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Interactions between pH-sensitive liposomes and model membranes

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Abstract

The structure and dynamics of two different pH-sensitive liposome systems were investigated by means of cryo-transmission electron microscopy and different photophysical techniques. Both systems consisted of dioleoylphosphatidylethanolamine (DOPE) and contained either oleic acid (OA) or a novel acid-labile polyethylene glycol-conjugated lipid (DHCho-MPEG5000) as stabiliser. Proton induced leakage, lipid mixing and structural changes were studied in the absence and presence of EPC liposomes, as well as in the presence of liposomes designed to model the endosome membrane. Neither DHCho-MPEG5000- nor OA-stabilised liposomes showed any tendency for fusion with pure EPC liposomes or endosome-like liposomes composed of EPC/DOPE/SM/Cho (40/20/6/34 mol.%). Our investigations showed, however, that incorporation of lipids from the pH-sensitive liposomes into the endosome membrane may lead to increased permeability and formation of non-lamellar structures. Taken together the results suggest that the observed ability of DOPE-containing liposomes to mediate cytoplasmic delivery of hydrophilic molecules cannot be explained by a mechanism based on a direct, and non-leaky, fusion between the liposome and endosome membranes. A mechanism involving destabilisation of the endosome membrane due to incorporation of DOPE, seems more plausible.

Keywords: Liposomes; Oleic acid; Membranes

1. Introduction

Phospholipid liposomes have during the last decade become increasingly popular as vehicles for systemic delivery of drugs, enzymes and genetic material. Due to the widespread use of sterically stabilised liposomes, most commonly achieved by incorporation of PEG-lipids in the lipid bilayer, the inherently short circulation times of conventional liposomes do no longer constitute a problem. Development of new loading technologies, furthermore, has ensured that the liposomes may be filled with large quantities of active substance. Great progress has recently also been made concerning targeting of liposomes to specific cells. By

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use of biomolecules, such as small proteins, vitamins or antibody fragments, it is possible to direct the liposomes towards receptors expressed on the surface of the target cells [1–5]. Providing that the receptor is capable of mediating endocytosis, the substance filled liposomes may after docking with the receptors also be internalised by the target cell [6–9].

When seeking to develop efficient liposomes for drug delivery, a low leakage of the encapsulated substance is often a prerequisite in order to minimise unwanted side effects while the liposomes are in circulation. However, once the liposomes have reached their destination, e.g. the interior of a target cell, the cargo needs, for most applications, to be quickly and efficiently released. The need for a quick release arises from the fact that (1) the active substance is normally prevented from exercising its therapeutic effect while still being enclosed in the lipid carrier and (2) liposomes internalised via the endocytotic pathway often end up in lysosomes where the action of degrading enzymes may decrease, or even destroy, the biological activity of the delivered substance [10].

Several approaches are employed in order to obtain liposomes that are non-leaky during circulation and yet capable of rapidly releasing their contents upon reaching their organ, tissue, or cell of destination. The rationale behind the approaches varies but they have generally one thing in common; the liposomes are made from lipid components, or lipid mixtures, that in response to a given change in conditions develop a propensity to form leaky or non-lamellar structures. Temperature- and light-sensitive liposomes, which release their cargo in response to an external stimuli, constitute interesting alternatives for therapies directed towards solid tumours and other spatially well defined targets [11–13]. However, in order to expand the use of triggered release beyond this rather narrow set of applications the discharge should ideally be prompted by a change in local environmental conditions. The gradual decrease in pH experienced by liposomes that are internalised via endocytosis [14–18] constitutes a potentially very useful intrinsic stimulus and several pH-sensitive liposome formulations based on this strategy have been developed and evaluated biologically [19–21].

Dioleoylphosphatidylethanolamine (DOPE) constitutes the major component in many of the formulations used to construct pH-sensitive liposomes suitable for drug delivery. At physiological salt and pH conditions, DOPE forms an inverted hexagonal (H_{II}) phase [22,23], but by inclusion of molecules that increase the spontaneous curvature, the mixture may be stabilised into a lamellar arrangement. Mildly acidic amphiphiles, such as oleic acid (OA) and cholesteryl hemisuccinate (CHEMS), have been commonly used as stabilisers. At high to near neutral pH, the headgroups of these amphiphiles are predominantly charged and their presence in the lipid mixture prevents the formation of H_{II} and other inverted phases. Liposomes that are stable at neutral pH may thus be made from mixtures of DOPE and the mildly acidic amphiphiles. Upon protonation of these amphiphiles, the stabilising effect is lost and structures with negative curvature are therefore induced when the pH drops below a critical value. During this process the liposomal structure is destroyed and material encapsulated in the aqueous core is released.

PEG-lipids constitute another interesting type of stabiliser [24–26]. Their inclusion in DOPE mixtures serves a dual purpose; liposome formation is facilitated and at the same time a compound offering steric protection is introduced in the bilayer. Upon removal of the PEG-lipids, a transition from lamellar to inverted hexagonal phase is induced at low to moderate pH. A similar effect may be achieved if the linkage between the PEG-moiety and the lipid anchor is specifically cleaved. Several formulations for triggered release have been constructed along these lines [27–32].

Although it is clearly established that the pH-sensitive liposomes collapse and release their content upon acidification, one problem still remains—the active substance must also be able to cross the endosomal membrane. A number of in vitro studies indicate that internalised DOPE-based pH-sensitive liposomes are indeed able to deliver hydrophilic substances to the cytosol of target cells [21,33]. The mechanisms behind the release process are complex, however, and far

from fully understood. Following proton induced destabilisation of the carrier liposome, the substance may—depending on its chemical properties—be able to cross the endosomal membrane by simple diffusion. In certain cases, the transport may be facilitated by specific membrane transporters located in the endosomal membrane, however, for most substances neither of the above processes are applicable and the release from the endosomal compartment must thus proceed via some alternative mechanism. Another possibility is that the liposomes fuse with the endosomal membrane after its acidification. Such direct fusion has been proposed to lead to a 'microinjection' of the encapsulated material into the cytosol [21]. It is experimentally well proven that DOPE liposomes stabilised by weakly acidic amphiphiles, such as OA and CHEMS, aggregate and subsequently perform lipid mixing with self-similar liposomes when the pH of the solution drops below a limiting value [14,19,34]. Experimental results have been presented, however, indicating that the liposomes become leaky and release their contents at an early stage during this process [19]. Furthermore, very few systematic studies concerning the interaction between stabilised DOPE liposomes and endosome-like model membranes have been carried out. In particular, no proof has been presented showing that the DOPE liposomes upon acidification are able to fuse with liposomes composed of mixtures of PC, cholesterol, sphingomyelin (SM), and PE in compositions resembling those of endosomal membranes. The possibility of a spontaneous and direct fusion between DOPE-based pH sensitive liposomes and the endosomal membrane thus remains to be thoroughly investigated.

A possible alternative is that the release does not require direct fusion but instead takes place in a stepwise manner. The liposomes may, as a result of the acidification, first collapse and release their contents into the endosomal compartment. The DOPE molecules, initially situated in the liposome may then, in a second step, interact with the endosomal membrane. It is plausible that incorporation of DOPE in the endosomal membrane could lead to a higher permeability and, if the incorporated amount is high enough, even result in large structural rearrangements. Verification of this mechanism requires systematic investigations of leakage and aggregate structure in relevant lipid mixtures.

A third possibility is that the observed cytosolic delivery cannot be explained based solely on lipid interactions but that the transport over the liposomal and endosomal membranes is dependent on the presence and action of specific proteins or other biomolecules.

The aim of this study was to increase the fundamental knowledge about the release of hydrophilic substances from DOPE-based pH-sensitive liposomes, and more specifically to collect experimental data to help discriminate between the different mechanisms described above. A second objective was to employ cryo-transmission electron microscopy (cryo-TEM) to directly visualise the structural transitions taking place in different pH-sensitive liposome systems upon acidification. Acquisition of detailed information regarding the transient structures formed in the pH-sensitive systems should also lead to a more complete picture of the sequence of events taking place during membrane fusion in general.

2. Experimental and materials

2.1. Materials

1,2-Distearoylphosphatidylethanolaine-*N*-[poly (ethylene glycol)-5000] (DSPE-PEG) and 1,2-DOPE, were purchased from Avanti Polar Lipids (Alabaster, AL). 8-Aminonaphthalene-1,3,6-trisulfonic acid disodium salt (ANTS), *p*-xylene-bis-pyridium bromide (DPX), lissamine[a] rhodamine B 1,2-dihexadecanoyl-*sn*-glycero-3-phosphatidylethanolamine triethylammonium salt (Rh-DHPE), and *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphatidylethanolamine triethylammoinum salt (NBD–PE), were obtained from Molecular Probes (Leiden, Netherlands). Dihydrocholesterol (DHCho) and OA of 99%+ purity were bought from Sigma-Aldrich (Stockholm, Sweden). All other salts and reagents were of analytical grade and were used as received.

2.2. Synthesis and purification of DHCho-MPEG5000

1′-(4′-Dihydrocholesteryloxy-3′-butenyl)-ω-methoxy-poly(ethylene glycol 5000)ate (DHCho-MPEG5000) was synthesized in 12% overall yield from DHCho, 1,4-butanediol (Aldrich, Milwaukee, WI), and M-PEG5000 (Shearwater Polymers, Huntsville, AL) using the method described by Boomer et al. [33], except that DHCho was used instead of cholesterol as starting material.

2.3. Sample preparation

Lipid mixtures were prepared by codissolving the lipids in chloroform, removing the chloroform by evaporation under vacuum, and thereafter redissolving the dried lipid film in a buffer solution (pH 9.5). The buffer solution used varied depending on the type of experiments that were to be performed. The redissolved lipids were subjected to freeze–thaw cycles, including freezing in liquid nitrogen and thawing in room temperature while vortexing. Room temperature was used since the DOPE containing samples formed precipitates at higher temperatures. The freeze–thawing continued until the lipid mixtures were completely dissolved into the buffer solution. Unilamellar liposomes were produce by multiple extrusion through polycarbonate filters with a pore size of 100 nm. All mixed lipid liposome dispersions are reported as molar ratios of the respective lipids.

The pH of the samples was changed by adding aliquots of the concentrated liposomal dispersion into prewarmed (25 or 37 °C) buffers of desired pH. The buffer solutions used, which contained EDTA to prevent possible phase transitions caused by Ca^{2+} ions [20,23,35], were selected from the following: ANTS/DPX, pH 9.5 (12.5 mM ANTS, 45 mM DPX, 20 mM glycine, 72.5 mM NaCl, 0.1 mM EDTA); glycine, pH 9.5 (20 mM glycine, 150 mM NaCl, 0.1 mM EDTA); sodium acetate, pH 4.5 (50 mM NaOAc, 100 mM NaCl, 0.1 mM EDTA); Hepes, pH 7.5 and 8.2 (20 mM Hepes, 150 mM NaCl, 0.1 mM EDTA); citrate, pH 5.5 and 6.6 (50 mM citrate, 100 mM NaCl and 0.1 mM EDTA).

2.4. Lipid mixing

The assay for liposome fusion involves resonance energy transfer between the fluorophore-labeled lipids NBD–PE and Rh–PE [19]. These lipid probes were incorporated into one population of liposomes at 1 mol.% each, a concentration at which there is significant NBD quenching by Rh. The labeled liposomes were mixed with liposomes containing no probe lipids, referred to as unlabeled liposomes, at a ratio of 1:9 in prewarmed (25 or 37 °C) buffers of desired pH. The total lipid concentration varied from 100 to 500 μM. Samples containing an appropriate amount of the fluorophores, corresponding to maximum fusion between the two populations, was also prepared. The percentage of lipid mixing was determined by the following equation

$$\%\ \text{lipid mixing} = \left((F^{530/590} - F_0^{530/590}) / (F_{\max}^{530/590} - F_0^{530/590}) \right) 100$$

where $F^{530/590}$ is the fluorescence intensity of the sample, $F_0^{530/590}$ is the fluorescence intensity at time 0 and $F_{\max}^{530/590}$ corresponds to the maximum fluorescence intensity observed at 530 and 590 nm upon complete mixing of the probe lipids.

Fusion was monitored by a SPEX-fluorolog 1650 0.22-m double spectrometer from SPEX Industries Inc. (Edison, NJ), using an excitation wavelength of 460 nm and measuring the emission at 530 and 590 nm. The samples were stirred and maintained at 25 or 37 °C during the measurements. All manipulations were performed under minimal light conditions to avoid photobleaching of the fluorophores.

2.5. Leakage measurements

ANTS/DPX buffer at pH 9.5 was used to redissolve the lipid film for the leakage measurements [36]. The extruded liposomes were separated from the nonencapsulated materials using a Sephadex G-25 column (Amersham Pharmacia Biotech, Sweden), equilibrated with glycine buffer, pH 9.5. The pH of the samples was changed by adding aliquots of the concentrated liposomal dispersion (pH 9.5) into prewarmed (25 or 37 °C)

buffers of the desired pH. Fluorescence measurements were initiated immediately after the addition. The percentage of leakage was calculated using the following equation

$$\%\ \text{leakage} = \left((F - F_0)/(F_{\max} - F_0)\right)100$$

where F is the fluorescence intensity of the sample and F_0 is the initial fluorescence intensity. $F_{\max}$ corresponds to the fluorescence intensity at maximum leakage and was obtained after adding Trition X-100, from a stock solution to the sample. All the samples had a lipid concentration between 25 and 500 μM. The leakage of ANTS from the liposomes could be followed, due to the relief of DPX quenching (excitation = 360 nm, emission = 530 nm). The samples were stirred and maintained at 25 or 37 °C during the measurements.

2.6. Turbidity measurements

Aggregation was monitored as an increase in turbidity at 350 nm using a Hewlett-Packard 8453 UV–visible spectrometer or by right angle light scattering (excitation and emission = 350 nm) using a fluorimeter. The samples were maintained at 25 or 37 °C during the measurements using lipid concentrations in the 100–500 μM range.

2.7. Thin layer chromatography

Analysis of DHCho-MPEG5000 degradation in buffers of different pH at 37 °C was performed on silica gel thin layer chromatography (TLC) plates (BakerFlex, J.T. Baker, Phillipsburg, NJ). DHCho-MPEG5000 was added to prewarmed (37 °C) buffer at pH 4.5. The solutions were incubated in 37 °C and aliquots at different time points were mixed with 10% concentrated NH_3 (28% w/w) in acetone to stop the hydrolysis. The mixture and standards, DHCho-MPEG5000 in $CHCl_3$ and PEG 5000 in $CHCl_3$, were spotted on TLC plates and developed in the solvent system $CHCl_3$/MeOH/NH_3 (100:15:1) and stained in a I_2 chamber to observe degradation products [28].

2.8. Cryo-transmission electron microscopy

A drop of the sample was placed onto a copper grid and the excess solution was removed by a filter paper, creating a thin sample film on the electron microscopy grid. The procedure was performed under controlled temperature (25 or 37 °C) and humidity conditions within a custom-built environmental chamber. Immediately after film preparation, the grid was plunged into liquid ethane held at a temperature just above the freezing point. The sample was then transferred, into a Zeiss EM 902A microscope, operating at 80 kV. To prevent sample perturbation and formation of ice crystals, the temperature was kept below 108 K during both the transfer and viewing procedure. All samples had a final lipid concentration of 2 or 3 mM depending on the system, and were vortexed before cryo-TEM investigation.

A more detailed description of the cryo-TEM technique can be found elsewhere [37].

3. Results and discussion

3.1. OA-stabilised DOPE liposomes

The weakly acidic amphiphile OA forms, depending on the pH and salt concentration, a variety of different structures in dilute aqueous solution [38,39]. At high pH, where OA carries a negative charge, globular or thread-like cylindrical micelles constitute the preferred structures. If the pH is lowered to values which roughly correspond to the apparent pK_a, OA self-assembles instead into aggregates of lamellar structure. Under acidic conditions where OA is fully protonated, particles of inverted hexagonal phase are formed. In solutions containing physiological salt concentrations, the first signs of inverted phase structure appear at pH values approximately 8 [38].

The mere fact that OA, at physiological salt concentration and pH, has a tendency to adapt inverted phase structure serves to indicate the limited use OA will have as a stabiliser for DOPE-based liposomes intended for in vivo applications. In model studies, however, OA constitutes one of the most commonly used stabilisers for pH-sensitive liposomes composed of DOPE [14,40–42]. Due to the large amount of available data, we choose to begin our investigations with the OA:DOPE system.

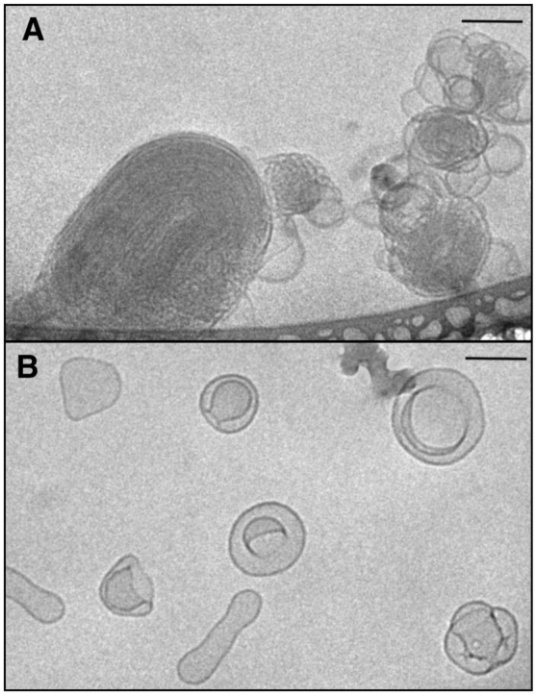

Fig. 1. Cryo-TEM micrographs of the OA/DOPE system at 25 °C and pH 8.2, at 2 mM total lipid concentration. (a) 12:88 OA:DOPE; (b) 44:56 OA:DOPE. Bar = 100 nm.

3.2. Structural investigation of aggregate structure in the liposome preparations

3.2.1. OA:DOPE liposomes

At pH 8.2, the zwitterionic DOPE carries no net charge and has a preference to form structures of negative curvature due to its inverted cone molecular shape, nonetheless, a lamellar phase can be stabilised by inclusion of OA into the host DOPE matrix. Due to the comparably high apparent pK_a of OA, relatively large amounts of the fatty acid are needed in order to prevent the formation of inverted phase structures. Fig. 1a shows the aggregate structure, as captured by cryo-TEM, in an extruded sample containing 12:88 OA:DOPE. Large aggregates with a dense inner structure, sometimes exhibiting striations indicating the long-range structural order typical of H_{II}-phases, were frequently observed in these mixtures. In addition, the micrographs reveal large clusters of tightly aggregated, liposome-like, structures. The latter display a very complex inner structure and probably constitute transition intermediates between L_α and H_{II}-phase. With increasing amount molar fraction of OA, the clusters and inverted phase structures became less frequent such that samples containing $\geqslant$40 mol.% OA were dominated by lamellar structures, however, some aggregation and possible interlamellar attachments (ILAs) [22] could still be observed (Fig. 1b). In order to make sure that the samples were completely lamellar and free of inverted phases, we chose to prepare 40:60 OA:DOPE liposomes at pH 9.5 where they are the sole phase in solution and show no signs of aggregation or ILAs (e.g. see Fig. 5b).

3.2.2. Mixtures of DOPE and EPC

It was important to establish for the purposes of our fusion studies how much DOPE could be tolerated in pure EPC liposomes before the first signs of inverted structures started to appear under acidic conditions. Fig. 2 shows some representative micrographs of liposomes prepared from lipid mixtures containing different molar ratios of EPC and DOPE at pH 7.4. The pH was then lowered to 4.5 and the samples examined by cryo-TEM. The pictures reveal that intact and well separated liposomes are formed in preparations containing up to approximately 50 mol.% DOPE. At higher DOPE concentrations, liposome aggregation and ILA formation can be detected (Fig. 2a and b).

3.2.3. Mixtures of OA, DOPE and EPC

The above investigations indicate that the products of a 1:1 fusion between EPC liposomes and 40:60 OA:DOPE liposomes would produce only lamellar mixed liposomes and no aggregates of inverted phase structure. To confirm this assumption, we examined premixed and extruded samples containing 20:30:50 OA:DOPE:EPC by cryo-TEM. As expected, there was no sign of aggregation or inverted phase structures in these preparations. At pH 4.5 the liposomes appeared very similar to those shown in Fig. 1b and Fig. 2c. This result is important for the interpretation of data reported below.

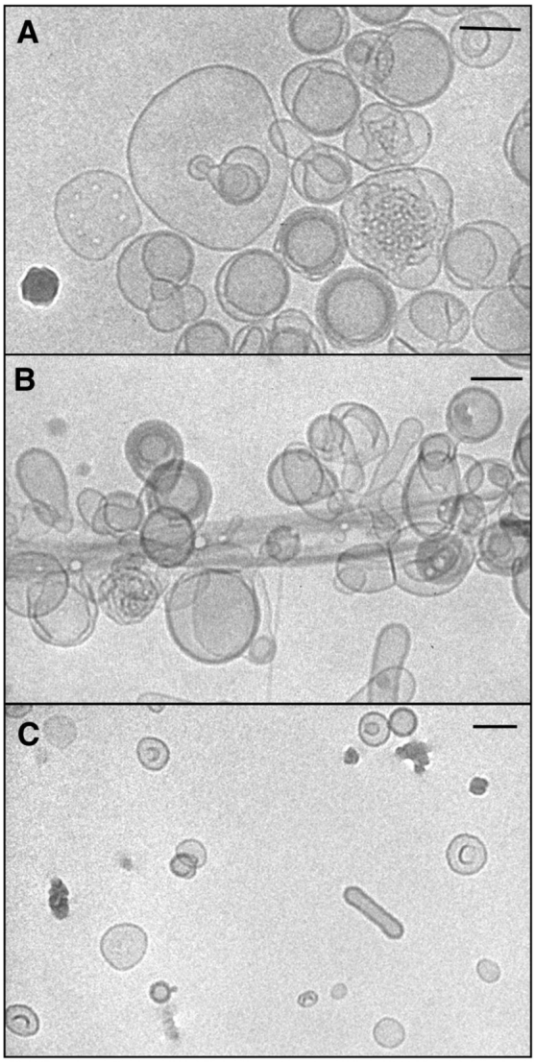

Fig. 2. Cryo-TEM micrographs of the EPC/DOPE system at 25 °C, pH 4.5, 2 mM total lipid concentration. (a) 25:75 EPC:DOPE; (b) 39:61 EPC:DOPE; (c) 50:50 EPC:DOPE. Bar = 100 nm.

3.3. Leakage, lipid mixing and turbidity

3.3.1. Pure OA:DOPE-systems

In order to characterise the interaction, and possible fusion, between OA:DOPE and EPC liposomes, we carried out a series of systematic studies. We began our investigations by examining the effects caused by acidification of pure OA:DOPE liposomes. As discussed earlier, the stabilising effect of OA is lost when the pH drops below a critical value. Below this value which is dependent on the OA:DOPE molar ratio the liposomes will aggregate and rearrange into large particles of inverted phase. The rate of this process increases with increasing lipid concentration and decreasing pH. At the comparably high lipid concentrations needed for cryo-TEM, aggregation and fusion was found to be too fast to allow for time-resolved investigations. Leakage, lipid mixing and turbidity measurements at lower lipid concentrations were therefore employed to monitor these interactions.

The proton-induced leakage of encapsulated ANTS/DPX from 40:60 OA:DOPE liposomes is shown in Fig. 3a. The same graph also displays the observed lipid mixing between self-similar liposomes. These data show that lipid mixing and liposome leakage proceed in a bimodal fashion, however, the initial rate of liposome leakage proceeds at a faster rate than lipid mixing. This result may suggest that the liposomes start to leak prior to aggregation and lipid rearrangement. To address this question, we carried out leakage measurements at varying lipid concentrations. As seen in Fig. 3b, the leakage rate is clearly concentration dependent, indicating that aggregation is indeed needed for leakage to occur. This finding is in agreement with results reported for pH-sensitive liposomes composed of DOPE/CHEMS [43].

In addition to the above investigations, the proton-induced interaction between OA:DOPE liposomes was followed by turbidity measurements. As expected, liposome aggregation and rearrangement into larger particles could, at short time-scales, be followed as a close to linear increase in turbidity (Fig. 3c). At later time points precipitation of large particular material gave rise to a decrease in turbidity and made further measurements meaningless.

In summary, our results show that pure OA:DOPE liposomes undergo rapid aggregation upon pH reduction from 9.5 to 6.6, leading to the onset of aqueous contents release and lipid mixing. The observed relative kinetics of these processes indicates that leakage appears on a faster time-scale than lipid rearrangements.

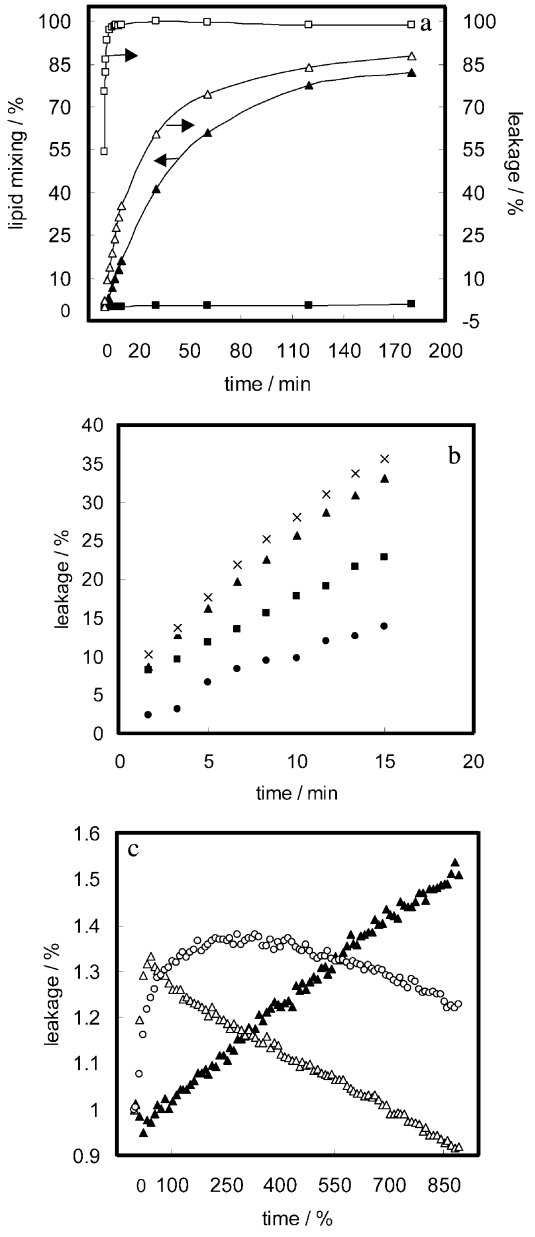

Fig. 3. Leakage, lipid mixing and turbidity of 40:60 OA:DOPE liposomes at 25 °C and pH 6.6. (a) Leakage (unfilled) and lipid mixing (filled) as a function of time in the presence of self-similar (triangles) and EPC liposomes (squares), at 250 μM total lipid concentration; (b) leakage as a function of time at ● 25 μM, ■ 50 μM, ▲ 100 μM and × 200 μM total lipid concentration; (c) Turbidity as a function of time, at 125 and 250 μM total lipid concentration, in the presence of ▲ self-similar (125 μM), △ EPC (250 μM) and ○ EPC/Cho (250 μM) liposomes.

3.3.2. Interactions between OA:DOPE and EPC liposomes

Phosphatidylcholine constitutes one of the main components of endosome membranes. In a first attempt to elucidate the fusogenic capability of pH-sensitive liposomes with the endosomal bilayer, we repeated the previous leakage and lipid mixing experiments in the presence of EPC liposomes. We were surprised to find that proton-induced leakage from OA:DOPE liposomes was substantially faster when EPC liposomes were present in the solution. Complete contents release of the encapsulated material occurs after only 2 min[1] (Fig. 3a). Within the same time span, a rapid precipitation of large particles could be observed by the naked eye. The high rate at which aggregation and formation of large particles took place was further verified by turbidity measurements (Fig. 3c).

According to our cryo-TEM investigations, no large aggregates of inverted phase structures, and thus no precipitation, should be present after complete fusion between the OA:DOPE and EPC liposomes. The precipitated material could therefore be expected to contain no, or only small amounts of, EPC. In line with this, lipid mixing experiments showed that there was virtually no exchange of lipid components between the OA:DOPE and EPC liposomes (Fig. 3a). Measurements carried out 3 days after the initiating pH-drop showed only approximately 15% lipid mixing. Extended investigations revealed that the lack of lipid mixing was most probably due to the fact that the EPC liposomes did not partake in the proton-induced aggregation process. Cryo-TEM investigations showed that a population of liposomes, presumably the EPC liposomes, remained well separated in the solution 40 min after the sample had been acidified (results not shown). In addition, hardly any leakage was observed in experiments using ANTS/DPX-filled EPC liposomes (results not shown). The increase in leakage and aggregation rate observed for OA:DOPE liposomes in the presence of EPC liposomes, therefore,

[1] Because of the rapid release the amount of leakage was calculated in an alternative way. Instead of subtracting the leakage at time 0 at pH 6.6, by which information in this case could be lost, the leakage at pH 9.5 was instead used.

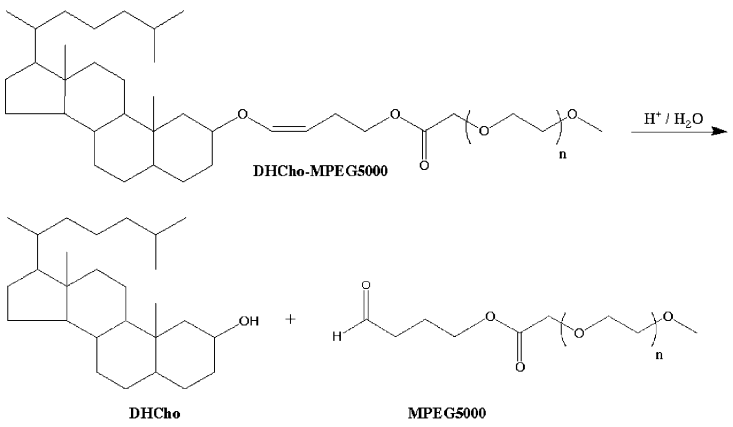

Fig. 4. Acid-catalyzed hydrolysis reaction of DHCho-MPEG5000. The number of PEG units, $n = 112$.

must be explained by a mechanism that does not rely on a direct interaction between the two different liposome populations. One possibility is that the pH-sensitive liposomes lose their stability faster in the presence of EPC liposomes due to OA transfer. Experimental data indicating rapid transfer of OA from pH-sensitive liposomes to both phospholipid liposomes [44] and cell membranes [14] have been published. We conducted a new round of turbidity measurements to elucidate whether OA transfer could explain the observed behaviour in our system as well. In these experiments, OA:DOPE liposomes were mixed in a 1:1 ratio with EPC liposomes containing 40 mol.% cholesterol. It is well known that cholesterol induces membrane lipid ordering [45,46] and resists the incorporation of surfactants [47] and alkanols [48] into PC membranes. OA may thus be expected to incorporate at a slower rate when the EPC liposomes contain cholesterol. As a consequence, OA:DOPE liposome destabilisation should also proceed at a slower rate. The turbidity measurements shown in Fig. 3c confirm the expected behaviour, therefore, we conclude that OA transfer constitutes a likely explanation for the fast destabilisation observed in the presence of pure EPC liposomes.

The collected results of our contents leakage, lipid mixing and turbidity measurements indicate that OA:DOPE liposomes, in agreement with some earlier reports [19,44], do not fuse readily with liposomes composed of primarily phosphatidylcholine.

3.4. DOPE-liposomes stabilised by DHCho-MPEG5000, a novel cleavable PEG-lipid

Investigations were conducted with PEG-lipid:DOPE liposomes to determine whether the behaviour observed for OA:DOPE liposomes could be generalised to include other types of DOPE-based systems. If the PEG is attached to the lipid anchoring moiety via an acid-labile linkage, it is possible to render the liposomes sensitive to a reduction in pH by PEG cleavage, thus inducing a lamellar–H_{II} phase transition.

DHCho-MPEG5000, a novel lipid composed of a hydrogenated cholesterol linked to a methoxy-PEG chain (MW 5000) by means of an acid-sensitive vinyl ether bond, was chosen as the acid-cleavable PEG-lipid. Upon acidification, DHCho-MPEG5000 is hydrolysed to give DHCho and a MPEG5000 derivative as shown in Fig. 4.

TLC analysis was employed to provide an initial estimate of the timescale of the DHCho-MPEG5000 cleavage reaction. Our results indicate that the hydrolysis is slow and still not complete after 5 days at pH 4.5. Although this finding suggests that DHCho-MPEG5000 may not be ideal for rapid destabilisation of liposomes intended for in vivo use, the slow hydrolysis kinetics are advantageous in that it provides a rare opportunity

to follow the proton-induced structural rearrangements, and to capture some of the normally very short-lived transition structures, by means of cryo-TEM.

3.5. Aggregate structure in the DHCho-MPEG5000:DOPE preparations

As in the case with the OA:DOPE liposomes, it was important to establish the aggregate structure of DHCho-MPEG5000:DOPE preparations at 37 °C prior to acidification using cryo-TEM. Fig. 5a shows that well-formed, predominantly spherical and non-aggregated, liposomes can be produced at pH 9.5 upon incorporation of 5 mol.% DHCho-MPEG5000. Comparison with pure DOPE liposomes (Fig. 5b) reveals that the presence of the long, flexible polymer chain protects the individual liposomes from achieving close contact of membranes from neighboring liposomes. Furthermore, incorporation of DHCho-MPEG5000 seems to reduce the formation of structures requiring a decrease in the aggregate mean curvature. Upon lowering the DHCho-MPEG5000 content to 1 mol.%, these protective properties were notably reduced. As evidenced by the micrograph shown in Fig. 5c, structures that likely represent intermediates between lamellar and inverted phases were now frequently observed in the samples.

3.6. Lipid mixing studies with DHCho-MPEG5000:DOPE liposomes

Lipid mixing experiments at pH 4.5 on samples containing 1, 3 and 5 mol.% DHCho-MPEG5000 were performed to determine the effects of acidification upon the interaction between the DHCho-MPEG5000:DOPE liposomes. At 37 °C and 100 μM lipid concentration, only the sample containing 1 mol.% DHCho-MPEG5000 showed appreciable lipid mixing within a time period of 5 days. We therefore chose this composition in our subsequent studies even though our cryo-TEM analysis indicated that these were not optimal dispersions.

Lipid mixing experiments were conducted at lipid concentrations between 100 and 500 μM to determine the effect of lipid concentration on lipid mixing behaviour. Fig. 6a shows that the interactions leading to lipid mixing between the liposomes are preceded by a lag-phase. The duration of the observed lag-phase is approximately 36–48 h (Fig. 6a, inset). Since the TLC analysis indicates that DHCho-MPEG5000 hydrolysis is a slow pro-

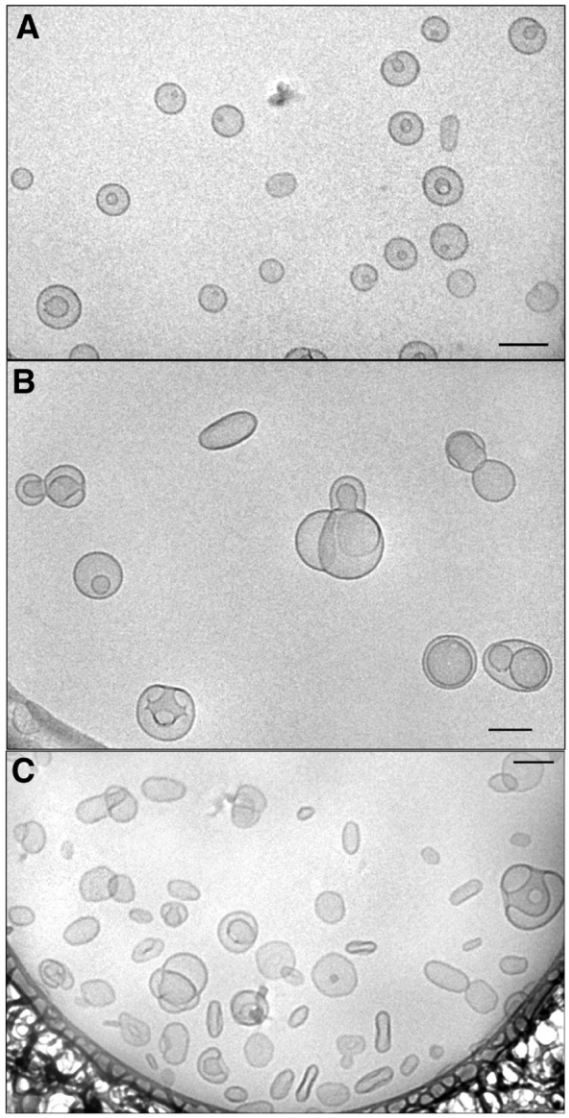

Fig. 5. Cryo-TEM micrographs of DOPE and DHCho-MPEG5000:DOPE liposomes at 37 °C, pH 9.5, 3 mM total lipid concentration. (a) 5:95 DHCho-MPEG5000:DOPE; (b) pure DOPE liposomes; (c) 1:99 DHCho-MPEG5000:DOPE. Bar = 100 nm.

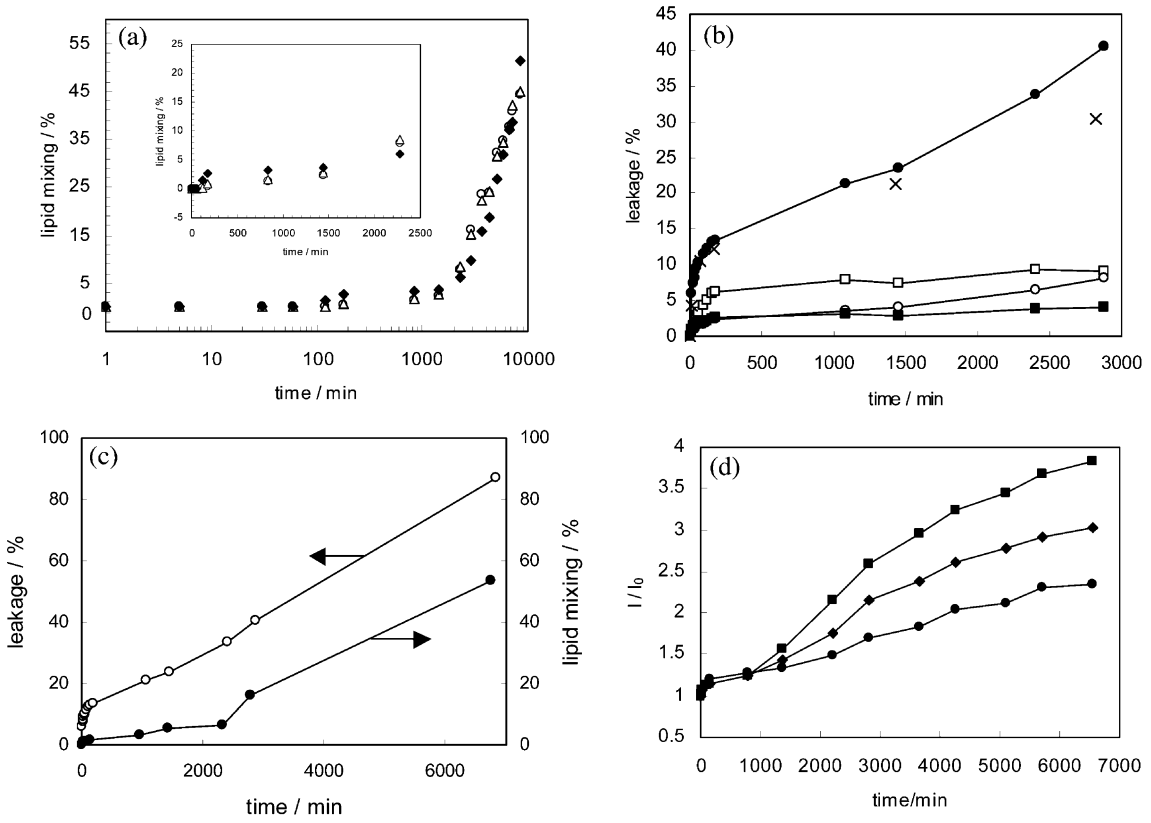

Fig. 6. Leakage, lipid mixing and turbidity of 1:99 DHCho-MPEG5000:DOPE liposomes at 37 °C, pH 4.5. (a) Lipid mixing as a function of time (log-scale) at ♦ 100 μM, △ 300 μM and ○ 500 μM total lipid concentration. The inset figure shows the lipid mixing within the lag-phase time; (b) leakage as a function of time at 100 μM total lipid concentration unless otherwise stated: ● 1:99 DHCho-MPEG5000:DOPE, × 1:99 DHCho-MPEG5000:DOPE (at 500 μM), □ 3:97 DHCho-MPEG5000:DOPE, ○ 5:95 DHCho-MPEG5000:DOPE 5:95 and ■ 1:99 DSPE-PEG_{5000}:DOPE; (c) Leakage ○ and lipid mixing ● as a function of time for 1:99 DHCho-MPEG5000:DOPE at 100 μM total lipid concentration; (d) Turbidity as a function of time at ● 100 μM, ♦ 300 μM and ● 500 μM total lipid concentration of 1:99 DHCho-MPEG5000:DOPE.

cess, these data suggest that the extended lag period observed is a consequence of residual intact PEG-lipid which blocks membrane–membrane contact, a step that must precede membrane fusion. The observed lag-phase also reveals that DHCho-MPEG5000 concentrations as low as 1 mol.% are sufficient to stabilise DOPE liposomes against fusion at low pH. This finding is in accordance with earlier reports concerning the stabilisation of DOPE liposomes with non-cleavable PEG-lipids [24,26,34].

The presence of a lag-phase has previously been detected in other pH-sensitive systems containing cleavable PEG-lipids [28]. In those studies, complete mixing of the lipid components was obtained within a comparably short period of time and the rate of the process was found to depend on the concentration of PEG-lipids, total lipid, and solution pH. The apparent concentration independence observed for the second phase of DHCho-MPEG5000:DOPE liposome lipid mixing (Fig. 6a) is somewhat surprising. Since the DHCho-MPEG5000 molecules are (1) not completely hydrolysed within the timescale of our measurements, and (2) are predominantly in the form of liposome aggregates (cf. Fig. 5c), we infer from

these results that fusion occurs from a preaggregated state that is gradually deblocked from membrane fusion as the PEG-lipid is hydrolysed.

Lipid mixing studies between 1:99 DHCho-MPEG5000:DOPE liposomes and EPC liposomes were also conducted at pH 4.5 and 37 °C using equal volumes of DHCho-MPEG5000:DOPE and EPC liposomes. Our results showed that no significant lipid mixing took place on the timescale where fusion between self-similar liposomes was observed.

3.7. Proton induced leakage

Leakage measurements were also performed to determine how the pH reduction affects the permeability of DHCho-MPEG5000 liposomes. Fig. 6b shows the release of encapsulated ANTS/DPX from DOPE liposomes containing 1, 3 and 5 mol.% DHCho-MPEG5000, respectively. In all cases, a rapid phase, lasting 2–3 h, was detected after acidification, followed by a much slower second phase of release. The data in Fig. 6b also show that the rate of release decreases substantially as the mole fraction of DHCho-MPEG5000 in the membrane increases, such that only ~5% of the encapsulated material is released at pH 4.5 after 48 h at 37 °C when either 3 or 5 mol.% DHCho-MPEG5000 was present. This leakage rate is only slightly faster than the nonhydrolyzable 1:99 DSPE-PEG_{5000}:DOPE control liposomes which display no significant leakage during the time of measurement (Fig. 6b). In addition, the fast, DHCho-MPEG5000 concentration-dependent, initial release behaviour is rationalized in the following manner. As the micrographs in Fig. 5 show, DOPE liposomes containing less than 5 mol.% DHCho-MPEG5000 are not fully stabilised. Fig. 5c, in fact, reveals that preparations containing 1 mol.% DHCho-MPEG5000 contain two different populations of liposomes—well-formed spherical liposomes that coexist with a population of less well-stabilised structures that display a complex morphology. It is likely that the rapid release observed during the initial stage of the leakage process is caused by destabilisation of these, poorly-formed liposomes. As the loading of DHCho-MPEG5000 increases, the population of poorly stabilised liposomes decreases, thus explaining the observed reduction in initial release rate.

The leakage rate in samples containing DHCho-MPEG5000:DOPE at different total lipid concentrations was also investigated. Several concentrations between 100 and 500 μM were analysed, but for clarity only the data obtained at the two extremes are displayed in Fig. 6b. In contrast to the results obtained for OA:DOPE liposomes, we could not establish a positive relation between the lipid concentration and the leakage rate.

Fig. 6c compares the leakage and lipid mixing results obtained for DOPE liposomes containing 1 mol.% DHCho-MPEG5000. The data clearly suggest that the leakage process occurs on a faster time-scale than lipid mixing with approximately 30% release of the encapsulated material occurring during the lag-phase observed for lipid mixing.

3.8. Turbidity measurements

The leakage and lipid mixing experiments described above were complemented by turbidity measurements. Fig. 6d shows the behaviour of three 1:99 DHCho-MPEG5000:DOPE samples having different total lipid concentrations. These experiments also displayed a concentration independent lag-phase of ~20 h before any significant turbidity changes were observed. Since turbidity measurements, in contrast to lipid mixing experiments, are sensitive to the aggregation steps that precede lamellar–H_{II} structural rearrangements, it is plausible that the turbidity changes occur on a faster timescale than membrane fusion. The observed changes in turbidity and leakage rate provide an interesting contrast. According to the data in Fig. 6b, close to 20% leakage has occurred before any aggregation can be detected by means of turbidity. This means that the hydrolysis of DHCho-MPEG5000 probably causes undetected changes in the organisation of the lipid membrane that affect its permeability relative to the nonhydrolyzable derivative, DSPE-PEG_{5000}.

The rate of aggregation is expected to increase with total lipid concentration. As seen in Fig. 6d, the increase in turbidity—and thus the rate of aggregation to form larger/denser structures—is

clearly dependent on the total lipid concentration. The fact that the initial rate of release did not increase with lipid concentration (Fig. 6b) is consistent with the finding that the liposomes start to leak before the onset of aggregation and lipid mixing.

3.9. Aggregate structure as revealed by cryo-TEM

Our initial intent was to determine the sequence of structural events as a function of the time after acidification. The cryo-TEM investigations revealed, however, that the aggregate structure in the samples was very polydisperse. In addition, investigations by means of light microscopy showed that aggregates too large to be visualised by cryo-TEM quickly formed in the samples (results not shown). Fig. 7 shows a gallery of micrographs, that taken together, give a good representation of the structures found in a 3 mM sample during the first 4 days after acidification. These transitions were observed to be highly concentration dependent such that the timescale for these transformations was short due to the high lipid concentrations used for cryo-TEM imaging.

Among the more interesting structures are the large, apparently hemifused liposomes shown in Fig. 7b and the diffuse, amorphous structures depicted in Fig. 7c. It is noteworthy that structures very similar to the former have been observed during pH-induced structural transitions in aqueous dispersions of OA/sodium oleate [38]. The diffuse assemblies in Fig. 7c, on the other hand, closely resemble structures captured by cryo-TEM during the transition from lamellar to cubic phase induced by phospholipase C [49]. The large electron dense particle shown in Fig. 7d displays structural characteristics typical of H_{II}-phase and analogous cryo-TEM images have for instance been observed in dispersions containing DOPE and the cationic surfactant cetyltrimethylammoniumchloride [50].

Although great polydispersity in both the size and structure of the aggregates were observed, the aggregates shown in Fig. 7c and d were undoubtedly more frequently observed in older samples.

3.9.1. Interactions in the presence of EPC liposomes

Leakage and lipid mixing experiments were carried out in order to explore whether the DHCho-MPEG5000:DOPE liposomes, in contrast to the OA:DOPE liposomes, possessed properties that made them prone to interaction and fusion with EPC liposomes. The leakage profiles recorded in presence of EPC liposomes, however, did not differ significantly from those obtained in the pure DHCho-MPEG5000:DOPE preparations (Fig. 6b). Furthermore, no exchange of lipid components between DHCho-MPEG5000:DOPE- and EPC liposomes could be detected by use of the lipid mixing experiments (results not shown).

3.10. Interactions between pH-sensitive liposomes and endosome-like membranes

The results presented so far indicate that no direct interactions take place between pH-sensitive OA:DOPE or DHCho-MPEG5000:DOPE liposomes and EPC liposomes. Endosome membranes, however, contain other lipids besides phosphatidylcholine. In order to determine whether the presence of these additional components affects their fusogenic behaviour, we set out to investigate the interaction between our pH-sensitive liposomes and membranes designed to have a composition more similar to that of endosome membranes.

Early endosomes are believed to contain an overall lipid composition similar to that of the plasma membrane [51]. The composition thus varies as a function of cell type [44,52,53], but normally includes PC, PE, SM, Cho and PS as major components. We wanted to avoid any possible complications due to the presence of charged PS components, therefore, extruded liposomes comprised of 40:20:6:34 EPC:DOPE:SM:Cho—referred to as endosome liposomes—were chosen as a standard composition for these studies.

3.11. Lipid mixing and leakage studies

We began our investigations by performing lipid mixing experiments involving liposomes composed of 40:60 OA:DOPE and 40:20:6:34 EPC:DOPE:SM:Cho. As shown in Fig. 8a, chang-

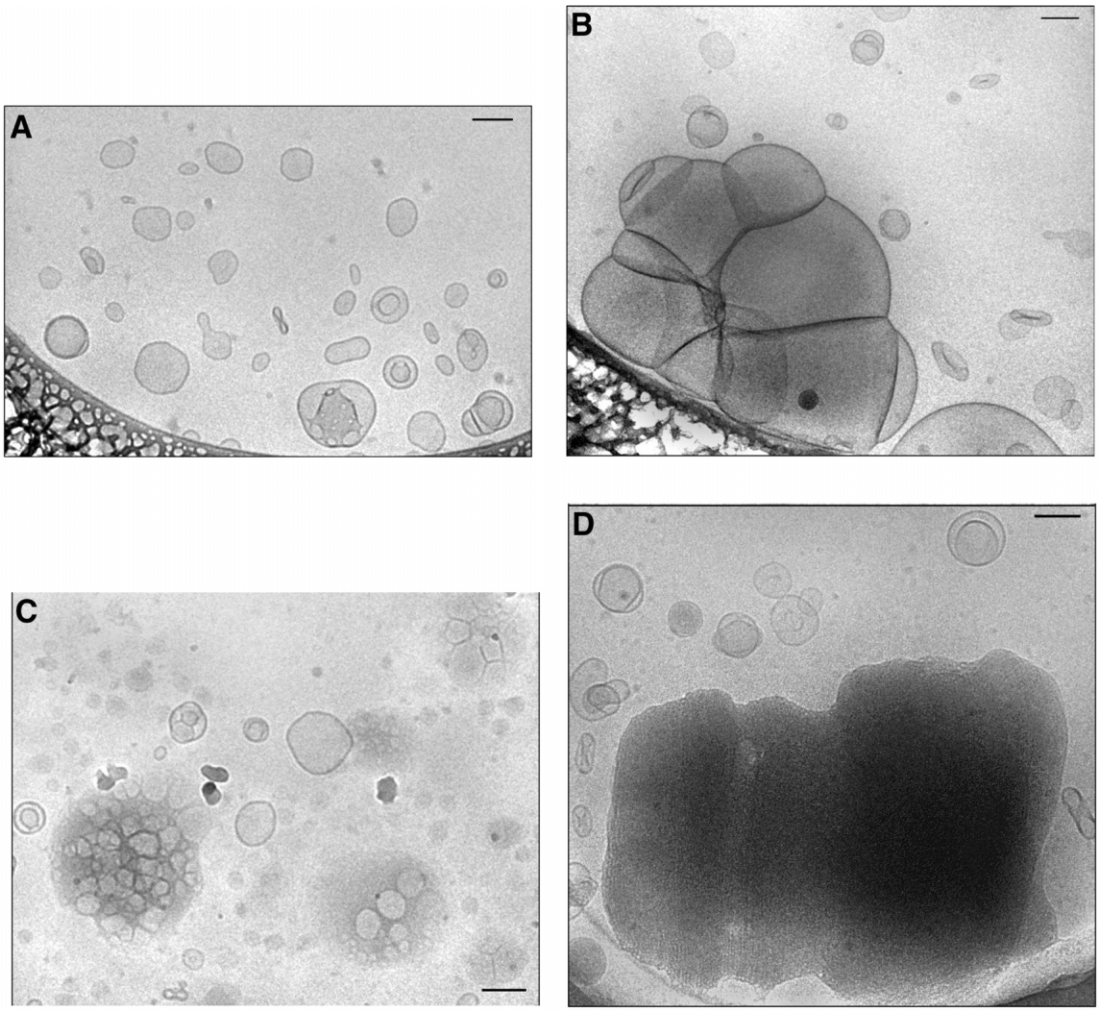

Fig. 7. Cryo-TEM micrographs of 1:99 DHCho-PEG/DOPE at pH 4.5, 37 °C, 3 mM total lipid concentration. Images (a)–(d) show examples of the many different types of transition structures present during acid-triggered liposome release. Bar = 100 nm.

ing the pH in the sample from pH 9.2 to 5.5 induced only a moderate amount of lipid mixing. Two days after their preparation, OA:DOPE liposomes produced less than 15% lipid mixing, indicating that they are unable to rapidly fuse with endosome liposomes. Very similar results were obtained when the OA:DOPE liposomes were exchanged for 3:97 DHCho:DOPE liposomes (Fig. 8a). Note that the DHCho:DOPE liposomes have a lipid composition corresponding to that found in pH-sensitive 3:97 DHCho-MPEG5000:DOPE liposomes after complete cleavage of the DHCho-MPEG5000 conjugate. The lack of significant lipid mixing after 2 days (Fig. 8a) shows that DOPE liposomes stabilised by means of the acid-labile DHCho-MPEG5000 are not likely to aggregate and fuse with endosome liposomes even after prolonged incubation at low pH.

These results suggest that neither OA:DOPE nor DHCho-MPEG5000:DOPE liposomes can be expected to directly and spontaneously fuse with the endosome membrane. It does not preclude

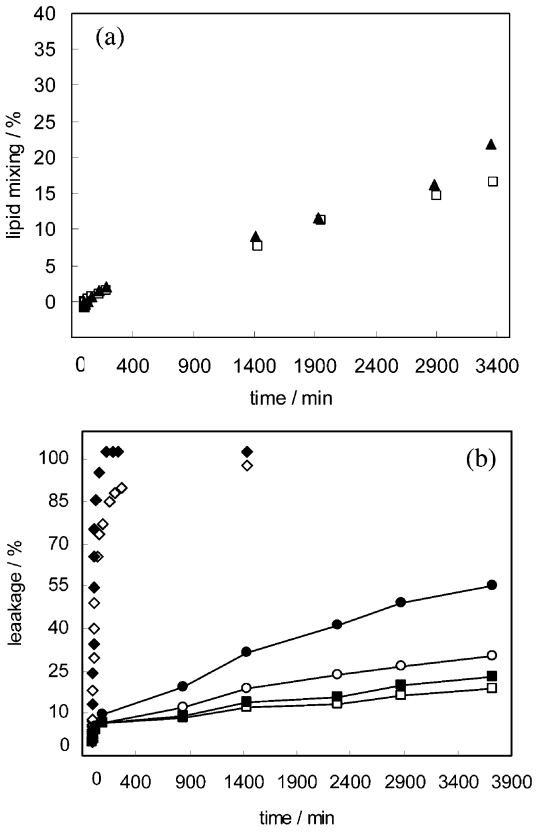

Fig. 8. Leakage and lipid mixing with endosome liposomes, 37 °C, pH 5.5, 250 μM total lipid concentration. (a) Lipid mixing as a function of time between endosome liposomes and: ▲ 40:60 OA:DOPE or □ 3:97 DHCho:DOPE liposomes; (b) Leakage as a function of time for ♦ sample 1, ◇ sample 2, ● sample 5, ○ sample 3, ■ sample 6 (endosome liposomes) and □ sample 4. Numbers 1–6 refer to the sample numbers used in Table 1.

other mechanistic possibilities for acid-sensitive endosomal escape, however. For example, it is conceivable that protein-assisted fusion or other related release processes may be mediated by endosome-specific biomolecules whose presence and function may currently be unknown. A second alternative is that contents escape from the endosomal compartment is based on membrane destabilisation rather than membrane fusion. It has been suggested that incorporation of lipids from the endocytosed liposomes into the endosome membrane may increase its permeability [14,54,55]. In this case, pH reduction would lead to liposomal contents release and formation of inverted phase structures that would not *directly* discharge the liposomal contents into the cytoplasm of the cell. Escape from the endosomal membrane may instead be due to a change in lipid composition of the endosomal membrane due to incorporation of lipids originating from the pH-sensitive liposome. In order to investigate this possibility, we compared the leakage from liposomes prepared from various lipid compositions that might result from lipid exchange between 40:60 OA:DOPE, 3:97 DHCho:DOPE, 1:99 DHCho-MPEG5000:DOPE, or 3:97 DSPE-PEG/DOPE liposomes and endosome liposomes (Table 1). Samples 1–4 in Table 1 have compositions corresponding to that obtained if a 1:1 mixture of the lipids from acid-sensitive liposomes and endosome liposomes are combined. Early endosomes have been reported to have an initial size in the range of 100 nm [56], thus, these compositions serve as models for the events taking place early during the endocytotic process. As a consequence

Table 1
Lipid composition[a] and size ratio of endosome:liposome mixtures

Sample	EPC	DOPE	SM	Cho	OA	DHCho	DHCho-MPEG	DSPE-PEG	SR[b]
1	21.9	38.1	3.3	18.6	18.1				1
2	22.8	53.1	3.4	19.4		1.3			1
3	21.1	57.2	3.2	18.0			0.5		1
4	23.2	52.3	3.5	19.8				1.3	1
5	31.7	35.9	4.8	27.0		0.6			3.5
6	40	20	6	34					n/a

[a] mol.%.
[b] SR is the size ratio, i.e. the size of the endosom/size of the liposome.

of several complex events taking place during the endocytic pathway, late endosomes have a considerably larger size than early endosomes [56,57]. In order to investigate the effect of size on liposome leakage, the composition in sample 5 was chosen to represent the mixture obtained by incorporation of the lipids from a 3:97 DHCho:DOPE liposome into a endosome liposome having a diameter that is four times larger.

As seen in Fig. 8b, the pure endosome liposomes release approximately 20% of the encapsulated material during the first 60 h of incubation at 37 °C and pH 5.5. The same slow release kinetics are observed also for sample 4, a composition that is similar to the endosome liposomes, except that a large quantity of additional DOPE and a small amount (1.3 mol.%) of nonhydrolyzable DSPE-PEG_{5000} is present. When the nonhydrolyzable DSPE-PEG_{5000} is exchanged for 0.5 mol.% acid-labile DHCho-MPEG5000, the resulting endosome liposomes display a slightly faster release (Sample 3), however, 70% of the material still remains encapsulated after 60 h of incubation at pH 5.5. This is not surprising since DHCho-MPEG5000 hydrolysis is slow, resulting in a large proportion of intact PEG-lipids that can stabilise the membrane even at long reaction times. Interestingly, liposomes that were completely devoid of PEG-lipids, such as those in sample 1 and 2, showed a very rapid release. Sample 1, containing endosome lipids augmented with DOPE and OA, reached maximum release within a couple of minutes. The rate of release was somewhat slower in sample 2; nevertheless, approximately 90% of the encapsulated material escaped during the first 4 h after the pH reduction. As expected, the release profile of sample 5 reveals that the rate of release decreases with decreasing amount of DOPE in the membrane.

The leakage experiments reported in Fig. 8b support the notion that incorporation of H_{II}-phase promoters, such as DOPE and OA, may increase the permeability of the endosome membrane. Further, it is clear that this effect is counteracted by the presence of low concentrations of PEG-lipid that stabilise the lamellar phase. In order to find out whether the large, and composition dependent, differences in leakage rate could be explained by changes in phase propensity, we investigated the different liposomal preparations by means of cryo-TEM.

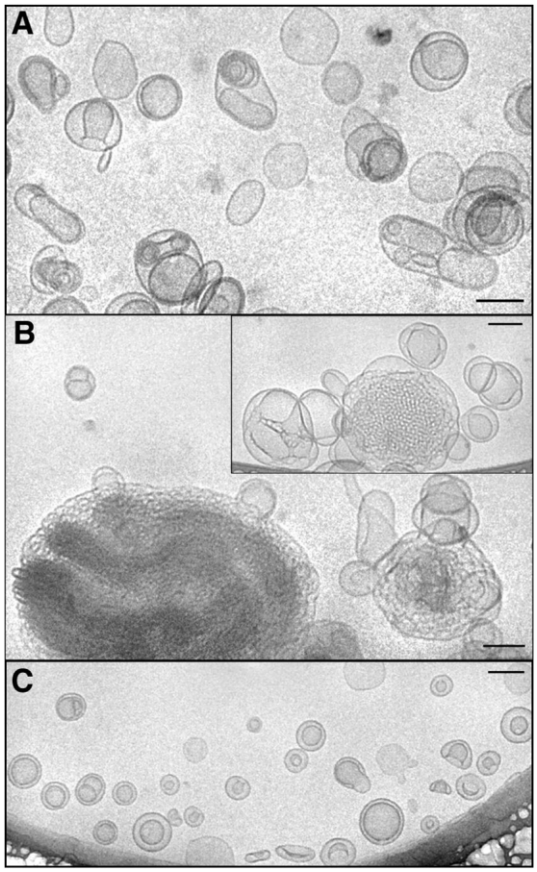

Fig. 9. Cryo-TEM micrographs of various liposome formulations imaged pH 5.5 at 37 °C (3 mM-total lipid concentration). (a) endosome liposomes; (b) sample 2; (c) sample 4. Numbers 1–4 refer to the sample numbers used in Table 1. Micrographs a and c were imaged >60 min and b within 15 min after acidification. Bar = 100 nm.

3.12. Aggregate structure as revealed by cryo-TEM

Cryo-TEM micrographs of the endosome liposomes reveal a polydisperse collection of structures at pH 5.5 and 37 °C (Fig. 9a), including spherical and elongated unilamellar liposomes that coexist with bi- and multilamellar structures that sometimes display intricate patterns of organisation. All

of these structures exhibit, however, a morphology consistent with lipids in a lamellar arrangement that lack obvious signs of inverted phase structure. In contrast, large aggregates displaying the complex morphology associated with the transformation from lamellar to inverted phase were frequently found in preparations corresponding to DHCho:DOPE-augmented endosome liposomes (sample 2 in Fig. 8b). Some typical examples of structures displaying ILA's and H_{II} characteristics were discovered in these samples (Fig. 9b). The same general appearance, indicating strong deviations from pure lamellar phase, was found also in preparations corresponding to OA:DOPE-augmented endosome liposomes (sample 1, Fig. 8b; results not shown). Thus, it is clear that incorporation of DOPE and other non-lamellar phase lipids from pH-sensitive liposomes into the endosome membrane may cause not only increased permeability, but also major alterations in the lipid organisation and phase propensity. The micrograph shown in Fig. 9c, which corresponds to the composition found in sample 4, confirms that only small quantities of PEG-lipids are needed to offset this tendency and help stabilise the lamellar arrangement of the lipid mixture.

4. Conclusions

The results reported in this study show that DOPE-based pH-sensitive liposomes do not interact or fuse readily with membranes containing EPC, DOPE, SM and cholesterol in compositions resembling that found in endosome membranes. Furthermore, the data collected for the pure OA:DOPE- and DHCho-MPEG5000:DOPE-systems indicate that acidification causes leakage before lipid mixing between the individual liposomes takes place. Liposomes taken up via the endocytotic pathway are thus believed to discharge their contents into the endosomal compartment upon pH reduction. Subsequent transport of the liposomal cargo across the endosomal membrane may then take place via a number of different mechanisms. The experimental evidence collected in the present investigation shows that the permeability of the endosomal membrane may increase significantly if its lipid composition is supplemented with extra DOPE. Cytosolic delivery of liposomal contents may, therefore, be aided by the transfer and incorporation of DOPE molecules from either the pH-sensitive liposomes or from precipitated particles of inverted hexagonal phase formed as a consequence of the endosomal acidification.

These investigations are of great importance for the future design and construction of effective pH-sensitive liposome formulations. Our findings suggest, for instance, that the time needed to achieve complete cleavage of the PEG moiety is a crucial parameter for any formulation based on acid-labile PEG-lipids. Slow cleavage kinetics will delay contents escape from the endosome and allow the maturation of early endosomes into larger, less DOPE-sensitive, late endosomal structures. Efforts are currently underway to enhance PEG-lipid cleavage rates to address this issue.

Acknowledgments

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