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Molecular structure and conformation in phospholipid vesicles sterically stabilized by (tri)-block copolymers investigated by multi-nuclear magnetic resonance techniques

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Abstract

Soybean lecithin was dispersed in water and sonicated to form unilamellar vesicles of 40–45 nm in diameter. (Tri)-block copolymer molecules of the A–B–A type (A is polyethylene oxide (PEO) and B is polypropylene oxide (PPO)) were added to enhance the stability of the vesicles. The hydrophobic (PPO) group of the copolymer was thought to be buried inside the vesicle bilayer after sonication of the phospholipids in the presence of copolymer. ^{13}C- and ^{31}P-NMR were used to investigate the specific localization of the block copolymer and its interaction with the phospholipids. Line broadening of the ^{31}P signals (phosphatidylcholine) and the ^{13}C NMR signals of the methyl groups (PPO) was observed for low molar ratios of copolymer:phospholipid. This effect was thought to be due to restriction of molecular mobility of the groups examined, suggesting incorporation of the copolymer inside the bilayer. Other studies investigated the thermotropic behaviour of the phospholipid aggregates formed and the consequences of copolymer presence in the vesicle systems. ^{31}P NMR offered a way to observe comparatively the effect of vesicle formation in the presence of copolymer molecules. According to this method of vesicle preparation (i.e. with copolymer present) the interaction between the lipid molecules and the copolymers is much more evident. © 1998 Elsevier Science B.V.

1. Introduction

Phospholipid vesicles (liposomes) have proved to be particularly interesting colloidal systems both as models for the study of biological membrane activity and as application tools, mainly as delivery systems. The aggregation of phospholipid molecules into closed bilayer vesicles of diverse structural characteristics, including size, lamellarity, surface properties, permeability, etc., have prompted extensive studies, using all possible experimental techniques, in order to describe and design optimal vesicle systems according to the needs of the particular application.

Nuclear Magnetic resonance (NMR) techniques have offered tremendous insight towards the structural description and characterization of phospholipid membranes. The main property identified with liquid-crystalline lipid bilayers is the anisotropic nature of the occurring molecular movements, both intramolecular reorientations of lipid functional groups (acyl chains and headgroups),

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and motions of the whole molecule (rotational and lateral diffusion, transbilayer transport, etc.) [1]. A vast amount of information has been compiled about the hydrophobic membrane interior [2], including packing of lipid chains, geometric conformations, gel→liquid crystalline phase transitions, etc., and also about the headgroup conformation and interaction with membrane proteins and metal ions [3], which are considered crucial factors deciding biological membrane function. Furthermore, the analysis of lipid polymorphism and aggregation behaviour has been greatly enhanced by NMR techniques (mainly ^{31}P through chemical shift anisotropy and ^{2}H through quadrupolar splittings) [4,5], which offer various advantages compared to other experimental techniques, like X-ray diffraction [3].

Most of the NMR studies carried out previously, placed more attention on studying multilamellar vesicles (MLVs), and hence lipid bilayers of low hydration levels, for various reasons: (a) anisotropic molecular motions are more pronounced and, therefore, more apparent effects can be observed; (b) the tumbling rates of multilamellar lipid structures are much slower (always as far as the NMR experimental time scale is concerned) than those of small unilamellar vesicles (SUVs) which are formed at high hydration levels and after high energy processes (i.e. sonication, extrusion, filtration, etc.). This results in very rapid diffusion of small vesicles, leading to averaging out of their quadrupolar splittings and/or their residing anisotropies, and thus to loss of vital sources of information; (c) lipid bilayers in MLVs are flat structures of low curvature and high elasticity/flexibility, resembling more the membranes of biological systems; (d) they are easier to prepare. Indeed, a great deal of biologically important membrane functions have been studied by using model membranes (MLVs) and NMR techniques, like fusion [6] and metal ion binding [7].

In this study, we try to elucidate the structural effect of adding block copolymer molecules to SUV systems. Small vesicles were preferred to multilamellar structures because of their considerable application as delivery vehicles of active ingredients (drugs, vitamins, contrasting agents, etc.) in pharmaceuticals, cosmetics and agrochemicals industries. Two methods of copolymer inclusion were designed that are believed to produce structurally different vesicle systems. Allowing the copolymer molecule to participate with the phospholipids in vesicle formation is thought to result in more efficient anchoring of the polymer molecule onto the bilayer surface, hence providing a more resistant steric barrier. Enhanced stability of these vesicles against monovalent and divalent cations is demonstrated elsewhere [8]. ^{31}P NMR is employed here to give a picture of the interactions taking place at the phospholipid headgroup region and of the effect of the presence of copolymer molecules to the phospholipid phase transitions. ^{13}C NMR is used to monitor directly the hydrophobic homopolymer segment (polypropylene oxide (PPO)) of the block copolymers, in order to draw further evidence of its exact location [9] in the vesicle structure.

2. Experimental

2.1. Materials

The vesicle systems were formed by using a mixture of soybean lecithin lipids (approximately 50% D-α-dimyristroylphosphatidylcholine (DPPC)), purchased from Sigma. The aqueous dispersion medium was always double distilled, deionized water. The A–B–A type (tri-)block copolymer used was of the Synperonic PE family, namely the PF127, where A is polyethylene oxide (PEO) and B is PPO, supplied by ICI Surfactants, Belgium. This polymer has the following structure:

$(EO)_{99}$–$(PO)_{65}$–$(EO)_{99}$

The numbers denote the monomer units in the polyethylene and polypropylene oxide blocks.

2.2. Preparation of vesicle systems

SUVs were formed by the sonication method [10] using a Kerry ultrasonic bath (50 Hz). Sonication for approximately 4 h was shown by photon correlation spectroscopy (PCS) [11] to produce minimum size liposomes of 20–25 nm radius. The temperature in the ultrasonic bath was

kept in a constant range between 25 and 30°C. The vesicle systems after preparation were filtered through 0.2 μm pore size filters (Millipore) to reduce the polydispersity of the samples. The pH of all samples fluctuated between 5 and 6.

Two methods have been used to include the A–B–A copolymers into the vesicles systems. According to the first method the vesicles were prepared (hydration and sonication steps) in the presence of copolymer molecules at the desired concentration. Following the second technique, the liposomes were formed, and after the sonication these were diluted with copolymer aqueous solutions in order to reach the required final concentrations. These systems were left to stand for at least 24 h before any measurement was carried out. For ease of presentation the samples denoted (I) refer to vesicle systems where the block copolymer was added initially, while the samples denoted (A) refer to the systems with addition of the polymer after the liposomes have been formed.

2.3. ^{13}C- and ^{31}P-NMR

^{13}C- and ^{31}P-NMR spectra were recorded on Jeol GSX400 and GSX270 spectrometers. Both types of spectra were acquired with 45° pulses, a 2 s repetition rate and with complete proton decoupling. The ^{13}C NMR spectra were acquired with 32-k data points, a 250 ppm spectral window and chemical shifts were referenced to external TMS at 0.00 ppm. The ^{31}P NMR spectra were acquired with 16-k points, a 50 ppm spectral window and chemical shifts referenced to external 85% H_3PO_4 at 0.00 ppm.

Line broadening determination was carried out by measurement of the instrument-provided signal intensity over the peak height, instead of the most commonly used linewidth at half height ($\omega_{1/2}$), since the possibility of side peaks due to artifacts could induce a large experimental error. These errors would affect considerably the linewidth results, since sonicated small vesicles exhibit subtle effects compared with those obtained from MLVs.

A preliminary study using sonicated liposomes was undertaken in order to investigate the determining phosphorus relaxation mechanism and, subsequently, the ^{31}P signal linewidths dependence. As expected, liposome ^{31}P linewidth broadened from 109 MHz to 160 MHz, and at 160 MHz the observed linewidth narrowed from 10°C to 60°C. Chemical shift anisotropy (CSA) is the only possible mechanism that could affect linewidth, dependent on both applied field frequency and temperature. These results confirmed that the primary relaxation mechanism for small unilamellar phospholipid vesicles is CSA, in accordance with numerous reports [12–14] studying relaxation mechanisms of multilamellar phospholipid dispersions.

3. Results and discussion

3.1. Line broadening experiments

The effect of increasing amounts of block copolymer molecules added to the vesicles was studied by monitoring the extent of line broadening occurring in the ^{31}P- and ^{13}C-NMR spectra. This investigation was based on the initial observations that the spectra of the vesicle system where PF127 molecules were added initially (I) at a molar ratio Lipid:Copolymer ~ 30 (Copolymer:Lipid 0.0163) exhibited evidently broader linewidths for the

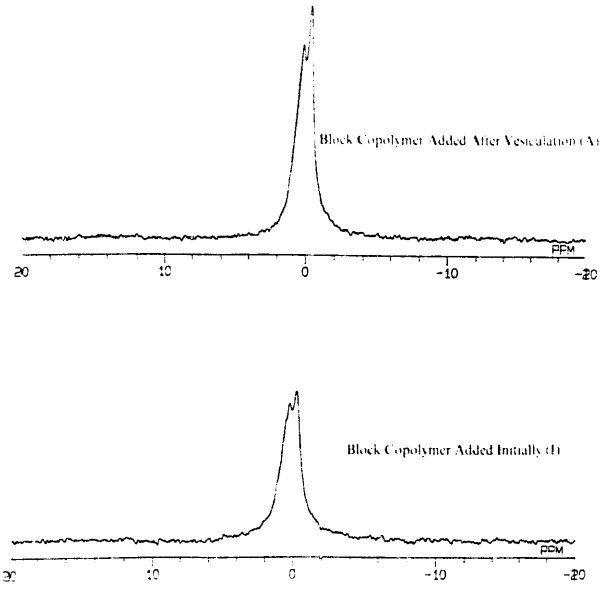

Fig. 1. ^{31}P NMR spectra showing line broadening when adding the block copolymer before (I) vesicle formation.

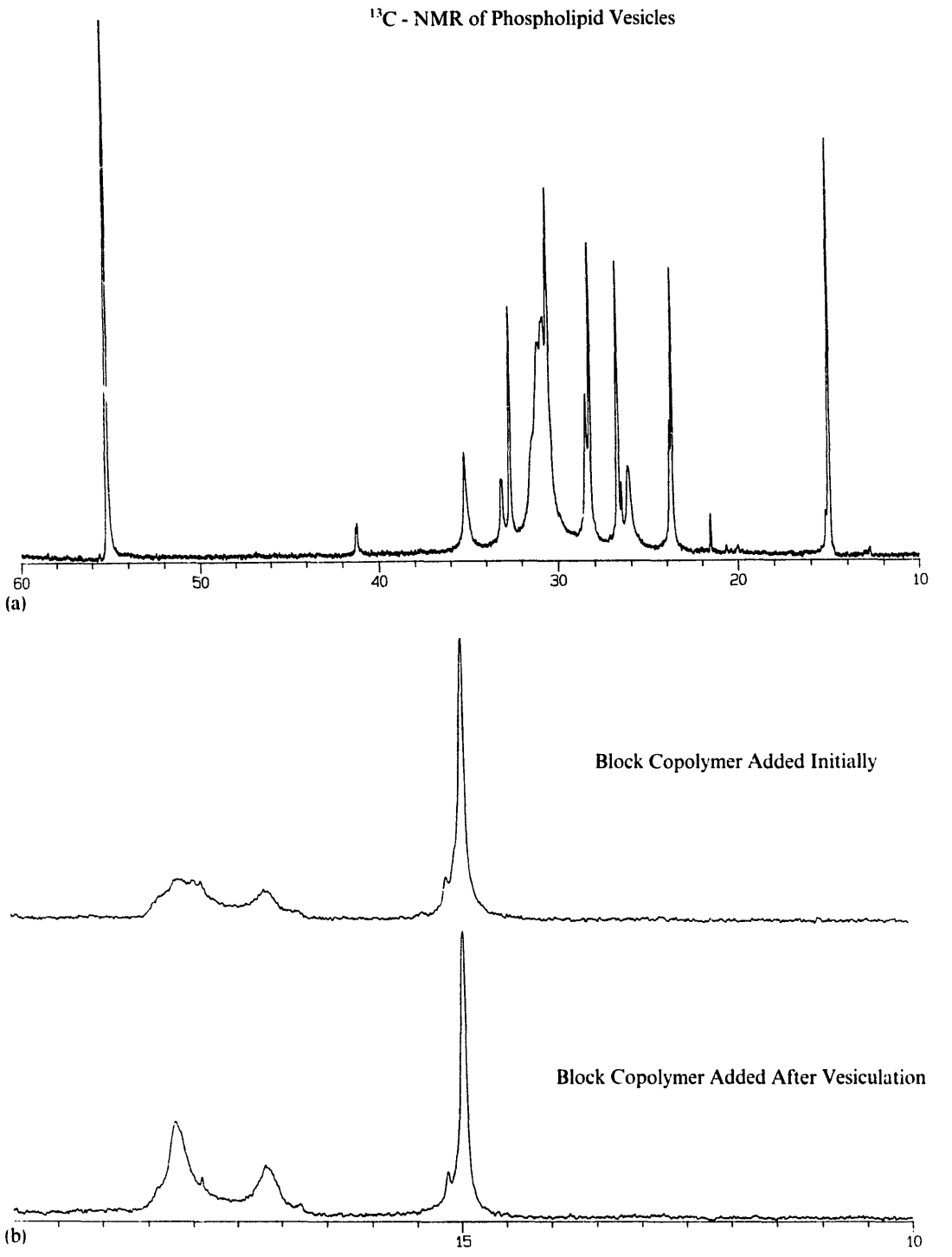

Fig. 2. (A) High-field area of the ^{13}C NMR spectra of sonicated small unilamellar vesicles. (B) Broadening of the PPO methyl signals when block copolymer is added initially (I) to the vesicles.

headgroup phosphorus (^{31}P), the copolymer PPO methyl carbons (^{13}C) and methyl protons (^{1}H), compared with the sharper lines obtained for the vesicle systems with block copolymer added after (A) vesiculation. Examples of the observed effects are shown in Fig. 1, where the ^{31}P spectra of (I) and (A) vesicle systems (molar ratios the same as mentioned above) are comparatively shown. In Fig. 2(A) the high field of the ^{13}C spectrum of liposomes is depicted. The peak at 15 ppm is due to the end methylene groups of the lipid chains and the one at 55 ppm shows the choline methyl [$^{+}N(CH_3)_3$] atoms, while the multiple peaks between 20 and 40 ppm are assigned to carbon atoms along the phospholipid molecule. The PPO methyl and methyl peaks were conveniently expected to lie between 17 and 18 ppm, in an area of the spectrum where overlapping with other peaks would not occur. Fig. 2(B) shows the broadening in the signal of the PPO peaks for the vesicle system in which the block copolymer was allowed to participate during vesiculation, compared with the vesicle system where vesiculation was carried out in the absence of polymer.

These observations were thought to indicate that the presence of block copolymer molecules during vesicle formation ((I) addition), was resulting in vesicle systems where both the phosphate groups and the hydrophobic block of the copolymer (PPO) were experiencing motional restriction (at least to some extent). The restricted motional freedom of the particular nuclei under examination produced broader lines (poorer reorientation to the applied magnetic field). On the other hand, the same nuclei in the (A) vesicle system seem to be able to undergo closer-to-isotropic motions, thus producing sharper lines.

Extensive studies monitoring the spin-lattice (T_1) relaxation time of the phosphate phosphorus of the choline group and the methyl carbon atoms of the PPO segment of the copolymer were carried out in the absence and presence of paramagnetic relaxation agents (Mn^{2+}) [9]. These studies provided further evidence of decreased molecular mobility in the case of (I) vesicles, and also ensured that the shorter lifetimes, resulting in rapidly decaying FIDs, and consequently broader lines for the above-mentioned nuclei, were due to structural effects rather than, for example, saturation. Moreover, further relaxation experiments involving spin-echo sequences are currently under way, in order to measure the spin-spin (T_2) relaxation times with greater precision and draw more information about the molecules' mobility in the slow tumbling region.

The structural interpretation of the above observations provides evidence of polymer incorporation inside the vesicle bilayer, manifested by the restriction in the mobility of the PPO block. Also, the reduction in the motional and/or orientational freedom of the phosphate group at the vesicle surface can be considered as evidence of a polymer-induced effect. It has certainly been shown that the more strongly the motion of the phosphate group region is restricted, the less effectively the ^{31}P chemical shielding tensor is averaged, and the larger the values of the effective CSA obtained [15] (and refs. [14–19] cited therein). Previous studies have reported motional restriction of bilayer-participating phospholipids when proteins were bound onto the bilayer's surface [16]. Other studies concerned with polymer macromolecules [17,18] were based on the determinant role of the mobility of polymer segments in their NMR relaxation times, in order to examine polymer adsorption on colloidal particles. The segments attached to the particle surface (trains) exhibited broader linewidths than the highly mobile loops and tails whose isotropic motion produced narrow lines.

The above observations prompted a further more systematic study of the interactions taking place between the block copolymer molecules and the phospholipids, based on the linewidth fluctuations of specific nuclei as the molar ratio Copolymer:Lipid was increased, comparing the two different vesicle preparation techniques followed. The nuclei to serve as molecular probes were (a) the lipid headgroup phosphorus, (b) the lipid headgroup *N*-methyl carbon atoms, and (c) the methyl carbon atoms of the PPO segment of the copolymer. The first two nuclei were thought to provide a picture of the vesicle headgroup region, while monitoring of the hydrophobic block of the copolymer was to give information as to whether incorporation inside the vesicle bilayer was occurring.

The linewidth values used were the intensity (from integration of the peaks concerned) over the peak height, as a proportion of the linewidth of the copolymer-free phospholipid vesicle spectrum.[1] This relative linewidth increases for the choline phosphate group irrespective of the way in which the polymer molecules were incubated in the systems (Fig. 3). The lines of the headgroup phosphorus are therefore broadened with increasing amounts of copolymer. The fact that the headgroup *N*-methyl carbon atoms of the (I) vesicle system showed broader lines than the (A) system (Fig. 4), can be attributed to the previously suggested conformation of the zwitterionic dipoles of the lipid headgroups lying parallel to the bilayer surface [19]. The resulting difference in the spatial orientation of the $^{-}PO_4$ and $^{+}N(CH_3)_3$ groups seems, from Figs. 3 and 4, to be demonstrated by the effect on their NMR linewidths that the presence of block copolymer molecules exert. The $^{+}N(CH_3)_3$ carbon atom lines look to be more affected by the way in which the polymers were included in the systems, suggesting a stronger interaction between that region of the lipid headgroup and the copolymer molecule.

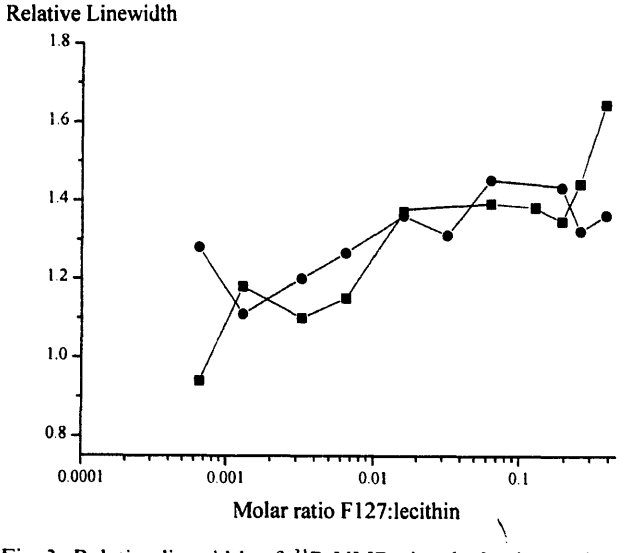

Fig. 3. Relative linewidth of ^{31}P NMR signals for increasing Copolymer:Lipid molar ratio; (●) are the (A) and (■) the (I) vesicle systems.

[1] In the case of the PPO linewidths, normalization was carried out relative to the PPO linewidth obtained from the F127 ^{13}C NMR spectrum (not shown).

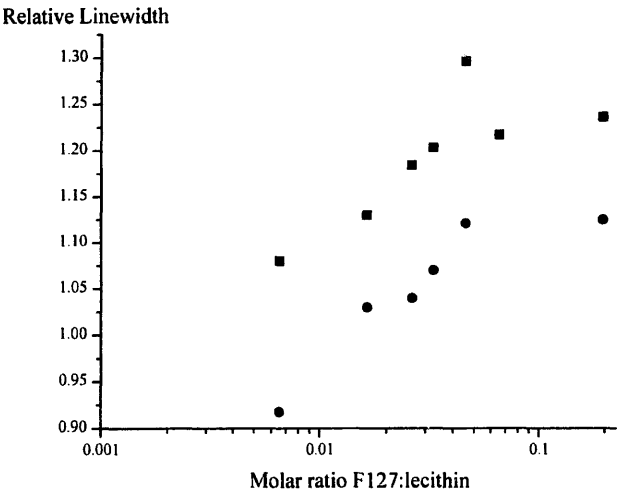

Fig. 4. Relative linewidth of ^{13}C NMR signals of the *N*-methyl carbon atoms of the choline group. Notation as in Fig. 3.

Nevertheless, both (I) and (A) vesicle systems manifest line broadening in the polar headgroup region with increasing F127:Lipid molar ratios (Figs. 3 and 4). Assuming the main source of line broadening to be vesicle tumbling (i.e. lateral diffusion of the phospholipid in the plane of the bilayer being slow enough as far as the experimental time scale is concerned), it can be confidently concluded that the addition of copolymer leads to an apparent confinement of the mobility experienced by the phospholipid headgroups. The reduction of the lipid polar headgroup tumbling rate, provides lucid evidence of vesicle coating by the block copolymer molecules. Either by reducing the ease with which the whole vesicle aggregate tumbles or by impeding the motions (flip-flop, inner/outer monolayer molecular exchange, translational movements, etc.) of the individual lipid molecules [20], the presence of the block copolymer at the vesicle surface is confirmed by the results obtained. Interestingly, the phosphorus linewidths examined did not show any significant overall narrowing at the high Copolymer:Lipid molar ratios, which would be indicative of highly mobile, isotropically tumbling phospholipid molecules.

The linewidth study of the PPO methyl signals (Fig. 5) shows that, for low Copolymer:Lipid molar ratios, broader lines were obtained for the (I) vesicle system. The significant divergence in

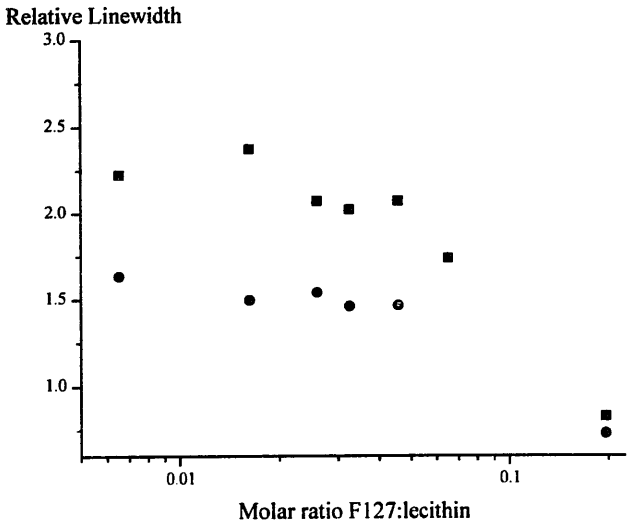

Fig. 5. Relative linewidth of ^{13}C NMR signals of the PPO methyl carbon atoms of the block copolymer. Notation as Figs. 3 and 4.

PPO linewidth between the two types of vesicle system prepared demonstrates the increased motional restriction that this segment of the polymer is experiencing when allowed to participate during vesicle formation. This effect can be attributed to the additional constraints in the mobility of the PPO block when buried deeper into the lipid bilayer (in the case of (I) vesicles), than just adsorbing onto the bilayer surface which is believed to produce the line broadening obtained from the (A) vesicles.

As the molar ratio was increased, two effects were observed: (a) the linewidth for both types of vesicle decreased, producing narrower lines; (b) the linewidth values between the (I) and the (A) vesicles converged gradually to the same value as that obtained for the F127 dispersion alone. The line narrowing observed can be explained by the fact that the mole fraction of associated (either incorporated or adsorbed) block copolymers with phospholipid molecules decreases for higher amounts of added polymer. This indicates that the PPO blocks of the copolymer molecules enjoy greater motional freedom at high Copolymer:Lipid molar ratios, as would be expected in the event of micelle formation. Indeed, the normalization standard used was an aqueous dispersion of F127 micelles, whose PPO linewidth was identical to the linewidths obtained at maximum F127:lecithin ratio for both types of vesicle system. Therefore, F127 micelle formation seems to occur at high concentrations of added copolymer. The case of mixed micelle formation between the copolymers and the phospholipids is certainly possible, as long as the resulting aggregate structures are consistent with the restricted phospholipid molecule mobility observed in Figs. 3 and 4.

In conclusion, the evidence obtained from the monitoring and a comparative study of the line broadening of particular nuclei in vesicle systems containing copolymers initially vs. vesicles with added copolymers after formation can be summarized as:

(A) the copolymer is present at the bilayer surface, coating it, causing restriction in the mobility of the phospholipid headgroup;

(B) the PPO signal for the vesicles formed in the presence of copolymer (I), shows clearly broader lines (at low Copolymer:Lipid molar ratio) than the equivalent/respective linewidths of the vesicles where copolymer addition was carried out to pre-formed liposomes (A). The incorporation of the hydrophobic PPO segment inside the bilayer could be responsible for this additional broadening effect observed in the (I) vesicle systems.

3.2. *Thermotropic behaviour*

The behaviour of the bilayer phase by addition of block copolymer molecules at the "optimum" [21] Lipid:Copolymer molar ratio of 30:1 was investigated by ^{31}P NMR for a series of temperatures (Fig. 6). Comparing the spectra of the bare liposome system, the vesicles with copolymer added after formation (A), and the vesicles prepared between phospholipids and copolymers (I), can provide a qualitative picture of the effect of increasing temperature. The narrow linewidth of the spectra (1.5–2 ppm) indicates that the liposome population consists mainly of SUVs, tumbling almost isotropically.[2] These structural implications from the ^{31}P NMR spectra agree with dynamic light scattering and cryogenic and negative staining

[2] Typical linewidths of multilamellar bilayer vesicles are between 25 and 40 ppm.

TEM studies [21] that were also performed on the vesicle systems at 25°C. Also, the observation that the copolymer-containing vesicle systems exhibit broader signals than the bare liposome system at 25°C correlates well with the line broadening experiments described Section 3.1.

Studying the bare liposome spectra a shoulder at positive parts per million values can be observed. As temperature is increased the shoulder gradually reverses its sign, depicting the occurrence of a phase transition, most probably due to lipid chain melting.[3] The same behaviour towards temperature increases is followed by the (A) vesicle system, where a phase transition seems to occur between 35 and 45°C. The phase transition for the bare phospholipid vesicles takes place both at higher temperatures and also at a slower rate, i.e. for the more extended temperature range (45–65°C). Contrary to both vesicle systems mentioned above, the vesicles formed between the phospholipids and the copolymers exhibit a shoulder at positive parts per million values at 25°C, but even at 75°C there is no clear reversal of the shoulder's sign (particularly when one compares the spectra of all three vesicle systems). Moreover, at this high temperature (75°C), much broader lines are obtained for this vesicle system, which was used as an indication of stronger interactions taking place between the polymer molecules and the phospholipid headgroups leading to further restrictions in their mobility. Generally, the different thermotropic behaviour that the (I) vesicle system demonstrates suggests a stronger interaction between the bilayer-participating components (i.e. phospholipids and copolymers).

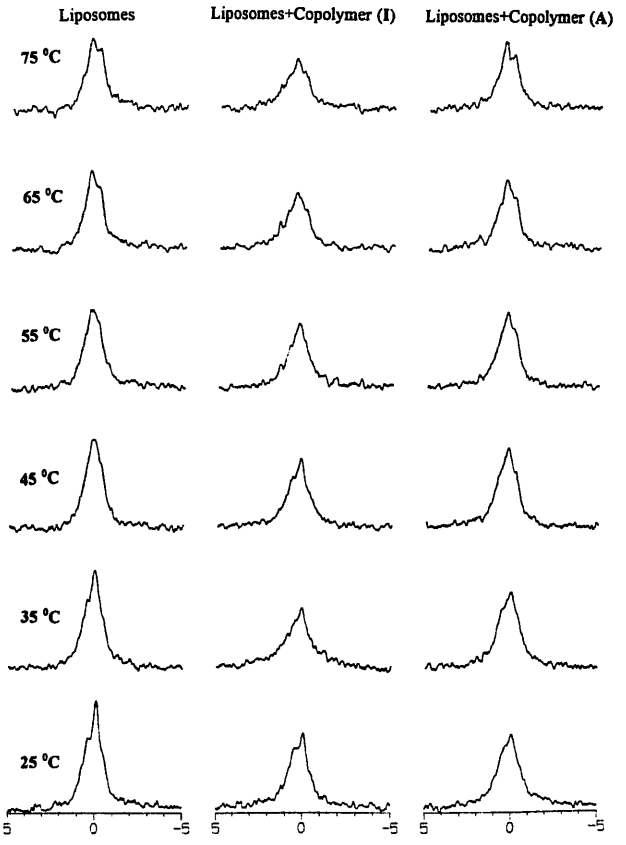

Fig. 6. Comparative presentation of ^{31}P NMR spectra for the three vesicles systems studied, for the temperature sweep 25–75°C.

4. Conclusion

The NMR resonance signals of particular nuclei were used as probes to study the effect of block copolymer addition to phospholipid vesicles. Polymer inclusion to the vesicle system was followed by two different techniques, namely addition to pre-formed liposomes and addition before vesicle formation. Both methods of copolymer addition result in coating of the vesicle surface. The participation of copolymer molecules in vesicle formation results in polymer incorporation inside the lipid bilayer as shown by the enhanced line broadening of the methyl carbon signals of the hydrophobic PPO block. Moreover, the thermotropic phase changes occurring in bare liposomes and liposomes with polymer added after their formation are altered in the vesicle system formed between phospholipids and copolymers. This effect

[3] There have been many reports describing the thermotropic phase behaviour of unsonicated MLVs based on the sign reversal of the residual CSA for the occurring bilayer→hexagonal phase transition [22]. The relevance of those effects to the present vesicle systems was considered highly unlikely, because: (a) the isotropic linewidths produced where vesicle tumbling averages the CSA, are contrary to the broad anisotropic envelopes characterizing the MLVs where CSA dominates the spectrum; (b) the high phospholipid hydration levels used (2 wt.%) prohibit the formation of a hexagonal (H_{II}) phase (low phospholipid hydration is necessary). However, the possibility of a phase transition to an inverse micellar phase (isotropic motion intact) cannot be ruled out.

provided further evidence of copolymer inclusion inside the bilayer through their apparent stronger interactions.

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