FARADAY

Steric stabilization of phospholipid vesicles by block copolymers

Vesicle flocculation and osmotic swelling caused by monovalent and divalent cations

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Steric stabilization of small unilamellar phospholipid vesicles (liposomes) following conjugation of (tri-)block copolymer molecules by physical means can be carried out either by allowing the copolymer molecules to participate in vesicle bilayer formation, or by adsorbing the copolymers onto preformed liposome surfaces. These systems and a bare liposome system have been compared. The degree of steric stability was determined relative to vesicle resistance against flocculation and osmotic swelling induced by cations. The pronounced affinity of monovalent (Na^+) and divalent (Mn^{2+}) cations for the phospholipid surface was exploited to cause flocculation of the vesicle dispersions. Moreover, the formation of an osmotic gradient by varying the electrolyte concentration in the outer and inner vesicle aqueous phases was used to produce swollen liposomes. Size determination by quasi-elastic light scattering (QUELS), surface characterization by laser Doppler electrophoresis (LDE), turbidity measurement by UV–VIS spectroscopy, NMR measurements and phase contrast video-microscopy were used to characterize and compare the vesicle systems. In both ways of copolymer addition the large hydrophilic polymer chains coat the phospholipid surface, as manifested by the considerable resistance to cation-induced flocculation. The presence of polymer at the vesicle surface offers increased protection, owing to the repelling forces due to polymeric steric barriers acting on vesicle close contact. Only the vesicles formed in the presence of copolymer molecules exhibited inert behaviour to osmotic swelling, confirming the alteration of the bilayer structure and its related properties. The advantages of constructing sterically stabilized vesicles by allowing the hydrophobic block of the copolymer to form an integral part of the bilayer are thus evident, since a robust surface coating is achieved by firmly anchored copolymer molecules rather than surface-adsorbed polymers, while avoiding the costly cumbersome chemical modification process of attaching polymer chains onto lipid headgroups.

Model membranes consisting of, or containing, acidic phospholipids, carry negative charges on their surface from the dissociation of the $-PO_4^-$ and $-COO_2^-$ groups. The effect of cations on phospholipid vesicles of varying lipid composition, carrying negative charges on their surface has been the subject of much research almost since the description of liposomes as simple model membrane systems.[1] The study of (mainly) Ca^{2+}-induced fusion of phospholipid vesicles, composed of phosphatidylserine (PS),[2] phosphatidylglycerol (PG),[3] and other phospholipids is important from a biochemical point of view for its obvious physiological implications. A number of techniques [*e.g.* differential scanning calorimetry (DSC)[4]] and novel assays based on the fluorescence properties of the complexes formed,[5] were used to determine and describe the fusion process between the vesicles. It was initially shown that Ca^{2+} induces release of vesicle contents,[6] triggering extensive studies and, principally, concluding that negatively charged liposomes are needed for Ca^{2+}-dependent fusion,[4] and that the fusion process has a time course of several hours.[7] The latter suggestion, together with observations of massive aggregation of PS small unilamellar vesicles (SUVs) induced by Ca^{2+},[8] led to another aspect of interest concerning the effect of cations on liposomes: the flocculation/aggregation of vesicles as a primary step in the fusion process. This area is studied mainly from a physicochemical point of view, owing to the theoretical and experimental implications it has on general colloid stability theories.

From the quite extensive investigations to date, it can be concluded that: (a) the flocculation of vesicles can occur with the addition of monovalent cations, without subsequent fusion; (b) the flocculation of liposomes by divalent cations proceeds through an inter-vesicle salt-linkage mechanism, dependent on the statistics of the distribution of the cation-occupied polar headgroups on the membrane surface,[9] leading eventually to vesicle fusion and exchange of their inner aqueous contents; (c) the stability of vesicles against divalent cations can be described by the DLVO theory, with the addition of repulsive hydration (short-range) and/or steric (longer-range) forces, according to the surface characteristics (*e.g.* the nature of the phospholipid headgroup, the presence of macromolecules) of the particular vesicle system.

In the present study, the flocculation of phospholipid vesicles in the presence of monovalent and polyvalent cations was used to provide valuable information on the degree of stabilization offered to vesicles when coated with A–B–A [where A is polyethylene oxide (PEO) and B polypropylene oxide (PPO)] block copolymer molecules. Various studies have dealt with the steric stabilization of phospholipid vesicles: by adsorption of glycolipids and water-soluble polymers (including block copolymers) onto the outer liposome surface;[10–12] by incorporation of glycolipids[13,14] and polyoxyethylene ether surfactants in the vesicles;[15,16] or by covalent attachment of polyethylene glycol (PEG) chains to the

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phospholipid headgroup.[17] However, very few studies[18,19] have been concerned with the systematic investigation of the steric stability offerred, particularly against flocculation of the vesicle dispersions. In this work, coating of the liposome surface was carried out in two different ways, resulting in two different vesicle systems, as described elsewhere.[20] The intermixing of phospholipid and polymer molecules, followed by hydration and SUV formation, was thought to lead to incorporation of the hydrophobic PPO blocks of the copolymer inside the lipid bilayer, while the addition of the copolymer molecules after vesiculation will result in weak Langmuirian-type adsorption onto the liposome. The effect of monovalent and polyvalent cations on the above vesicle systems was compared by dynamic light scattering, turbidimetry, optical microscopy and ^{13}C NMR spectroscopy.

Experimental

Materials

The vesicle systems were formed from a mixture of soybean lecithin lipids [*ca.* 50% D-α-dimyristroylphosphatidylcholine (DPPC)] from Sigma. The aqueous dispersion medium was always doubly distilled, deionised water. The A–B–A type (tri-) block copolymer used was of the Synperonic PE family, namely PF127, supplied by ICI Surfactants, Belgium, with structure $(EO)_{99}$–$(PO)_{65}$–$(EO)_{99}$. The lecithin and block copolymer concentrations mentioned are given in wt.% with respect to the total sample weight, unless otherwise stated.

The salts used in this study NaCl and $MnCl_2 \cdot 4H_2O$ were from Sigma. Both the monovalent and divalent cations were of standard laboratory grade and were used without further purification. Mn^{2+} was used in order to offer alternative information on the effect of divalent cations other than Ca^{2+} (by far the most extensively investigated cation). Moreover, the paramagnetic nature of Mn^{2+} offered possibilities of studying the interaction with the phospholipid surfaces by nuclear magnetic resonance (NMR).

Methods

Preparation of vesicle systems. The formation of SUV was performed by sonication of hydrated lipids[21] using a Kerry ultrasonic bath (50 Hz). There was no effect on the mean vesicle size when the lipids were dispersed in chloroform–methanol (4 : 1) mixture prior to hydration with the aqueous phase. Sonication was shown to produce minimum-size liposomes of 20–25 nm radius. The temperature in the ultrasonic bath was kept constant 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, without causing any major implications. The standard phospholipid concentration used to produce the vesicles was 2 wt.%. The electrolytes were added to the vesicles by dilution of the 2 wt.% dispersions, always resulting in final lecithin concentrations of 0.02 wt.%.

Two methods have been used to include the A–B–A copolymers into the vesicle systems. First, the vesicles were prepared (hydration and sonication steps) in the presence of copolymer molecules at the desired concentration, and secondly, the liposomes were formed and, after sonication, 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 to ensure optimum interaction between the copolymer molecules and the phospholipid surface. Samples denoted (I) refer to vesicle systems where the block copolymer was added initially and samples denoted (A) refer to the systems with addition of the polymer after the liposomes had been formed. Further information and analysis on the structural and morphological characteristics of the resulting vesicle systems has been published previously.[22]

Dynamic light scattering. During a dynamic light scattering experiment, the coherent light of a laser beam passes through the colloidal sample. The particles move at random owing to their Brownian motion, the latter being characterized by the diffusion coefficient. Scattering occurs and the scattered light is collected by a photodetector at a certain angle from the incident light. The photon signal detected is photomultiplied and sent to a digital correlator where the correlation process takes place. This process consists of the temporal correlation of the detected photocount, which, according to certain assumptions (monodisperse, spherical, homogeneous particle dispersions) is a single exponential whose time constant is the characteristic time of the particles' Brownian motion, *i.e.* the diffusion coefficient according to Stoke's equation. Consequently, from the diffusion coefficient and other independently measured properties (*e.g.* viscosity, refractive index) the particle hydrodynamic radius can be deduced from the Stokes–Einstein equation:

$$D = \frac{kT}{6\pi\eta R_H} \quad (1)$$

where k is the Boltzmann constant, T the absolute temperature, η the viscosity of the dispersion medium and R_H the mean vesicle radius.

A Malvern (UK) 4700 PCS apparatus, with an Argon laser beam (50 mW) emitting at a wavelength of 488 nm was used. The mean vesicle diameter was calculated by measuring the diffusion coefficients by correlation at a 90° angle and 25 °C. At least three series of ten measurements were performed for each sample. The pinhole of the photomultiplier was normally adjusted to 100 μm, to obtain optimum photon counts. The viscosity (0.8905 mPa s) and the refractive index (1.337 at 488 nm) for water were input for all measurements, unless stated otherwise. All samples were measured at 0.02 wt.% lipid concentration, to eliminate any artifacts responsible for deviations from eqn. (1). The mean vesicle diameter and the polydispersity index of the sample were automatically provided by the instrument using the cumulants method of analysis.[23,24] The estimated error of the dynamic light scattering results (from the standard deviation) presented in this study was *ca.* 1–2% of the mean vesicle diameter value obtained directly from the instrument.

Electrophoresis. The electrophoretic mobility was measured by laser velocimetry, with a Zetasizer 4 (Malvern, UK) operating with an He–Ne laser beam (5 mW). The principle of operation is that two laser beams of equal intensity are allowed to cross at a particular point within the cell containing the suspension of vesicles. At the intersection of the two beams, which is focused at the stationary layer, interferences of known spacing are formed. The particles, moving through the fringes under the influence of the electric field, scatter light, whose intensity fluctuates with a frequency that is related to the mobility of the vesicles. From the electrophoretic mobility of the vesicles the ζ-potential can be calculated using the Hückel relation:

$$\zeta = \eta \frac{u_E}{\varepsilon\varepsilon_0} \quad (2)$$

where η is the viscosity of the dispersion medium, ε_0 is the permittivity of free space, ε the relative permittivity and u_E the electrophoretic mobility of the particle.

Turbidity. Relative turbidities were determined *vs.* the bare liposome system (*i.e.* no copolymer nor electrolytes added) under physiological buffer conditions. Variations in the amount of scattered light at 450 nm were, in this way, used to monitor the flocculation and swelling of the vesicle systems. All turbidity measurements were carried out using a Pye-Unicam SP1700 UV spectrophotometer.

Video optical microscopy. A Leitz Diaplan light microscope was used to obtain the optical micrographs of the vesicle dispersions. The microscope was connected to a Cohu high-performance CCD camera and video micrographs were processed and printed in a Mitsubishi video copy processor. The samples of the vesicle systems were placed on a microscope slide while directly observing the flocculated and/or osmotic swollen vesicles under the cover slip. All video-micrographs were taken using the 40x objective lens under phase contrast.

^{13}C NMR. ^{13}C NMR spectra were recorded on a JEOL GSX400 spectrometer. The spectra were acquired with 45′ pulses, a 2 s repetition rate, 32 k data points, a 250 ppm spectral window, with complete proton decoupling and chemical shifts were referenced to external TMS at 0.00 ppm. Glucose (Laboratory Grade, Aldrich) was also used. All NMR experiments were carried out in D_2O (Aldrich) at 2 wt.% total lipid concentration.

Results

Vesicle surface characteristics

The addition of block copolymer molecules before and after the high-energy sonication step and the formation of small unilamellar liposomes, is thought to result in two different vesicle structures. The incorporation of the more hydrophobic PPO block of the copolymer inside the liposome bilayer, will lead to an improved, sterically stabilized vesicle system, compared with the alternative system, where the block copolymer molecule is adsorbed on the vesicle surface in a rather flat configuration. Improvement of the dispersion stability is expected because of improved physical anchoring of the polymer molecule on the vesicle surface.

The mean vesicle diameter and the vesicle surface ζ-potential (Fig. 1 and 2), illustrate the differences between adding the block copolymer molecules before and after liposome formation. The mean particle diameter for the systems prepared in the presence of polymer molecules (I) shows a considerable increase, reaching a plateau value of *ca.* 75 nm, for a range of added copolymer concentrations, and then decreasing to lower values, most probably owing to solubilization of the bilayer. On the other hand, addition of block copolymer molecules after the formation of the vesicles (A) gives a Langmuirian-like adsorption, with a plateau value of *ca.* 48 nm, above 0.01 wt.% (copolymer : lipid molar ratio = 0.034) of added polymer (Fig. 1).

Surface characterization was obtained by monitoring the ζ-potential at the vesicle surface, which decreases from −51.7 to *ca.* −20 mV after addition of block copolymer molecules, irrespective of the way in which they are added. The presence of the copolymer molecules coating the vesicle surface was thus evident in both types of addition. The ζ-potential at the surface of the particles decreases on coating, because of an outer shift of the shear plane caused by the long polymer chains. The experimental observations can be explained in terms of the conformations attained by the PEO chains at the surface of the vesicle. However, the subtle differences do not allow for definite explanations.

From the above investigations on the structural and surface characteristics of the vesicles, two important conclusions were drawn: (a) two structurally different vesicle systems seem to occur, depending on the way in which the block copolymer molecules are added and; (b) the vesicle surface, in both cases, is coated with copolymer molecules, which most probably will attain different chain conformations. More systematic studies offering a comprehensive view of the structural differences between the vesicle systems prepared are presented elsewhere.[22] The above conclusions are meant to serve as descriptions of the vesicle systems. The primary aim of the present study was to compare the stability of these vesicle systems against flocculation and swelling occurring in the presence of salts.

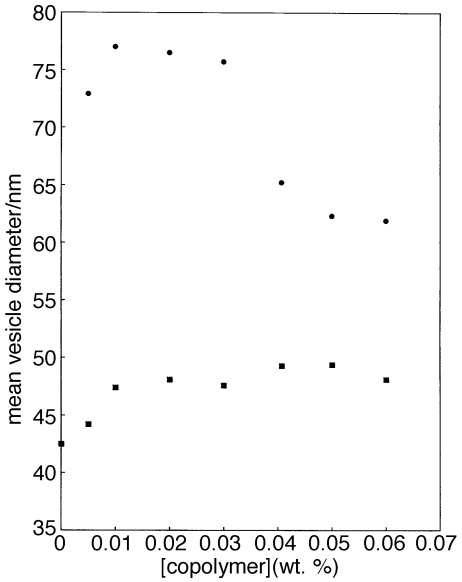

Fig. 1 Mean vesicle diameter as a function of added PF127 concentration; (A) vesicle systems (■) and (I) vesicle systems (●). Bulk lipid concentration 0.02 wt.%.

Preliminary stability studies of vesicles

Series of samples were stored at room temperature (*ca.* 20 °C) in air-tight glass vials for 2 months after preparation, in order to test their physical stability. N_2 was bubbled through the samples for at least 5 min before storage. The (A) samples were unstable after the storage period, as evidenced by floc

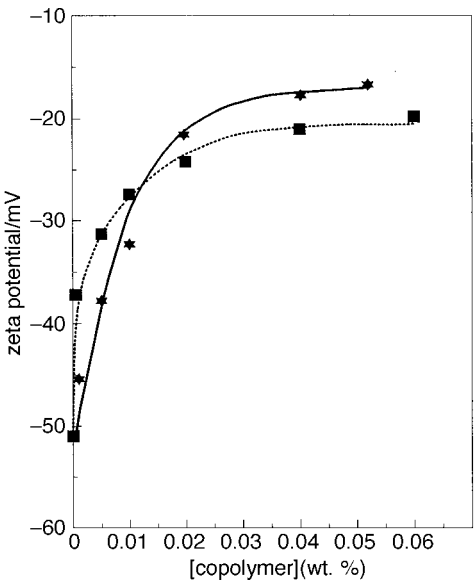

Fig. 2 Vesicle ζ-potential as a function of added PF127 concentration; (A) vesicle systems (■) and (I) vesicle systems (★). The high copolymer concentration points for the (I) vesicles are not included, because vesicle destruction is thought to occur. Bulk lipid concentration 0.02 wt.%.

formation. However, the (I) samples seemed to have a greater long-term stability, as evidenced by the clear solutions obtained after the 2 month storage period. The bare liposome system was the least stable vesicle population, resulting in a thick slurry. This preliminary study provided serious indications that the presence of block copolymer molecules during vesiculation affected the bilayer structure, increasing its physical durability.

Effect of monovalent cations (Na^+)

The bare liposome system (0.02 wt.% lipid), and the (I) and (A) samples were compared. The mean vesicle size was monitored while increasing the NaCl concentration in the dispersion medium (outer aqueous phase). As can be seen from Fig. 3, with the bare liposome system flocculation started between 0.1 and 0.5 M NaCl, while the two block copolymer-containing systems demonstrated higher resistance to the cations, with increase in their mean size occurring between 2 and 3 M NaCl. The flocculation of the bare liposome system by Na^+ was reversible; on gradual dilution of the sample, deflocculation occurred.

Preparing the vesicle systems in high electrolyte aqueous buffer, followed by dilution and therefore reduction of the NaCl concentration only in the external aqueous phase, provided information on the water permeability through the bilayer and the extent of vesicle osmotic swelling, as water tends to efflux in the inner vesicle aqueous phase. From Fig. 4, it can be seen how the mean vesicle diameter changes with a decrease in the NaCl concentration from 1 to 0.001 M. There appears to be some deflocculation as the mean vesicle size decreases with dilution for all three systems. However, the final vesicle sizes, obtained after dilution of the external aqueous phase to 0.001 M NaCl, for the bare liposomes and (A) samples increased remarkably in size (70 and 75.4 nm, respectively) compared to those in a normal aqueous buffer (42.5 and 48.8 nm, from Fig. 3). In contrast, for the (I) system, where the polymer may be incorporated inside the bilayer, there was hardly any increase in mean diameter (82 nm *ca.* to 77.6 nm in pure water).

Effect of divalent cations (Mn^{2+})

Studies using $MnCl_2$ were undertaken to investigate the interaction with vesicles. The concentration of electrolyte was

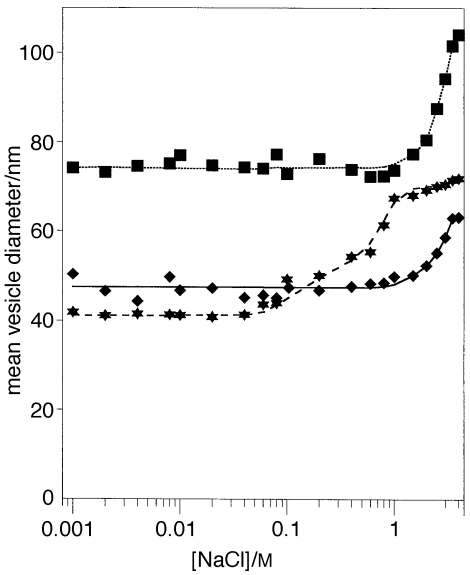

Fig. 3 Mean vesicle diameter determination by dynamic light scattering for increasing concentration of NaCl in the external aqueous phase. The final lipid concentration was 0.02 wt.% at all times. (★) bare liposomes; (◆) (A) vesicle systems; (■) (I) vesicle systems. This notation is kept throughout.

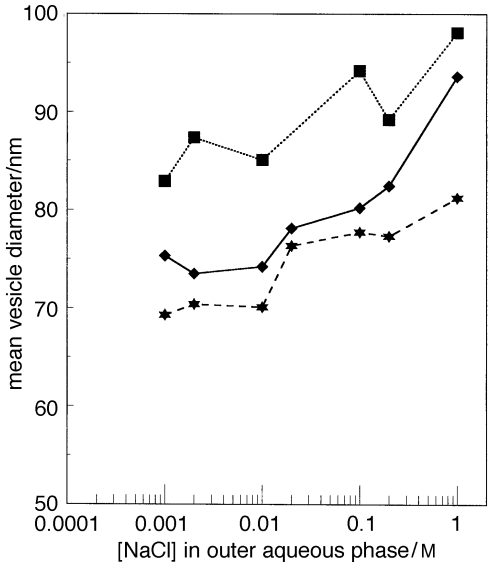

Fig. 4 Mean vesicle diameter of vesicles when diluting the outer aqueous phase. Vesicles prepared in 1M NaCl (*i.e.* follow the sizes from right to left).

increased in the outer aqueous phase, and the behaviour of the different systems was examined by turbidity, PCS, optical video-microscopy and ^{13}C NMR. Note that much lower concentrations were used with the divalent cations than with NaCl, because of the much higher affinity that these ions exhibit for the liposome surface (see Discussion).

When the electrolyte concentration in the vesicle outer aqueous phase was increased, the turbidity of the bare liposome system increased sharply (Fig. 5). The copolymer-containing vesicle systems, however, exhibit different behaviour from the bare liposome system, with turbidity values reaching a plateau above 0.01 mM Mn^{2+}. The mean vesicle diameter determination for the three vesicle systems gave a very similar result to the turbidity measurements (Fig. 6). The main effect is that the copolymer-containing vesicle systems show particle size increases around 0.5 mM $MnCl_2$, whereas the bare liposomes seem much more susceptible to flocculation with increasing electrolyte concentration.

These observations were followed by a more systematic examination of the flocculation of the liposomes by Mn^{2+}, using optical microscopy and ^{13}C NMR. The bare liposome

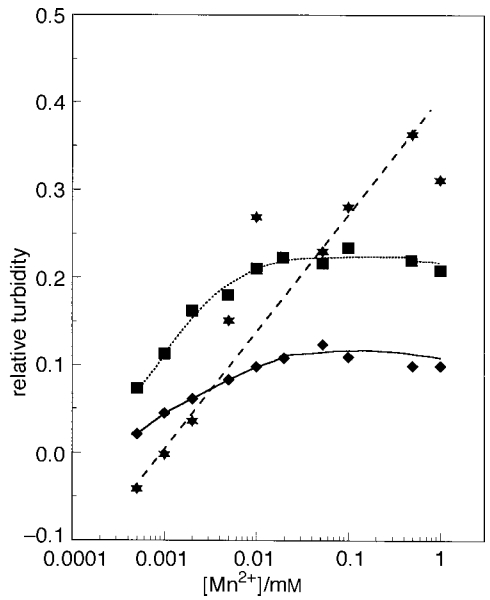

Fig. 5 Turbidity relative to bare liposome system in pure water for increasing concentrations of $MnCl_2$ at the external aqueous phase

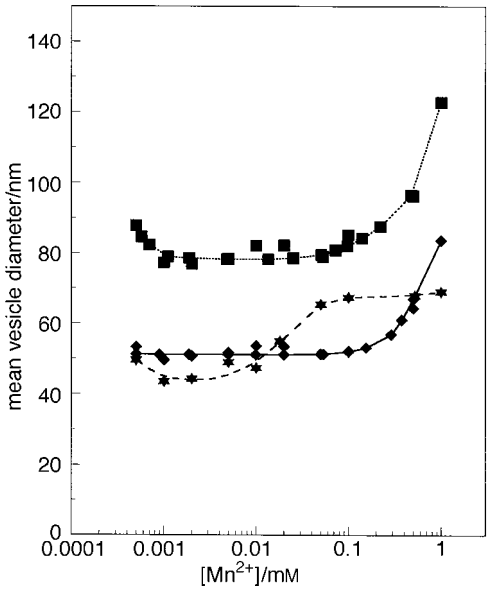

Fig. 6 Mean vesicle size for increasing concentrations of $MnCl_2$ in the external aqueous phase

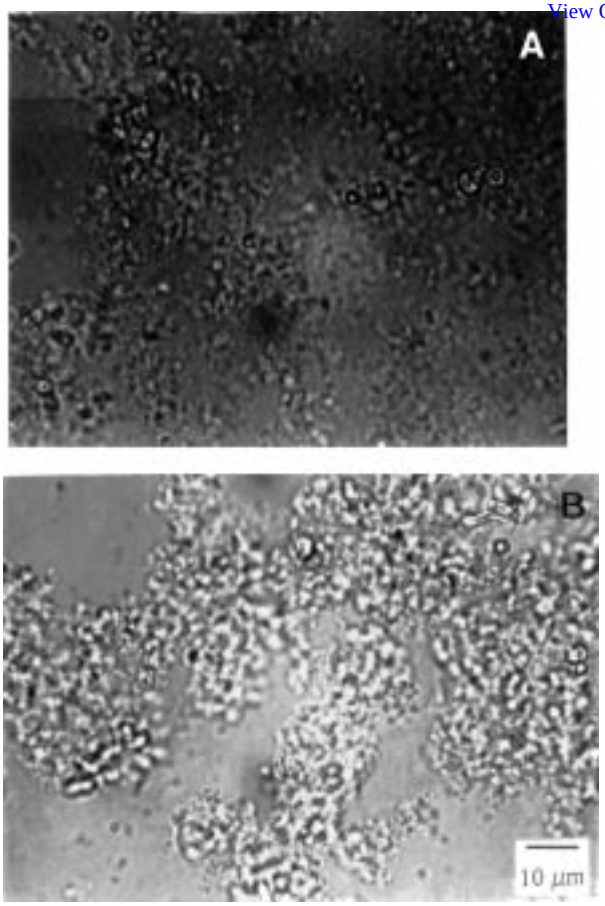

Fig. 7 Optical micrographs of the upper layer of a flocculated bare liposome sample before (A) and after (B) 5 × dilution with pure water. Bulk lipid concentration 2 wt.%; $MnCl_2$ concentration 0.1 mM.

system separated into two layers 24 h after salt addition (0.1 mM $MnCl_2$). The top layer consisted of strongly flocculated liposomes that, even after 5 × dilution did not deflocculate (Fig. 7A and B). The lower layer consisted of weakly flocculated liposomes that exhibited similar behaviour to the reversible flocculation caused by NaCl. Gradual dilution resulted in a dispersed liposome system (Fig. 8A–D). The differences between the two layers of the flocculated liposome sample are thought to occur because of the binding mechanism and kinetics between Mn^{2+} cations and the phospholipid headgroups (particularly the phosphate group).

Stronger evidence for the interaction between Mn^{2+} cations and the phospholipid surface was provided by NMR. The ^{13}C NMR spectrum of the bare liposome system is shown in Fig. 9. The peak of interest at 55.11 ppm depicts the $^{+}N(CH_3)_3$ methyl carbons of the phospholipid headgroup, while the 12 sharp signals from 62–105 ppm result from the sugar (glucose) molecules included in the sample. Note that these sugar molecules were added specifically and solely for the purpose of the particular experiment, exactly prior to performing the NMR experiment, thus minimising their interaction with the vesicle components under study. The experiment was based on the ability of Mn^{2+} metal ions to broaden the resonance signals of nuclei which are in close proximity to the cations.[25] ^{13}C NMR spectra of the liposome systems containing Mn^{2+} cations only in the external aqueous phase, and in both outer and inner aqueous phases, are compared with the spectrum without Mn^{2+} (Fig. 10). The observation that more than half the signal is broadened in the presence of Mn^{2+} at the outer aqueous phase, and almost disappears when cations are present in both the inner and outer water phases (in contrast to the practically constant sugar signals), was used as solid evidence of strong Mn^{2+} binding onto the headgroup of the liposomes. If binding was not taking place, then the Mn^{2+} ions would be expected to broaden, at least partly, the sugar molecule carbon peaks in their vicinity.

Preparation of the vesicles in high $MnCl_2$ concentration, provided further information on their flocculation and response to osmotic effects. The electrolyte concentration in which the vesicle systems were formed was 5 mM $MnCl_2$ and the final dilution step resulted in 0.05 mM $MnCl_2$ at the external aqueous phase. From both the turbidity and the size data obtained (Fig. 11 and 12), the (I) samples exhibited distinctly different behaviour from the other two systems. They seemed to be almost completely resistant to the effects of Mn^{2+}. Turbidity values remained unchanged throughout the 100-fold decrease in the electrolyte concentration of the vesicle outer aqueous phase (Fig. 11), while the mean vesicle diameter increased minimally (*ca.* 88 nm) compared to values obtained in pure water (77.6 nm, Fig. 12). However, the liposome system and the (A) samples, demonstrated almost identical behaviour; much higher mean diameter values (*ca.* 140 nm *cf.* 42.5 and 48.8 in pure water) were obtained after 100 × dilution.

Therefore, the response of the three vesicle systems to the osmotic gradient produced after dilution agrees with the observations from the study of monovalent cations. The (I) samples displayed increased stability. These effects were further investigated using optical microscopy. Interestingly, when diluting the 5 mM Mn^{2+} dispersions 10-fold in water, different behaviour is observed (Fig.13 A–F). The flocculated bare liposome system dispersed almost immediately on dilution (A and B) forming vesicles of large mean diameter. The (A) samples also redispersed into large vesicles (C and D). The large vesicle size observed is believed to be due to osmotic swelling of the vesicles as the inner aqueous phase Mn^{2+} concentration was 5 mM, while the outer water phase is diluted 10 times (0.5 mM $MnCl_2$). However, the (A) samples on dilution with water seem to produce a well dispersed system of low mean diameter (E and F), thus exhibiting considerable resistance to swelling.

Discussion

Since flocculation of liposomes can be seen as a prerequisite of bilayer fusion, numerous studies have been concerned with this effect. Monovalent cations have been reported to cause

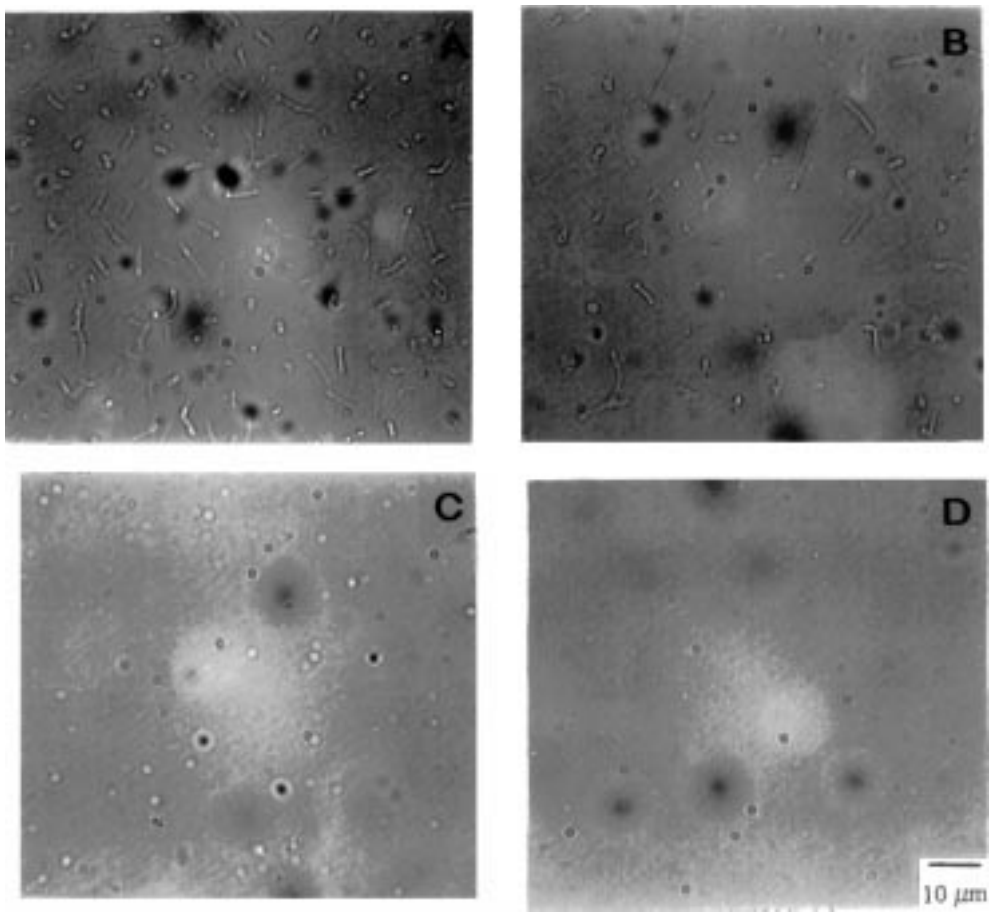

Fig. 8 Optical micrographs of the lower layer of the same flocculated bare liposome sample of Fig. 7, before (A), and after 5 × (B), 50 × (C), and 100 × (D) dilution with pure water buffer

aggregation of acidic phospholipid vesicles without being able to induce any fusion,[26,27] owing to the low binding affinities of these ions for the phospholipid surface, compared with those of divalent or polyvalent cations. This was attributed to the compression of the double layer.[28,29] It is widely accepted that the aggregation behaviour of vesicles, induced by divalent cations, involves a much more complicated process, not well explained to date. A particularly important effect occurring when divalent cations are added to a liposome dispersion causing the reduction of the electrostatic repulsive forces, is surface ion binding, where the cations can bind to the ionizable groups and decrease the apparent negative charge density on the membrane surface.[30] Such an effect was clearly demonstrated in the present study, when Mn^{2+} cations were bound to the phospholipid headgroups (Fig. 7–10).

The binding of multivalent cations may well lead to reversal of the sign of the ξ-potential. The first report of such behaviour for membrane systems was by Bangham *et al.*[31] who showed that human erythrocyte membranes formed stable suspensions possessing a ξ-potential of -14 mV, but continued to be stable even after the addition of 20 mM Ca^{2+}, resulting in the reduction of the ξ-potential to almost zero. Such behaviour, that does not follow from classic DLVO theory of colloidal stability, can be attributed to additional steric repulsive forces due to the presence of proteins, sugars or other macromolecules at the membrane surface. These forces are responsible for the observed stability of the copolymer-coated vesicles against flocculation caused by

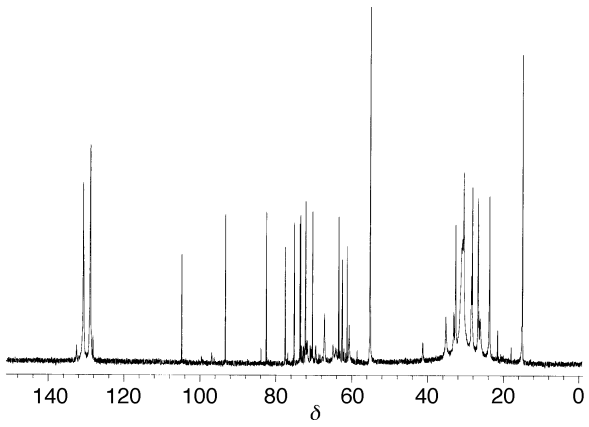

Fig. 9 ^{13}C NMR spectrum of a bare liposome system, immediately after addition of an arbitrary amount of sugar (glucose) molecules. Bulk lipid concentration 2 wt.% (see text for peak assignment).

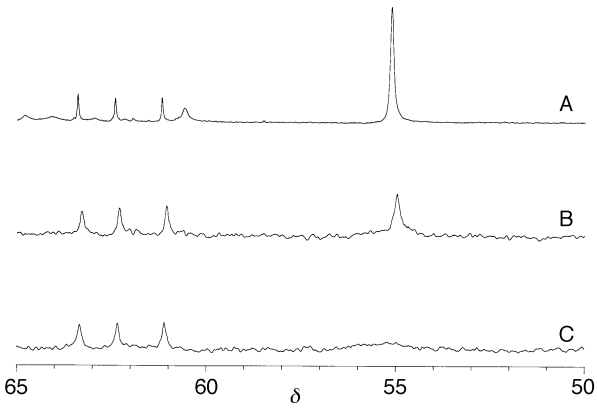

Fig. 10 Region 50–65 ppm of the ^{13}C NMR spectrum of Fig. 9. (A), after the addition of $MnCl_2$ to the external (B), and to both internal and external (C) vesicle aqueous phases. $MnCl_2$ concentration 0.01 mM. The experiments were run immediately after $MnCl_2$ addition, to avoid destruction of the vesicle system.

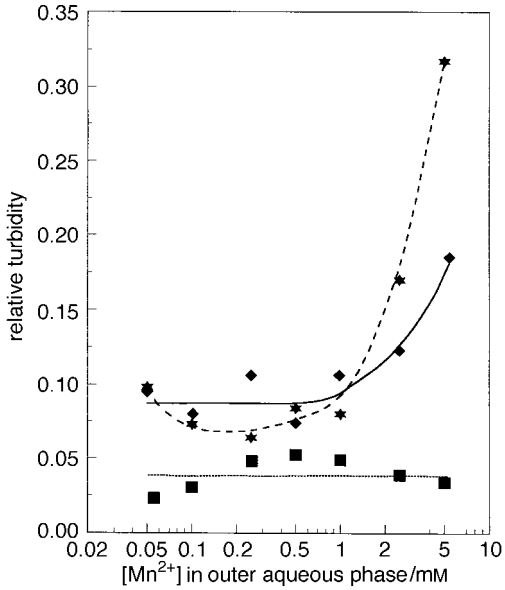

Fig. 11 Relative vesicle turbidity when diluting the outer vesicle aqueous phase. Vesicles prepared in 5 mM $MnCl_2$.

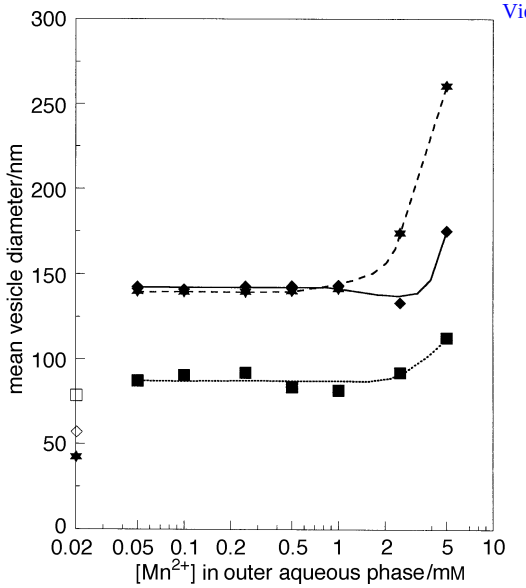

Fig. 12 Mean vesicle size when diluting the salt concentration in the outer aqueous phase. Note that the open symbols on the *y*-axis indicate the mean vesicle diameter for each vesicle system prepared in pure distilled water. Bulk lipid concentration was kept constant at 0.02 wt.%, prior to measurement, at all times.

cations in the outer aqueous environment (Fig. 3, 5 and 6).

Other studies have been concerned with the dependence of the flocculation of liposomes on various factors, such as the concentration of divalent cations, the vesicle concentration and the use of cations of different valency and nature.[30,32] There is a notable selectivity in the binding of divalent cations on the membrane surface: $Mn^{2+} > Ca^{2+} > Mg^{2+}$ [33,34] which is consistent with more recent data,[35,36] showing that binding increases with ion charge and that ions of the same charge can differ in their affinity for a liposome surface.‡ All this information was considered critical to drawing conclusions about the effect of Na^+ and Mn^{2+} cations on the bare and copolymer-coated vesicles compared in this study. As expected, the presence of Mn^{2+} exhibited considerably higher affinity towards the phospholipid surfaces (hence leading to easier vesicle flocculation and binding) than the Na^+ cations.

Bare liposome system

From the present studies, interesting information was provided about the bare liposome dispersion. The presence of Na^+ ions at the outer aqueous phase (Fig. 3) resulted in weak flocculation (increase in size), from the depression of the electrostatic double layer at the liposome surface, *i.e.* the 'screening effect' of the cations. This finding agrees with previously reported effects of monovalent cations on acid surface liposomes.[29] When Mn^{2+} ions were present at the outer aqueous liposome phase, stronger flocculation of the bare liposomes occurred (Fig. 5 and 6), resulting in separation of the sample into two distinct layers. The optical microscopic study (Fig. 7 and 8) suggested that, at the top layer, bridging flocculation of phospholipid vesicles was occurring *via* surface-bound cations, therefore deflocculation was not possible on dilution with a physiological aqueous buffer. The lower layer, on the other hand, consists of a vesicle system where binding of Mn^{2+} cations is occurring, therefore weak flocculation is mainly observed owing to the cations 'screening effect.' These observations suggest that the binding of Mn^{2+} cations onto phospholipid vesicle surfaces and the subsequent bridging flocculation caused, are governed by kinetic constants, rather than occurring instantly. Indeed, these results agree with previous reports that divalent cations (Ca^{2+}) bind strongly to liposomes, leading to aggregation. The strong and specific binding of the cations onto the phospholipid headgroup was confirmed by the ^{13}C NMR study (Fig. 9 and 10). Binding of the Mn^{2+} onto the headgroups of the phospholipids led to almost complete removal of the cations from the aqueous phase, and strong bridging flocculation of liposomes. This finding affirms previous suggestions of specific Ca^{2+} cation binding onto the phosphate groups of the phospholipid, as the initial step in the liposome fusion process.[7]

When liposomes were formed in a high electrolyte aqueous buffer, so that cations (Na^+ and Mn^{2+}) were present in both aqueous phases, flocculated dispersions were obtained. On dilution with pure water, these liposome systems initially displayed some deflocculation (decrease in size), but the mean liposome diameter reached at high dilution was significantly higher (70 nm for Na^+ and 140 nm for Mn^{2+}) than the mean diameter of the liposome system prepared in pure water (42.5 nm) (Fig. 4 and 12). This effect was attributed to swelling of the liposomes due to the osmotic gradient between the different electrolyte concentrations in the outer and inner liposome aqueous phases, as confirmed by the optical microscopic study (Fig. 13).

(A) Systems

The preliminary physical stability study indicated that destruction of the (A) vesicle systems could not be avoided (at least over a 2 month period), as for the bare liposome system. However, in the presence of monovalent (Na^+) and divalent (Mn^{2+}) cations at the outer vesicle aqueous phase (Fig. 3, 5 and 6), the addition of block copolymers to the pre-formed liposomes increased their stability against flocculation. Hence, coating of the liposome surface by the polymer molecules results in improvement of the vesicle stability towards aggregation caused by the cation-associated collapse of the surface electrostatic double layer. This effect demonstrates the steric repulsive forces acting between the tri-block copolymer-coated liposomes. Such forces have been previously described by others studying vesicles formed with the inclusion of pegylated lipids,[37] and polyoxyethylene ether surfactants.[18]

The conformations attained by the polyethylene oxide chains at the vesicle surface could not be confidently determined from the experimental data. However, more detailed analysis of the vesicle surface characteristics is published elsewhere.[38] What is evidently occurring is that, by increasing the cation concentration up to a critical point, the extending

‡ Binding constants values of 31 200, 1408 and 438.8 $dm^3\ mol^{-1}$, respectively, were reported for the series $La^{3+} > Ca^{2+} > Mg^{2+}$.

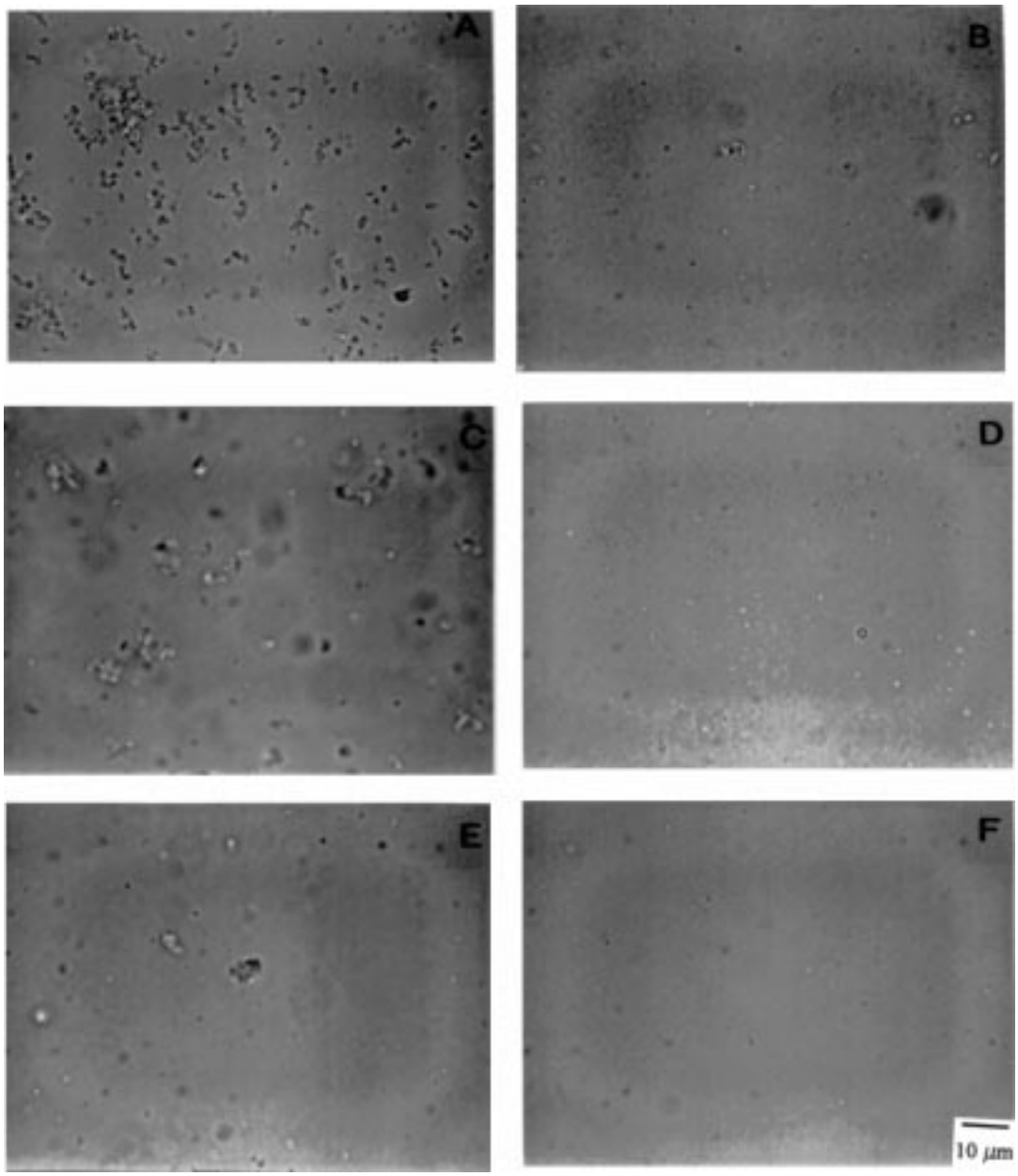

Fig. 13 Optical micrographs of the three vesicle systems depicted in Fig. 11 and 12. The bare liposome system before (A) and after (B) 10 × dilution; the (A) vesicle system before (C) and after (D) 10 × dilution; and the (I) vesicle system before (E) and after (F) 10 × dilution.

polymer chains become ineffective steric barriers owing to their inadequate hydration and, hence, solubility in the dispersion medium. Such electrolyte solutions will thus be theta solvents, or worse, for the polyethylene oxide chains. Consequently, the stabilizing moieties at the vesicle surface will acquire less extended conformations,§ hence the effectiveness of the steric barriers against flocculation will be decreased with increasing electrolyte (NaCl or $MnCl_2$) concentration. These effects are thought to cause the eventual vesicle flocculation observed at high electrolyte concentrations, despite the presence of polymer molecules at the liposome surface. Particularly in the case of Mn^{2+} (at the outer aqueous phase), coating with block copolymers (irrespective of the way in which the addition of the polymer was carried out), resulted in more resistant vesicle systems, because the presence of polymer molecules at the vesicle surface delays and/or inhibits binding of the cations to the phospholipid headgroups. Such behaviour can also explain the very recently reported findings that Ca^{2+}-induced[40] and phospholipase C-induced[41] LUV fusion is inhibited when pegylated lipids and gangliosides, respectively, were included in the vesicles.

The (A) samples however, exhibited almost identical behaviour to the bare liposomes when cations were present in the inner and outer aqueous phases (Fig. 4, 11 and 12). The degree of osmotic swelling was identical (a mean diameter of 140 nm after dilution). This similarity demonstrates the identical bilayer characteristics of the two systems, providing further evidence that the addition of block copolymer molecules to pre-formed liposomes does not affect the membrane bilayer structure, and the properties associated with it (rigidity, permeability of water or ions *etc.*).

(I) Systems

The physical stability study provided evidence of prolonged vesicle durability for the systems where the block copolymer was present during vesicle formation. The size of the (I) vesicle systems appeared quite constant after 2 months at room temperature.

The prolonged stability of the (I) vesicles against cation-induced flocculation (Fig. 3, 5 and 6), proved the presence of

§ Reduction in the layer thickness of block copolymer-coated particles with increasing electrolyte concentration has been reported.[39]

the long copolymer chains at the vesicle surface. Thus, the copolymer-coated liposome surfaces resist aggregation due to the steric repulsions between the PEO chains of approaching vesicles, as for the (A) vesicle systems. The most notable difference between the (I) vesicle systems and the other two types of vesicles, however, was found in their response to osmotic effects caused by the gradients in electrolyte concentration in the outer and inner vesicle aqueous phases. Almost no size or turbidity change was observed for the (I) vesicle system, throughout the gradual dilution shocks (Fig. 4, 11 and 12), contrary to the evident swelling of the bare and (A) vesicles. These results were interpreted as conclusive evidence that the hydrophobic PPO block is indeed buried inside the alkyl lipid phase.

The irresponsive behaviour to the osmotic gradients, can be explained by two possible mechanisms, based on the fact that the copolymer hydrophobic block is incorporated as an integral part of the vesicle bilayer. The first is due to the construction of a more 'rigid' bilayer, less prone to osmotic swelling. The advantageous effect of incorporating substances within the liposome bilayer, is based on the so-called 'condensing' or 'stiffening' effect; in other words, the increase of the bilayer rigidity, due to restrictions of the lipid acyl chain mobility. Cholesterol-containing liposomes have proven to be more stable[42] and with greater content retention,[43] and are used regularly when studying release rates, *in vivo* drug delivery *etc.* Also, liposome incorporated gangliosides greatly reduce their susceptibility to lysis and content leakage induced by plasma components.[44] The PPO block present inside the bilayer, sandwiched between lipid molecules, can result in a similar 'rigidifying' effect that is responsible for the observed resilient behaviour against osmotic swelling. Alternatively, it can indeed be argued that the constant vesicle sizes observed for the (I) system are due to free flow of ions through channels in the bilayer, opened up by the incorporated blocks (PPO) of the copolymer molecules:¶ therefore, no osmotic gradient exists between the inner and outer vesicle aqueous phases. In such a case, the increased leakage of entrapped vesicle contents could be compensated by inclusion of molecules (cholesterol) inducing the bilayer 'condensing' or 'stiffening' effect. In the present study, however, such experiments were not carried out, in an attempt to keep complexity to a minimum, in order to characterize the interaction between the lipid and the copolymer molecules as optimally as possible.

Nevertheless, the incorporation of the hydrophobic segment of the block copolymer molecules inside the bilayer is an uncontested fact, resulting from the present studies. It is seen to be responsible for the inert behaviour of the (I) system to osmotic shocks. This incorporation of the polymer hydrophobic block inside the lipid bilayer is also relevant, and will surely provide further insight and information on the effect of protein inclusion (*e.g.* glycophorin)[45,46] inside lipid bilayers, used towards the construction of cell membrane models. In the present study membrane-spanning polymer hydrophobic blocks are found to exert significant bilayer stabilization against osmotic swelling.

The pronounced advantages of association of the PPO block as an integral part of the bilayer are evident in the present work. By anchoring the polymer molecule inside the lipid bilayer, surface coating is achieved in a much more efficient way than by copolymer adsorption. The steric barriers are much more robust and less susceptible to desorption (caused by interparticle collisions, dilution effects, *etc.*), thus offering a permanent and resistant polymer coating to the vesicle. This is attainable by physical means; in this way, avoiding any chemical manipulation of the liposome-participating lipids (*e.g.* pegylation of the phospholipid headgroup). The benefits of being able to construct a sterically stabilized vesicle system physically, purely by taking advantage of the hydrophobic interactions, are thought to act synergistically towards the optimization of vesicle constructs.

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¶ Conductivity measurements were carried out in order to monitor the leakage rate of cations to the outer aqueous phase. Unfortunately, the bulk salt concentration at the outer aqueous phase changes only slightly, so insignificant conductivity changes occurred and no valid conclusions could be drawn.

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