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# Nuclear magnetic relaxation dispersion and $^{31}$P-NMR studies of the effect of covalent modification of membrane surfaces with poly(ethylene glycol)

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Covalent attachment of methoxypoly(ethylene glycol) (MPEG) 5000 to the surface of unilamellar liposomes composed of egg phosphatidylcholine and dioleoylphosphatidylethanolamine (DOPE) (8:2) containing paramagnetic chelates, either entrapped within the interior volume of the liposomes, or associated with the membrane surface, had no effect upon the measured spin-lattice relaxation rates ($1/T_1$) for water in these systems. $^{31}$P-NMR studies indicate no destabilization of dioleoylphosphatidylcholine (DOPC)/(DOPE) (1:1) vesicles following attachment of MPEG. However, in DOPC/DOPE (1:3) mixtures, covalent modification with MPEG results in a destabilization of multilamellar vesicles into smaller vesicular structures. These results indicate that covalent attachment of poly(ethylene glycol) to liposomal magnetic resonance agents may prove a useful method for increasing their utility as vascular MR agents by extending their lifetime in the circulation, without decreasing the relaxivity of paramagnetic species associated with the liposome, but that the presence of PEG covalently attached to the membrane surface may modify the polymorphic phase behavior of the lipid system to which it is covalently linked.

## 1. Introduction

Once injected into the circulation, liposomes are subject to numerous interactions with plasma components which, for certain lipid compositions, results in lipid exchange between liposome and albumin or lipoproteins with concommitant release of liposomal contents [1]. Because liposomes, by virtue of their size, are relatively confined to the vasculature, they are rapidly accumulated by the reticuloendothelial (RES) system and delivered primarily to liver, spleen and bone marrow. In order to facilitate sustained release and delivery of liposome-associated drugs, various methods have been employed to increase the lifetime of liposomes within the circulation including the use of saturated lipids [2] or by hydrophilic modification of the the surface of the liposome with gangliosides [3], glycosides [4] and or synthetic polymers such as poly(ethylene glycol) (PEG) [5–8].

In addition to drug delivery applications, liposomes with either entrapped or surface-incorporated paramagnetic species are of interest as both vascular and RES contrast agents for the improved detection of hepatosplenic metastases by magnetic resonance (MR) imaging [9]. In systems with encapsulated paramagnetic contrast agent, or those in which surface-associated paramagnetic species are associated with the inner surface of the membrane, water must cross the lipid bilayer in order to sample the paramagnetic species inside the liposome. Any factor which decreases the water permeability at a given temperature such as increased acyl chain order [10], may decrease the measured relaxation rate and effectiveness of the contrast agent [11,12]. For this reason the use of saturated lipid species is not the best approach to the design of liposomal MR agents with extended lifetime in the circulation. Because PEG when bound to the liposome surface can significantly prolong the circulation half-life of liposomes [5–8], we have investigated whether PEG

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has any effect upon the relaxivity of paramagnetic species either trapped within the interior aqueous space of liposomes or attached via a ligand to the surface of the lipid bilayer.

It is reasonable to suggest that the covalent attachment of a relatively large hydrophilic polymer such as PEG to a lipid molecule would result in a PEG-lipid complex with a higher cmc than the non-modified lipid. A PEG-modified lipid would therefore be more likely to exchange away from the vesicles bilayer, although it is not obvious that this would necessarily decrease the stability of the vesicles to release of internal contents, and may also affect the hydrocarbon packing constraints within the lipid bilayer. Within this context we have examined by $^{31}$P-NMR whether the presence of covalently attached PEG can indeed affect lipid packing within a membrane by modifying the polymorphic phase behavior of lipid systems.

## 2. Materials and Methods

*2.1. Materials.* Gadolinium-1,4,7-tricarboxymethyl-1,4,7,10-tetraazacyclodecane (Gd-DO3A) was a gift of Squibb Diagnostics. Trinitrobenzenesulfonic acid (TNBS) and Triton X-100 were obtained from Sigma (St. Louis, MO). Egg phosphatidylcholine (EPC), dioleoylphosphatidylethanolamine (DOPE) and dioleoylphosphatidylcholine (DOPC) were obtained from Avanti Polar Lipids (Birmingham, AL) and used without further purification. The stearyl ester diethylenetriaminepentaacetic acid-gadolinium complex (SE-Gd) was synthesized as previously described [13]. Monomethoxy PEG (MPEG) 5000 was derivatized with tresyl chloride as described elsewhere [14] to yield tresyl-MPEG (TMPEG).

*2.2. Preparation of unilamellar liposomes and PEGylation.* Unilamellar 100 nm diameter liposomes composed of EPC/PE (8:2) mole ratio containing 100 mM Gd-DO3A were prepared by freeze-thaw extrusion [15] of multilamellar liposomes followed by chromatography on Sephadex G-50 to remove untrapped paramagnetic chelate using 55 mM sodium phosphate (pH 8.5) as eluant. Unilamellar 100 nm diameter liposomes composed of EPC/PE (8:2) containing 5 mole percent of the lipophilic paramagnetic chelate SE-Gd were prepared by freeze-thaw extrusion in 55 mM sodium phosphate (pH 8.5) as above. Coupling of PEG was achieved by addition of a 2:1 mole ratio of TMPEG to external PE on the liposomes followed by incubation at room temperature for 30 min. For TNBS assays, unilamellar EPC/PE (8:2) liposomes were prepared in 50 mM boric acid, 100 mM $NaHCO_3$, pH 8.5. After PEGylation as above, unreacted TMPEG was removed by chromatography on Sephadex G-50F using borate buffer as eluant. For $^{31}$P-NMR studies, DOPC/DOPE (1:1) and DOPC/DOPE (1:3) mixtures were prepared at a concentration of 100 $\mu$mol/ml in 100 mM borate (pH 8.5) by vortex mixing at room temperature. TMPEG was dissolved in a minimal volume of 100 mM borate buffer (pH 8.5) and added to the samples in the NMR tube. For controls MPEG was substituted for TMPEG. Efficiency of coupling was determined by partition of 0.3–0.4 $\mu$mol of [$^3$H]dipalmitoylphosphatidylcholine-labelled liposomes for 25 min in a 5%/5% w/w PEG 8000/Dextran T-500 phase system in 0.15 M NaCl buffered with 0.01 M sodium phosphate at room temperature according to established methods [16]. In the absence of covalently attached MPEG, the liposomes partitioned primarily to the lower dextran-rich phase and also to the interface whereas when MPEG was coupled to the membrane surface, the liposomes partitioned (> 90%) into the upper PEG-rich phase of the two-phase system.

*2.3. TNBS assay.* A 50 mM solution of TNBS in borate buffer was prepared immediately before use and 3 ml of this solution placed in the sample cuvet of a dual beam spectrophotometer. The reference cuvet contained 3 ml of borate buffer. To both the sample and reference cuvets was added 100 $\mu$l of PEGylated liposomes (0.2–0.4 $\mu$mol of lipid) after chromatography and the absorbance at 420 nm followed for 30 min at ambient temperature. After 30 min, 200 $\mu$l of 10% Triton X-100 in water was added to both cuvets and the absorbance monitored for a further 30 min. Controls with non-PEGylated liposomes showed that the increase in absorbance at 420 nm was the same before and after addition of detergent, i.e., under these conditions, 50 percent of the total PE of the content could be detected on the surface of liposomes. After PEGylation, the increase in absorbance after addition of detergent was always greater than before addition of detergent, indicating masking of the surface PE. It is assumed that PE covalently coupled to PEG would not react with TNBS and would therefore not be detected. Because of possible steric hindrance to TNBS labelling imposed by the surface-attached polymer, it must be considered that this assay may overestimate the extent of PEGylation.

*2.4. Miscellaneous methods.* Nuclear Magnetic Relaxation Dispersion (NMRD) $1/T_1$ profiles at 35°C were determined over 0.02–50 MHz as previously described [17] using a custom-built field cycling relaxometer. $^{31}$P-NMR spectra were obtained using a Bruker MSL 200 spectrometer operating at 81.0 MHz for $^{31}$P using a modified Hahn-echo pulse sequence with WALTZ decoupling, 5 s interpulse delay and 50 kHz sweepwidth for a total of up to 640 transients. An exponential multiplication corresponding to 10–30 Hz line-broadening was applied to the free induction decays prior to Fourier transformation. Gadolinium concentrations were determined by inductively-coupled plasma spectrophotometry following acidification of the samples

with nitric acid. Liposome sizes were determined by quasielastic light scattering using a Nicomp Model 270 particle sizer (Goleta, CA).

## Results and Discussion

### *NMRD profiles*

$1/T_1$ NMRD profiles at 35°C for liposomes containing surface-associated and entrapped paramagnetic ions, in the absence and presence of covalently attached PEG, are shown in Fig. 1. For unilamellar liposomes with surface-associated gadolinium, there is a peak in the dispersion profile to high field at approximately 20 MHz. Such a maximum is known to occur for systems in which a paramagnetic chelate is attached to particles greater than approximately 50 kDa, e.g., Mn electrostatically bound to phosphatidylserine liposomes [18] or Gd attached to proteins [19] and reflects a field-dependent increase in the correlation time for the electron–proton interaction [20]. By comparison, for liposomes with entrapped gadolinium, the dispersion profile is virtually identical with that of the free paramagnetic chelate in solution indicating that the measured relaxation rates are not limited by water exchange across the liposome membrane under the experimental conditions [11,21]. After covalent attachment of poly(ethylene glycol) to the surface of the liposomes, there was no significant change in the relaxation rate for either surface-attached or entrapped gadolinium at any field strength. NMRD profiles obtained at 6°C (Fig. 2) show that while the dispersion profile for liposomes with encapsulated gadolinium

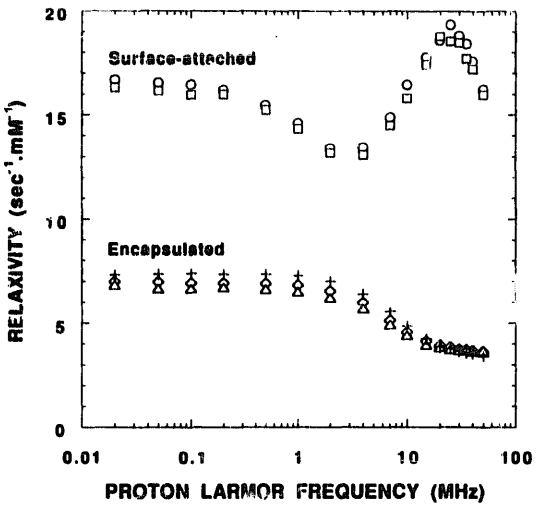


Fig. 1. NMRD ($1/T_1$) dispersion profiles at 35°C for 100 nm diameter unilamellar EPC/PE (8:2) liposomes containing 100 mM Gd-DO3A encapsulated within the liposome interior (◇) before and (△) after coupling PEG 5000 to the membrane surface, together with dispersion profile for (+) 1 mM Gd-DO3A in solution by comparison. Also shown are dispersion profiles for EPC/PE (8:2) liposomes containing 5 mol percent of the surface attached paramagnetic chelate SE-Gd (○) before and (□) after attachment of MPEG.

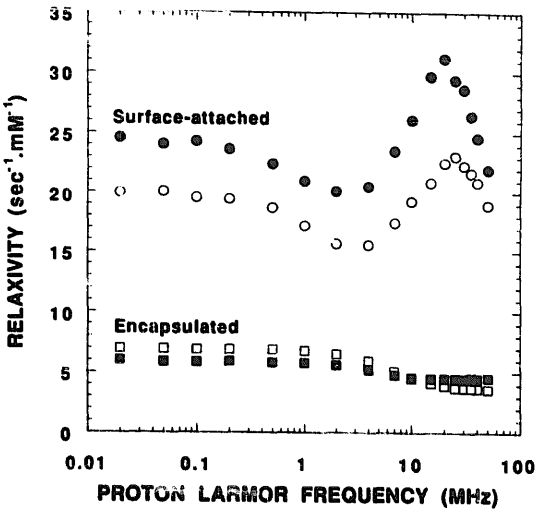


Fig. 2. NMRD ($1/T_1$) dispersion profiles for 100 nm unilamellar liposomes (without surface-attached poly(ethylene glycol)) containing Gd-DO3A at (□) 35°C and (■) 6°C. Also shown are dispersion profiles for liposomes containing 5 mol percent SE-Gd at (○) 35°C and (●) 6°C.

approximates the exchange-limited condition [21], the relaxation rate for surface-attached gadolinium increases at all field strengths. This implies that the relaxation rate observed for surface-attached systems at higher temperature (35°C) is not exchange-limited because otherwise the decrease in temperature would have caused a decrease not an increase in relaxation rate. If the relaxation rate is not limited by water exchange across the vesicle membrane, this implies that gadolinium was detected attached both to the outer and inner surfaces of the lipid bilayer.

The observation that relaxation rates were unaltered after coupling of PEG to the membrane surface may be rationalized by considering the relative timescales for water diffusion between liposomes in dispersion and water transport across the lipid bilayer. The mean free path $L$ for liposomes in dispersion may be approximated from the relation $L = 1/(\sqrt{2}\pi d^2 N)$ where $d$ is the liposome diameter in cm and $N$ the number of particles per $cm^3$. Assuming a surface area per lipid of approx. 0.6 $nm^2$, liposomes of diameter 100 nm, $d = 10^{-5}$ cm, contain approx. $10^5$ lipid molecules. At a lipid concentration of 5 $\mu$mol/ml (Fig. 1) there are approx. $3 \cdot 10^{13}$ liposomes per $cm^3$ and $L$ is approx. $10^{-4}$ cm. An estimate for the time required for water to diffuse $10^{-4}$ cm can be calculated from the three-dimensional solution for the Fick diffusion equation, i.e., the diffusion time $t = L^2/6D$ where $L$ here is $10^{-4}$ cm and $D$ is the diffusion coefficient for water ($3 \cdot 10^{-5}$ $cm^2\ s^{-1}$), whence $t$ is approx. 50 $\mu$s. Diffusion of water between the lipid vesicles is therefore rapid compared to water permeation across the lipid bilayer. Because it is the latter, slower process that limits the ability of water to sample paramagnetic species on the inside of

the liposome, small changes to the former may have little or no effect upon the measured relaxation rates. While our results cannot exclude the possibility that the distributed presence of the polymer on the surface of the membrane does indeed influence the ability of water in the bulk phase to approach the membrane surface, the results of Fig. 1 indicate that even if this does occur, it is a process which has no detectable effect upon the rate determining step for water transport across the lipid bilayer in these liquid-crystalline systems.

Light scattering measurements upon an EPC/DOPE (8:2) liposome preparation indicated a small increase in average diameter from 115 ± 15 nm to 144 ± 28 nm after coupling PEG to the surface of the liposome, suggesting that the polymer may contribute an approx. 15 nm deep layer external to the liposome. We note that the volume of a PEG 5000 molecule in aqueous solution is approx. 17 $nm^3$ [22], thus in order to form a polymer coat 15 nm deep, the polymer would have to project out from the surface of the liposome rather than approximating a globular form on the membrane surface. For particles with long flexible polymers attached to their surfaces, it is entropically disfavored to bring two particles together because the polymers on the surface would have to be bent aside and their motion restricted. Such steric repulsion would prevent rather than induce aggregation [23,24] hence it is unlikely that the apparent increase in average liposome diameter after PEGylation represents vesicle aggregation. TNBS labelling studies indicated that for an EPC/DOPE (8:2) liposome only approx. 35% of the exterior PE on the liposome surface (7% of the total surface lipid) could be PEGylated using a TMPEG to PE molar ratio of 3:1, consistent with other findings [7]. One possible explanation is that the presence of polymer on the membrane surface represents a steric barrier to further attachment. Assuming an average surface area of 0.6 $nm^2$ per lipid, a liposome of diameter 115 nm will have approximately $6.5 \cdot 10^4$ lipid molecules in its outer surface. For a EPC/PE (8:2) liposome, $1.3 \cdot 10^4$ of those will be PE molecules and of those, only 35% (~ 4000 lipids) could be PEGylated as determined by TNBS labelling. Assuming a PEG molecular weight of 5000, the total weight of PEG attached to each liposome is approx. $(4000 \cdot 5000/6.023 \cdot 10^{23}) = 3.3 \cdot 10^{-17}$ g. Assuming a spherical liposome, the volume of the 15 nm deep polymer coat on the liposome surface is readily calculated to be $7.5 \cdot 10^{-16}$ ml, thus the approximate surface concentration of the PEG on the liposome would be 0.04 g/ml or 4% by volume. Given the nature of the errors and assumptions in this crude calculation this value could quite reasonably be off by a factor of two or more, however the value calculated (4%) would be consistent with the observation that there was no change in water permeability as a result of polymer-induced changes in lipid acyl chain order [25].

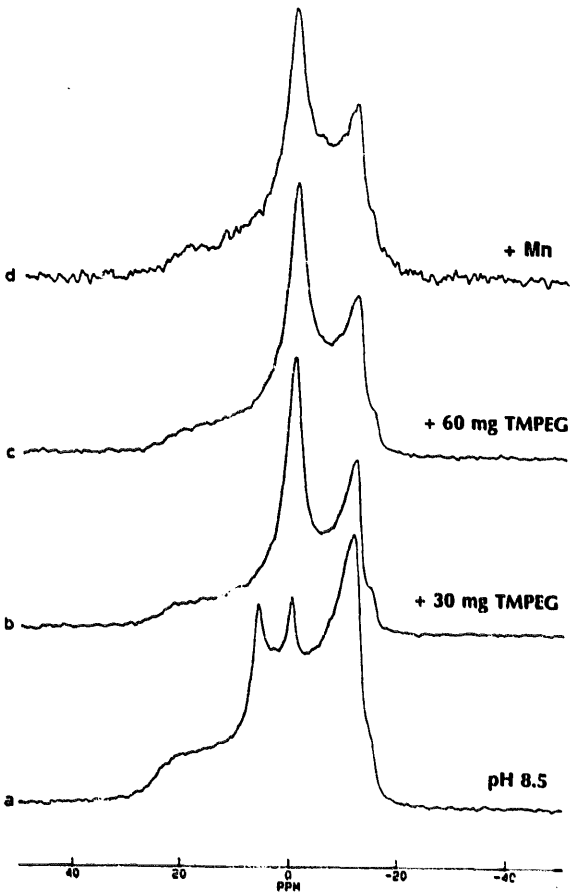


Fig. 3. 81 MHz $^{31}$P-NMR spectra of DOPC/DOPE (1:3) mixtures at 40°C and pH 8.5 (a) in the absence of tresyl-MPEG (TMPEG) 5000, (b) + 30 mg of TMPEG, (c) + 60 mg of TMPEG, and (d) (c) + 5 mM $MnCl_2$.

### *$^{31}$P-NMR*

For a mixture composed of DOPC/DOPE (1:3) at pH 8.5, $^{31}$P-NMR revealed that this lipid mixture exhibited a complex lineshape with three superimposed resonances (Fig. 3). There was a component with an upfield peak and downfield shoulder arising from lipids undergoing rapid symmetric partially-averaged motion on the NMR timescale, consistent with lipid in a lamellar phase [26]. Note the presence of two upfield shoulders because of small differences in the effective chemical shift anisotropy ($CSA_{eff}$) for DOPE and DOPC. The relative height of the two shoulders reflects the 3:1 ratio of DOPE and DOPC in the mixture. There was also a relatively small isotropic component and also a spectral component with reversed asymmetry and reduced chemical shift anisotropy compared to the lipid in the lamellar phase. Numerous previous studies have indicated that for this lipid system, this last resonance correlates with lipid in the hexagonal $H_{II}$ phase (reviewed in Ref. 27). Upon covalent modification with

TMPEG there was a significant change in the appearance of the spectrum with a greatly increased signal at the position of the isotropic resonance coincident with elimination of the resonance corresponding to lipid in the $H_{II}$ phase. Controls established that addition of MPEG which does not covalently bind to the lipid membrane, produces only a broadening of the spectrum consistent with a increase in viscosity, but no change in the phase behavior of the lipid system. The phase changes observed in Fig. 3 therefore occur as a result of the covalent modification of the lipid system rather than an absorption of polymer at the membrane surface.

After modification with TMPEG the visual appearance of the sample changed from opalescent to translucent suggesting that the dispersion contained smaller scatterers than before addition of polymer. Addition of $MnCl_2$ to a concentration of 5 mM resulted in a decrease in the overall signal to noise ratio and a slightly greater decrease in the intensity of the isotropic resonance compared to the underlying broader resonance. This result suggests that the isotropic resonance is unlikely to be due to the presence of either micelles of short $H_I$ tubes because in both cases the phospholipid headgroups would be completely exposed to the aqueous environment and so addition of Mn would eliminate rather than merely decrease the signal intensity from such structures. Taken together, these results suggest that the presence of the isotropic resonance may represent the occurence of smaller oligolamellar vesicles. This would be consistent with the change in the optical appearance of the dispersion as well as the decrease but not elimination of isotropic signal with addition of manganese.

The results presented in Fig. 3 may be considered in terms of an ensemble property of the lipid system, the equilibrium radius of curvature ($R_0$) of the lipid mixture [28] which is decreased when the relatively bulky MPEG molecule is attached to the membrane surface.We have shown that the addition of TMPEG has no effect upon the polymorphic phase behavior of pure DOPE (results not shown) which, in isolation, adopts the $H_{II}$ phase. Presumably this lack of effect arises simply because the TMPEG cannot access the DOPE headgroups which are arranged on the interior of the hexagonally packed water tubes of the 'inverted' $H_{II}$ phase. Thus it should not be considered for the system described in Fig. 3, that the presence of MPEG covalently attached at the vesicle surface modifies the phase properties of only a portion of the lipid so that it prefers to adopt a lamellar rather than a hexagonal $H_{II}$ phase, rather the presence of MPEG on the membrane shifts the equilibrium for the entire lipid mixture such that a lamellar phase better accomodates the changes in $R_0$ for the system as a whole. These results indicate the presence of MPEG covalently linked to a membrane surface can indeed affect the phase behavior of the lipid system. Whether or not polymer-induced changes in curvature will be expressed clearly depends upon the extent of modification with polymer as well as the composition of the system as illustrated in Fig. 4.

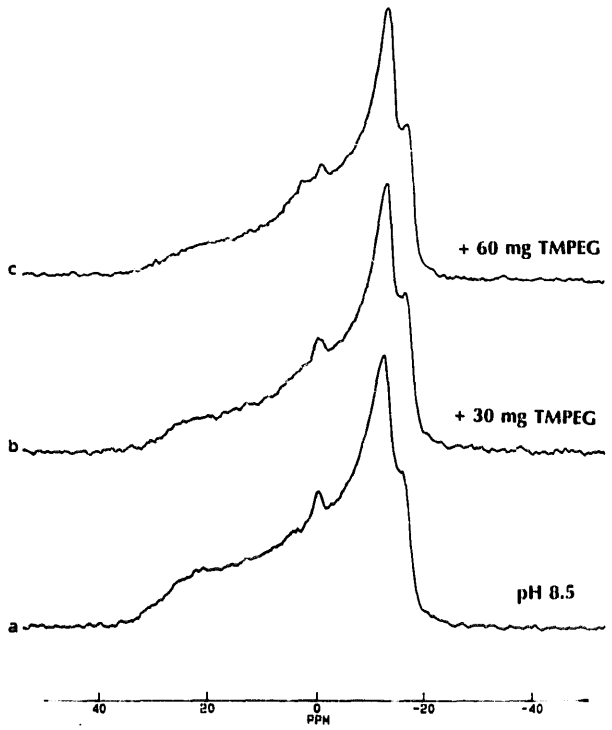


Fig. 4. 81 MHz $^{31}$P-NMR spectra of DOPC/DOPE (1 : 1) mixtures at 40°C and pH 8.5 (a) in the absence of TMPEG, (b) +30 mg of TMPEG, and (c) +60 mg of TMPEG.

In the absence of TMPEG, DOPC/DOPE (1 : 1) multilamellar vesicles at pH 8.5 exhibit an asymmetric $^{31}$P-NMR lineshape with an upfield peak and downfield shoulder consistent with lipid in a liquid crystalline lamellar phase (Fig. 4). Again two upfield shoulders were noticeable due to slight differences in the $CSA_{eff}$ for DOPE and DOPC. There was also a very small isotropic resonance which may arise from a variety of sources such as small vesicles or inverted micelles amongst others [29]. Following modification with TMPEG at two TMPEG/lipid ratios there was a very slight change in the relative height of the upfield peak and downfield shoulder that may reflect small changes in viscosity, however there was no significant change in the effective chemical shift anisotropy nor overall change in the appearance of the spectrum. Addition of MPEG alone caused no change in the $^{31}$P-NMR spectrum. Certainly there was no evidence of a greatly increased intensity of the isotropic resonance as might have been expected if the TMPEG has induced formation of small vesicular structures or micelles [29] or of a resonance with reversed asymmetry as might have been expected if the MPEG caused a lamellar to hexagonal $H_I$ transition [29]. Thus, in this lipid system, covalent

modification with MPEG does not cause any apparent change in the polymophic phase behavior of the system. This should however not be taken to imply that covalent attachment of PEG to the membrane system has no effect on the $R_0$ for the system, merely that the changes in $R_0$ can be accomodated within the existing extended multilamellar phase. Similar considerations will presumably apply to unilamellar vesicles although the caveat must be given that, because of hydrocarbon packing differences between unilamellar and multilamellar systems, quantitatively different behaviour may be observed. It is quite conceivable that at high degrees of modification, the lipid system shown in Fig. 4 may be destabilized to form smaller oligolamellar vesicles (as in Fig. 3) or even micelles.

## Conclusions

Covalent attachment of poly(ethylene glycol) to the surface of unilamellar liposomes has no detectable effect upon the relaxation enhancement produced by paramagnetic species either entrapped within the interior aqueous space of the liposome or attached to the membrane surface.. Covalent attachment of PEG to liposomal MR agents may therefore prove an effective method for increasing circulation half-life without decreasing their effectiveness as contrast agents. $^{31}$P-NMR studies demonstrate unequivocally that covalent attachment of PEG to a membrane surface can influence the equilibrium radius of curvature ($R_0$) and hence phase behavior of the lipid system as a whole. Whether or not such polymer-induced changes in $R_0$ are actually expressed depends upon both the extent of polymer modification as well as the composition of the lipid membrane to which the polymer is covalently attached.

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