$^+$−DMPC	6.7
2SBD-T$^+$−DMPC DMPA-Na (40%)	6.7

dendrimers. This diminishes the repulsion exerted by DMPA-Na on the hydrophobic 5DSA. The restructuration of the vesicles upon interaction with the dendrimers is also proved by the mobility variations in terms of the correlation times for rotational motion and the order parameter. It is proposed that the vesicle binds to the dendrimer surface and the phospholipid headgroups interact with the dendrimer surface amino groups, giving rise to a bilayer-like structure. However, the ordering of the chains is lower for the mixed vesicles compared to the DMPC vesicles. This indicates that the charged phospholipids perturb the ordering effect due to more direct interactions between the negatively charged headgroups of DMPA-Na and the positively charged protonated amino surface groups of the dendrimers.

The increased rotational freedom of the probes in DMPC vesicles is attributed to the increased hydration of the polar heads upon interaction with the dendrimer surface groups which is in line with the results previously found for similar systems in ref 2. The increased hydration of the surface is in part responsible for the higher freedom

of motion of 5DSA into the mixed vesicles compared to the DMPC vesicles.

The addition of the positively charged dendrimer probably does not induce a redistribution of the probes in the vesicle structure, suggesting that the 5DSA aggregates are not destroyed, since the neutralization of the positive dendrimer charges by the negative vesicle charges is not complete and the repulsion charge effect on the hydrophobic 5DSA still holds. The increase in both mobility and polarity is expected on the basis of the partial swelling of the vesicle structure upon interaction with the acidic surface such as that of 6SBD$^+$. A direct comparison between the DMPC\6SBD$^+$ system and the mixed DMPC−DMPA-Na\6SBD$^+$ system reveals a different mechanism of interaction; that is, the DMPC chains are partially ordered at the 6SBD$^+$ surface, whereas the ordering effect almost disappears when DMPA-Na is present. However, the 5DSA aggregates further demonstrated formation far from DMPA-Na and surrounded by DMPC, since the neutralization of DMPA-Na by the positively charged amino groups does not destroy the 5DSA aggregates and the excess external positive charge also prevents redistribution of the probes. In line with the above findings, the quenching in mobility of the radicals is higher for the mixed vesicles than for the DMPC vesicles when in contact with 6SBD$^+$.

The positively charged CAT16 groups inserted in the DMPC vesicles do not monitor the dendrimer/DMPC vesicle interactions since they do not interact with the positively charged amino groups of the dendrimers. On the contrary, the CAT16 radicals monitor the dendrimer/mixed DMPA-Na + DMPC vesicle interactions. Indeed, the probes in the mixed vesicles interact with DMPA-Na molecules through electrostatic interactions between the positively charged heads of CAT16 and the negatively charged heads of the phospholipids. When the dendrimers interact with the mixed vesicles, the negatively charged heads of DMPA-Na are neutralized by the positively charged amino groups. As a consequence, the probes are no longer affected by the presence of DMPA-Na and their behavior resembles the behavior of pure DMPC vesicles interacting with the dendrimers.

Complementary information with respect to that obtained with the probes was obtained by using the labeled dendrimers interacting with the vesicles. The variation of the diffusion tilt axis and angle indicated that the N−O group of the labeled dendrimers in the absence of vesicles is directed toward the more polar SBD surface and, in the presence of the vesicles, it inserts in the less polar hydration layer at the vesicle surface, where the local viscosity is higher, due to the electrostatic interactions between the positively charged groups at the dendrimer surface and the negatively charged groups at the vesicle surface.

The addition of DMPA-Na decreases the ordering of the carbon chains in the vesicles interacting with the dendrimers, indicating a looser packing density of the bilayer structure. This is expected since the charged headgroups of these phospholipids repel each other. As a consequence, the labels at the vesicle/dendrimer interface experience a less viscous environment, as found with the probes, but more easily orient in the direction of the vesicle surface (tilting in the $z−x$ direction). Also, the average polarity of the vesicle surface increases.

The dendrimers at a high level of protonation show a larger variation in mobility compared to dendrimers at a lower level of protonation (for both generations). This result is expected since a high level of protonation of the dendrimer surface favors electrostatic interactions with
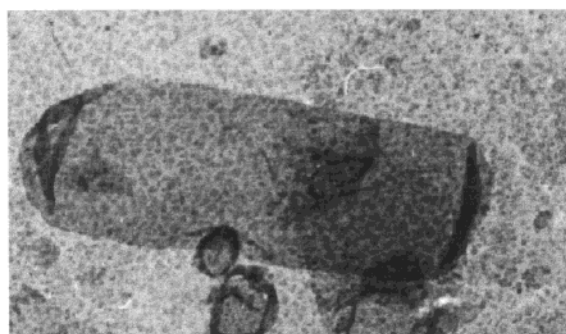
1 μm

Figure 5. Transmission electron micrographs of the pure DMPC vesicles (a) and mixed vesicles (DMPA-Na 40%) (b). These micrographs are obtained by means of the negative staining procedure using uranyl ions as staining agents.

the vesicle surface. We expect an enhanced electrostatic interaction in the presence of the negatively charged DMPA-Na, but the structural variation of the bilayer of the mixed vesicles toward an increase in the chain–chain distance and looseness of packing does not allow the labels to correctly monitor the dendrimer–mixed vesicle interactions.

An interesting result, not obtained by using the probes in the vesicles, is about the different interacting behavior between small (2SBD-T) and large (6SBD-T) labeled dendrimers: on one side, the large dendrimers (late generation) are more affected by the structural variations of the bilayer, but on the other side, the small dendrimers (early generations) are more sensitive, at the label level, to the presence of DMPA-Na, that is, to the electrostatic interactions between the charged groups of the dendrimers and the vesicles. This indicates that the interactions between the dendrimer and the vesicle are different with respect to early and late generations. First of all, the addition of DMPA-Na to DMPC modifies the vesicle shape from spherical to rodlike, as shown by the transmission electron micrographs of the pure DMPC vesicles and the mixed vesicles (DMPA-Na 40%) in parts a and b of Figure 5, respectively. These micrographs are obtained by means of the negative staining procedure using uranyl ions as staining agents. A recent paper[30] has shown that the uranyl ions are completely sequestered by the dendrimers and the vesicles become invisible by TEM in the presence

of the dendrimers. As a consequence, the interactions between the dendrimer and the vesicles could not be analyzed by TEM and only vesicles without dendrimers provide clear TEM features.

On the basis of the EPR and TEM results, we suggest that the vesicles in the rodlike shape wrap around the large dendrimers (6SBD), but in such way that the direct interaction between the charged groups of the dendrimers and the vesicles is prevented, as recently shown by polynucleotides wrapping around these dendrimers.[16,17] In contrast, the small and flexible dendrimer (2SBD) directly interacts with the vesicle surface favoring the electrostatic interactions between the charged groups but modifying less the vesicle structure. In this respect, the spin label technique resulted in much more information than the spin probe technique, where the line shape variations were very small from small to large dendrimers. However, both spin probe and spin label methods provided clear indications of the variation of the vesicle structure upon vesicle/dendrimer interactions, toward a bilayer-like structure.

Conclusions

Employing electron spin probe and spin label methods, polyamidoamine SBDs were demonstrated to interact with mixed vesicles, constituted by DMPC and DMPA-Na in various relative ratios (from 0% to 40% of DMPA-Na). The computer-aided analysis of the EPR spectra allowed for extraction of mobility and structural parameters.

Higher hydration of the charged surface and swelling of the structure were found on the basis of EPR results from 5DSA probes in mixed vesicles compared to pure DMPC vesicles. The addition of 6SBD to the mixed vesicles provokes the ordering of the phospholipid chains. However, the ordering of the chains is lower for the mixed vesicles compared to the DMPC vesicles due to the direct interactions between the negatively charged headgroups of DMPA-Na and the positively charged protonated amino groups of the dendrimers. A partial ordering of the DMPC chains is retained at the 6SBD$^+$ surface, whereas the ordering effect almost disappears when DMPA-Na is present.

The CAT16 probe monitored well the formation of SBD–mixed vesicle adducts also indicating the occurrence of the electrostatic interactions between DMPA-Na and the protonated amino groups at the dendrimer surface.

Analysis of the EPR spectra of labeled dendrimers indicated that the N–O group is initially directed toward the more polar SBD surface and then inserts in the less polar hydration layer at the vesicle surface. Mainly, the charged headgroups of DMPA-Na repulse each other, also an indication of a structural modification of the vesicles. As a consequence, the labels at the vesicle/dendrimer interface experience a less viscous environment. The highly protonated dendrimers show a larger interaction with the mixed vesicles, compared to those low protonated, since the high protonation of the dendrimer surface favors the electrostatic interactions with the vesicle surface. The large dendrimers are more affected by the structural variations of the bilayer with respect to the small dendrimers, but at the same time, the small dendrimers are more sensitive to the electrostatic interactions between the charged groups of the dendrimers and the vesicles. These results suggest a different interacting model between the small and the large dendrimers and the vesicles.

(30) Ottaviani, M. F.; Favuzza, P.; Bigazzi, M.; Turro, N. J.; Jockusch, S.; Tomalia, D. A. *Langmuir* **2000**, *16*, 7368.

However, in line with the EPR results, transmission electron micrographs show that the addition of DMPA-Na to DMPC modifies the vesicle shape from spherical to rodlike. We propose that the rodlike vesicles wrap around the large dendrimers, whereas the small dendrimers interact directly with the vesicle. This hypothesis is also based on previous EPR results obtained with polynucleotides interacting with the labeled dendrimers[17,18] and is in line with the results obtained with pure DMPC vesicles interacting with dendrimers,[1,2] indicating the modification of the vesicle toward a bilayer-like structure upon interaction with the dendrimers.

Scheme 1 depicts schematically the main results obtained in the present study: (a) the disordering effect played by the charged phospholipid when added to the DMPC vesicles, corresponding to a variation in mobility of the probes and to the shape variation of the vesicles toward a rodlike structure; (b) the partial ordering effect on the bilayer played by the dendrimers when interacting with the vesicles; (c) the different interacting behavior of small and large dendrimers, with the large dendrimers being wrapped up by the vesicles whereas the flexible small dendrimers adapt to the vesicle surface.

These results provided a further piece of information about the properties of the biosystem in which the dendrimers play the role of gene carriers and go to interact with the cell membranes, whose structure is nicely mimed by the mixed vesicles.