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Temperature- and pH-controlled fusion between complex lipid membranes. Examples with the diacylphosphatidylcholine/fatty acid mixed liposomes

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Abstract

The fusion capability of complex lipid bilayers and its pH as well as temperature sensitivity have been studied by optical and spectroscopic means. The aggregation and fusion efficiency of such lipid membranes can be optimized by controlling the phase characteristics of the individual membrane components. For a practically relevant illustration, the stoichiometric 1:2 (mol/mol) mixtures of phosphatidylcholines and fatty acids are used. Perhaps the most interesting liposomes of this kind, which are made of dipalmitoylphosphatidylcholine/elaidic acid (DPPC/ELA-COOH (1:2)), undergo a chain-melting phase transition between 42°C and 48°C, depending on the bulk pH value. The highest chain-melting phase transition temperatures are measured with the fully protonated fatty acids at pH $\leq$ 5.5 and involve a change into the non-bilayer high-temperature state. Upon increasing pH, this transition reverts into an ordinary gel-to-fluid lamellar phase change and occurs at 42°C, by and large. Simultaneously, the rate and the efficacy of fusion between the PC/FA and PC/FA$^-$ mixed vesicles decreases. The fusion efficacy of the PC/FA$^{(-)}$ mixed liposomes at pH $\geq$ pK(FA) $\approx$ 7.5 is practically negligible. This is largely due to the increased interbilayer repulsion and to the relatively high water-solubility of the deprotonated fatty acid molecules at high pH. While the pH-variability chiefly affects the efficacy of the intermembrane aggregation, the vesicle fusion itself is more sensitive to temperature variations. It is most likely that the temperature dependence of the intramembrane defect density is chiefly responsible for this. Optimal conditions for the fusion between DPPC/ELA-COOH (1:2) mixed vesicles are thus 3.5 $\leq$ pH $\leq$ 5.5 (6.3) (aggregation maximum) and $T \geq 41.5°C = T_m$(DPPC) (defect density and fusion maximum). Under such conditions the average size of PC/FA (1:2) mixed vesicles in a 1 mM suspension increases by a factor of 10 over a period of 10 min. Interbilayer fusion can also be catalyzed by the mechanically induced local membrane defects. Freshly made liposomes thus always fuse more avidly than aged vesicles. This permits estimates of the kinetics of membrane defects annihilation based on the measured temporal dependence of the maximum fusion-rate. From such studies, a quasi-exponential decay on the time scale of 1.2 h is found for the thermolabile fusogenic DPPC/ELA-COOH liposomes.

Keywords: Lipid vesicle; Phase behavior; Membrane fusion; Phosphatidylcholine; Fatty acid; Drug carrier; Mixed bilayer

1. Introduction

Membrane fusion is vital for all living organisms [1]. It plays an important role, for example, in the transport of fluids and particles between the different cell compartments. It is also involved in the material exchange between the cells and their environment. Detailed understanding of fusion, consequently, is important in biology and also as a basis for the rational design of fusogenic drug carriers.

Lipid bilayer fusion, in general, involves two steps at least. The first is intervesicle aggregation; this is preceded by the (partial) dehydration of both adjacent lipid bilayers

Abbreviations: DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; ELA-COOH, elaidic acid (octadecenoic-9-*trans*-acid); FA, fatty acid; FE, fusion efficacy; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid); MA, myristic acid (tetradecanoic acid); NBD-PE, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl); PA, palmitic acid (hexadecanoic acid); PE, phosphatidylethanolamine; Rh-PE, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-rhodamine; R18, octadecylrhodamine B chloride; SA, stearic acid (octadecanoic acid); TVAC-COOH, *trans*-vaccenic acid (octadecenoic-11-*trans*-acid).

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at the sites of closest approach (and future fusion). The second step is the reorganization of lipid molecules at such contact sites. This terminates in a complete merger and in the subsequent reformation of both initially separated bilayers into a single membrane after the fusion event [4]. If the site of membrane-fusion is non-leaky vesicle contents simultaneously intermix. Such mixing of aqueous compartment is diagnostic of vesicle fusion but may be absent in the process of membrane fusion.

Regulation of intermembrane fusion thus depends on two types of external controls, at least. The first pertains to membrane aggregation; this can be achieved by affecting the protonation [8,9] and/or dehydration [10] state of the participating bilayers. The second is the command of membrane fusion itself: this can be accomplished, for example, by modifying the number of defects in the membranes. Extreme bilayer curvature [11,12], chemical fusogens [13], or domain incompatibility all induce such defects. In addition to this, near the bilayer chain-melting phase transition temperature [3] numerous defects are created, especially at the boundaries between the coexisting gel- and fluid lipid domains.

Such thermally induced density fluctuations occur in simple as well as in complex membranes [28]. The latter are much more versatile than the former, however. This has prompted us to investigate the properties of phosphatidylcholine-single chain amphiphile mixtures [3,16], since the fluctuations in such systems, and thus the fusogenicity can be controlled relatively accurately.

The aggregation and phase behaviour of diacylphosphatidylcholines mixed with homologous fatty acids in a molar ratio of 1:2 have been analyzed previously by several groups including ours [3,17–19]. PC/FA vesicles are thus known to undergo a spontaneous phase transition from the lamellar gel ($L_{\beta'}$) into an inverted hexagonal (H_{II}) phase upon hydrocarbon chain-melting. In a series of preliminary studies we have shown [16] that the dimyristoylphosphatidylcholine/myristic acid (DMPC/MA 1:2 (mol/mol)) vesicles not only exhibit a lamellar to nonlamellar transition near 50°C, but also become fusogenic at 23°C. Owing to this, they can be used for the drug delivery into cells in vitro. In vivo, such vesicles can only be used in the closed compartments, such as urinary bladder, etc. [16], where the region of fusion is confined by the existing anatomical barriers. For any general, systemic drug application it is, therefore, essential to find a different sort of vesicles and a means for restricting the fusion to the region of interest by an external 'pointer'. Local hyperthermia is useful for this purpose if combined with the lipid mixtures which have the primary fusion maximum between 42°C and 45°C. Several diacylphosphatidylcholine/fatty acid (1:2) mixtures fulfill this criterium.

To date, a variety of liposomes has been introduced for the temperature-controlled site-specific drug delivery in vitro and in vivo. Yatvin et al. [2], for example, have suggested that the thermolabile mixed phosphatidylcholine (DPPC/DSPC) vesicles in combination with a local hyperthermia are useful for this purpose. Such vesicles, indeed, release their payload in the heated body but the final success of this approach depends on the membrane partition coefficient of the drug and on the agent's passive uptake by the cells.

Fusogenic liposomes are advantageous in this respect as they are, in principle, capable of bringing the drugs into the intracellular rather than into the extracellular compartment. Several attempts have therefore already been made to develop such liposomes [3,14]. Most of these have been based on the assumption that the crucial step for membrane fusion is the non-bilayer phase generation in the carrier vesicle [5], such phases having been proposed to play a pivotal role in the process of membrane fusion. Phosphatidylethanolamine, for example, forms a nonlamellar, inverted hexagonal phase spontaneously. Hapten-stabilized phosphatidylethanolamine vesicles have thus been tested in vitro and in vivo and found to be only moderately efficient drug carriers (for a recent review, see [6]). The fusion efficiency of such vesicles was too low and the drug transfer into the non-phagocytic cells, consequently, rather inefficient.

We have therefore decided to develop thermolabile liposomes which become fusogenic upon heating above a selected critical temperature. The concept of rational membrane design has helped us approach this goal.

In this paper we present the corresponding results for various stoichiometric phosphatidylcholine/fatty acid (1:2) mixtures. These data support the view that most such mixtures fuse near the phase transition temperature of the pure phosphatidylcholine component. This suggests that the direct transition into a non-bilayer phase is not essential for the intermembrane fusion in spite of the fact that such a transition is inevitably associated with massive membrane unification. 1:2 Dipalmitoylphosphatidylcholine/elaidic acid mixed vesicles (DPPC/ELA-COOH (1:2) illustrate this nicely. Data measured with such system indicate that the close proximity of the chain-melting phase transition temperature of the individual lipid components (T_m(DPPC) = 41.5°C; T_m(ELA-COOH) = 46°C) and the proximity of a non-bilayer phase transition are convenient for the temperature-controlled fusogenicity. We argue that the increased fusion propensity in the therapeutically significant hyperthermia region $T = 42$°C is due to the formation of (probably nonlamellar) domains within the DPPC/ELA-COOH bilayers. Similar domains are much more rare in the DPPC/PA (1:2) mixed bilayers [3], with the bulk chain-melting and the lamellar-to-non-lamellar phase transition temperature of $T_m = 62$°C, or in the DMPC/MA (1:2) mixed bilayers with $T_m = 49$°C.

In our next papers the development of pH-independent thermolabile fusogenic vesicles and the use of both types of such vesicles for the drug targeting in vivo will be discussed. Preliminary results of these studies have been reported before [20].

2. Materials and methods

2.1. Chemicals

1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC, 99% +) was purchased from Boehringer (Mannheim, Germany) or Fluka (Neu-Ulm, Germany). DMPC, puriss., was also obtained from Fluka. Elaidic acid (ELA-COOH, 99%) as well as *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid) (Hepes) were products of Sigma (Neu-Ulm, Germany). The fluorescent label octadecylrhodamine B chloride (R18) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-rhodamine (Rh-PE) were bought from Molecular Probes (Eugene, OR). 1,2-Dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) (NBD-PE) was from Avanti Polar Lipids, Alabaster, AL). *trans*-Vaccenic acid (TVAC-COOH) was from Sigma (Neu-Ulm, Germany). Na_2HPO_4, NaH_2PO_4, CH_3OH and $CHCl_3$ were all p.a. and obtained from Merck (Darmstadt, Germany). All phosphatidylcholines were shown to be pure by thin-layer chromatography on silicic acid plates using a solvent system of $CHCl_3/CH_3OH/33\%NH_3$ (65:35:5, v/v) with molybdenum blue staining and subsequent sulfuric acid charring and were used without further purification. NaOH, HCl, and NaCl were products of Merck (Darmstadt, Germany) and p.a.

2.2. Vesicle preparation

To prepare mixtures with a well defined phosphatidylcholine/fatty acid ratio appropriate amounts of the dry lipids were weighed into 2 ml vials and dissolved completely in a chloroform/methanol (1:2, v/v) mixture. To prepare fluorescently labelled liposomes the appropriate lipophilic fluorescent labels dissolved in chloroform were added to the lipid solution. (In the case of R18 the final label concentration was 5 mol%, relatively to the total lipid. For the Rh-PE and NBD-PE labels, molar ratios between 2.5:0.5 and 0.5:0.5, respectively, and the total label concentrations between 1 mol% and 3 mol% were used.)

The resulting lipid mixture was first dried under a gentle stream of nitrogen. Then, the remnants of the organic solution were removed in vacuo ($< 10^{-2}$ Pa) overnight. To obtain a 5 weight% lipid suspension an appropriate amount of 0.1 M Hepes buffer was added to the dry lipid film. Sonication with a tip-sonicator (Branson Sonifier B12, Danbury, CT) above the chain-melting phase transition temperature of lipids (45–50°C) produced vesicles with an average diameter below 100 nm. The vesicle diameter was determined immediately after the sonication by means of the dynamic light scattering, using a Malvern Zetasizer 2C instrument (Malvern, UK) run with the multi-tau software.

Lipid suspensions for the turbidimetric membrane fusion assay were prepared by mixing the dry PC/FAc (1:2) mixture with a warm electrolyte solution of ionic strength $J = 0.0025$ so that the final lipid chain concentration was 3 mM and the starting bulk pH-value was approx. 10 (at room temperature). The resulting suspension of rather large liposomes was homogenized with ultrasound (Biosonik III, Bronwill, USA; setting 30) for 15 min at room temperature. The average vesicle size in the resulting optically clear suspension ($A_{400\ nm} \leq 0.03$ for 1 cm light-path) was approx. 80 nm. (This value was determined either by means of gel chromatography on a Sephacryl 200 column or by the dynamic light scattering.) A freshly made suspension was used for each series of experiments.

2.3. Phase transition measurements

Typically, a cuvette with 5 μl of fluorescently labelled vesicles in 1 ml of 0.1 M buffer was placed in a thermostated holder of a Perkin-Elmer LS 3B fluorescence spectrometer (Überlingen, Germany). (Phosphate buffer was used in the range of pH 7.2 to 5.8; for the lower pH values acetic acid was used.) Heating-rate was set to 1 K/min. The sample temperature was measured with a thermocouple inserted directly into the suspension. For the R-18-labelled vesicles, the excitation wavelength was set to 520 nm; for the Rh-PE and NBD-PE labelled liposomes the excitation and emission wavelengths were 530 and 470 nm, respectively. For the measurements with R18, a 550 nm cut-off filter (Schott, Mainz, Germany) was inserted into the emission path to minimize the contribution from the scattered light. The change in fluorescence was always measured at 590 nm as a function of temperature. Temperature and fluorescence data were recorded simultaneously by a personal computer and then analyzed by a self-written software.

During the late stage of this investigation, a differential scanning calorimeter MC2 (MicroCal, Amherst, MA) became available to us. This was used with the original data acquisition and analysis software (ORIGIN). For the cooling-scan measurements the calorimeter was connected to a computer-controlled refrigerated bath (F3C, Haake, Germany). DSC samples were heated and cooled at scanning rates of 30 K/h and 20 K/h, respectively.

2.4. Vesicle fusion measurements

The efficacy of intervesicle fusion as a function of temperature was measured turbidimetrically as described in Ref. [3]. In brief, an aliquot of the stock lipid suspension with pH $\approx$ 10 was heated in the cuvette in the spectrometer to the desired temperature. Subsequently, a sufficient amount (typically approx. 400 μl) of a 0.025 molar HCl solution was injected into the cuvette under constant stirring to lower the pH-value to approx. 3.5 $\cdots$ 4 within less than 1 s. (The final ionic strength of such samples was $0.003 \leq J \leq 0.08$.) The temporal evolution of absorbance A_{400} was simultaneously plotted on a strip-chart recorder.

After 10 min the bulk pH value was increased to $9 \leq pH \leq 10$ to reverse the acid-induced intervesicle aggregation. The efficacy of membrane fusion was taken to be identical to the irreversible sample turbidity change:

$$FE_{turbidimetric} = A_{400,final}/A_{400,starting} - 1$$
$$\simeq A_{400,final}/A_{400,starting}$$

This is an ad hoc and qualitative, but nevertheless trustworthy, definition. It is justified by the observation that no vesicle aggregation or non-lamellar phases are seen at $pH \geq 9$ in our experimental system. It also has a limited value at the temperatures higher than the lamellar-to-non-lamellar phase transition temperature, however. At $T \geq T_h$ a considerable proportion of the total aggregated and/or fused lipid namely starts to float on the liquid surface or else gets stuck onto cuvette walls. Even after the terminal pH value increase it is then necessary to mix samples vigorously in order to bring such lipid aggregates into suspension. This stirring probably fragments lipid aggregates and fusionates considerably. The relative fusion efficacy measured for $T \geq T_h$, consequently, only gives a lower limit for this value.

Alternatively, the efficacy of membrane fusion was determined by the modified fluorescence assays of Hoechstra and co-workers [21]. In the first of these, 1 μl of the R18 labelled vesicle suspension was injected into 1 ml of 0.1 M buffer with appropriate pH and temperature. After the stabilization of fluorescence signal, 10 μl of the non-labelled vesicle suspension was added into a cuvette. The change of fluorescence intensity at 590 nm was then recorded with a digital computer. The calibration of fluorescence data was based on the fluorescence intensity measured with a vesicle suspension containing 0.45 mol% R18. This latter bilayer composition mimics the situation expected to be found when all the labelled vesicles (1 μl, 5 mol%) are fused with the non-labelled vesicles (10 μl, 0 mol%). This latter fluorescence value has thus been taken to represent 100% fusion. In the second fluorescence-based fusion assay (NBD/Rh-assay) the change in the efficacy of the energy transfer between both types of the membrane-bound labels, $K = (F_{530,start}/F_{590,start})/(F_{530,end}/F_{590,end})$, was measured. A homogeneous marker distribution throughout the membranes was always assumed.

In order to determine the values of fusion rate and efficiency the measured fluorescence change data was numerically fitted by an exponential:

$$F(t) = F_0 + F_1 \exp(-t/\tau)$$

The fusion efficacy FE was then defined as the ratio of initial and final fluorescence intensity. The characteristic fusion half-time was given by $t_{1/2} = \ln 2 \cdot \tau$.

The results of our fluorescence fusion-assays was also confirmed by an independent qualitative method: the change of the average vesicle diameter, as measured by the dynamic light scattering. In a typical such experiment, 10 μl of the fusogenic vesicle suspension were mixed with 1 ml of 0.1 M phosphate buffer (pH 7.2) or with 1 ml of 0.1 M acetate buffer (pH 3.9). Each sample was then incubated for 10 min at 37°C or 42°C in a water bath. Subsequently, the bulk pH of the suspension was titrated back to neutral, if required, in order to reverse the acid-induced intervesicle aggregation. The final size of lipid vesicles was always measured ten times for 10 s at 25°C with a Zetasizer 2C instrument (Malvern Instruments, Malvern, UK) equipped with a thermostated (± 1 degree) sample cell. Data were analyzed by the multi-tau procedure from the Autosizer software package. This ensured a better than 20% reproducibility for the distributions with one main peak (high pH-range or low temperatures) and a reproducibility of 30% in the mean-value for the broader distribution (low pH and high temperatures).

The results of such measurements may have been slightly affected by the residual aggregation of lipid vesicles. (We estimate the potential error to be $\leq 20\%$.) They are certainly not biased by the presence of the non-lamellar phases, however, which do not exist in any of the investigated lipid mixtures at 25°C.

3. Results

We have used various methods to investigate the colloidal and phase behaviour of phosphatidylcholine/fatty acid mixtures as a function of chain-length, position of double bonds, pH value, temperature, and age after the preparation.

3.1. Phase behaviour

By turbidimetry, we have previously shown that the stoichiometric 1:2 (mol/mol) mixtures of dipalmitoylphosphatidylcholine and palmitic acid (DPPC/PA (1:2)) undergo a phase transition at 62°C [3]. Independent X-ray diffractometric studies [23] have confirmed that this chain-melting phase transition is from a lamellar gel ($L_{\beta'}$) into a fluid non-lamellar H_{II}) phase [18].

In the cooling scan only one phase transition is always observed. An immediately following heating scan also reveals just one phase transition. The first heating scan measured with the samples that were aged a low temperatures, however, regularly unveils several peaks. The first of these coincides with the chain-melting phase transition temperature of the pure phospholipid or fatty acid component, depending on which of the two has a lower value. For the DPPC/PA (1:2) mixtures such peak is thus seen at approx. 42°C. Related dimyristoylphosphatidylcholine and myristic acid mixtures (DMPC/MA (1:2)) also show a similar phase behaviour with a reproducible transition at approx. 50°C. This latter transition may involve an intermediate cubic phase [22], however.

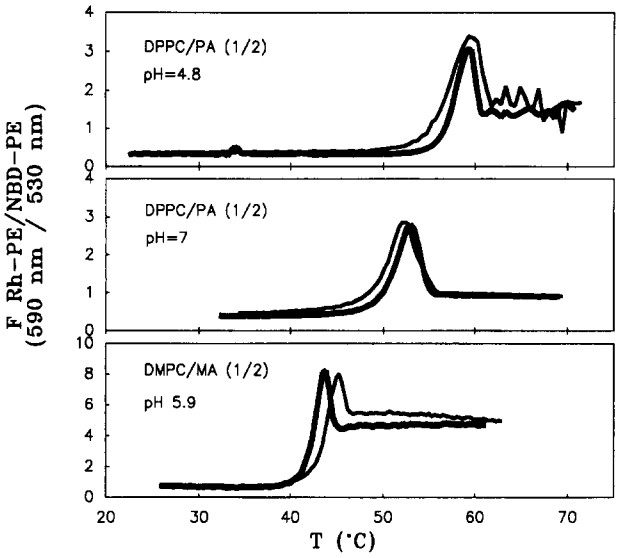

Fig. 1. Chain-melting phase transitions of DPPC/PA (1:2, mol/mol) and DMPC/MA (1:2) mixed membranes as measured means of the resonance energy transfer between Rh-PE and NBD-PE for a 1 mM bulk suspension.

Data measured by means of fluorescence probes are in qualitative agreement with these results (Fig. 1). Quantitatively, however, the perturbation of lipid bilayers by the fluorescence markers is reflected in a downward shift of the phase transition temperature by 3 to 5 degrees. The results of fluorimetric membrane fusion experiments (see further discussion) must be corrected correspondingly [2].

In order to determine the effect of the lipid phase transition on the extent of intervesicle aggregation and fusion we have measured the irreversible increase of the average vesicle diameter after a transient acidification of each lipid suspension at different temperatures.

3.2. Temperature dependence of the colloidal properties of PC / FA mixed systems

The chain-melting phase transition temperature and the accompanying creation of non-bilayer structures at $T = T_h$ results in a collapse of all PC/FA (1:2) suspensions (cf. Fig. 2). After a short initial period of vesicle flocculation the original acidic mixed lipid suspension then segregates into a sticky (non-lamellar) lipid subphase and an aqueous subphase. The lipid composition of the former is identical to that of the original suspension: PC/FA = 1:2. (Azeotropy thus plays no role in this system, unlike in the related mixed lipid membranes investigated before [24].) Fusion

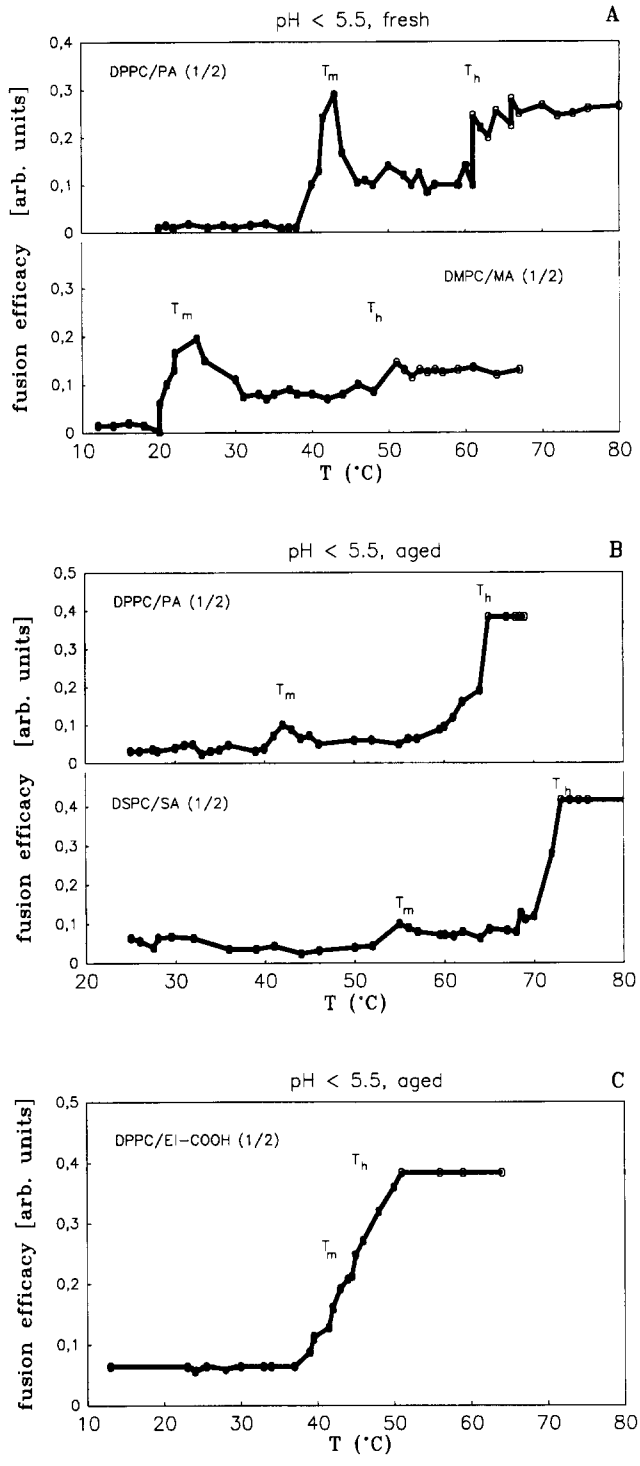

Fig. 2. Temperature dependence of the fusion efficacy of freshly prepared ($t \leq 1$ h, A) and aged ($t \geq 24$ h, B) DPPC/PA (1:2) and DMPC/MA or of the aged DPPC/El-COOH (1:2) mixed vesicles (C), measured by the turbidimetric assay. Fusion maxima are observed near and at the chain-melting phase transition temperature, T_m, of the phosphatidylcholine component. Above the lamellar-to-nonlamellar phase transition temperature, T_h, massive lipid aggregation and fusion occurs. Values at $T \geq T_h$ (open symbols) thus only represent the lower limit of the actual, temperature-induced bilayer fusion. For the DPPC/El-COOH mixtures (panel C) both decisive temperatures nearly coincide; this precludes the identification of the fusion peak at T_m.

[2] Differential scanning calorimetric data (not shown) indicate that this fluorescence-measured shift is only an apparent one. The actual bulk phase transition temperature of the labelled and non-labelled vesicles is almost the same. This suggests that the difference in T_m data given in Figs. 1 and 2 or 4, 5 and 6 is not due to the general but just a local membrane perturbation by the fluorescently labelled phospholipids.

efficiency values measured for $T \ll T_h$ and shown as closed symbols in Fig. 2 are quite reliable. In contrast to this, the open symbols in Fig. 2 which pertain to the data measured above the lamellar-to-nonlamellar phase transition temperature are quantitatively unreliable. Massive lipid reorganization in the non-bilayer phase region, which destroys the vesicular structures, precludes the quantitative accuracy of such data.

Some samples begin to aggregate and fuse at temperatures below $T = T_h$ already. The resulting enlarged lipid bodies are not as sticky as the non-bilayer, H_{II} lipid phase and also show no other characteristics of such phase. They are thus different from this type of phase.

Vesicle fusion below T_h is most clearly observed with the freshly made vesicle suspensions (Fig. 2A). It typically happens near the chain-melting phase transition temperature of the pure phosphatidylcholine component, this is, at T_m = 23.5°C for DMPC, 41.5°C for DPPC, and 55.5°C for DSPC.

Vesicle aging at pH ≫ pK and at $T \gg T_m$, suppresses such low-temperature interbilayer fusion. Fully equilibrated PC/FA (1:2) preparations only show a small hump at $T = T_m$ (Fig. 2B). Pure phosphatidylcholine vesicles of comparable size do not fuse on this time scale at all, irrespective of their age (data not shown).

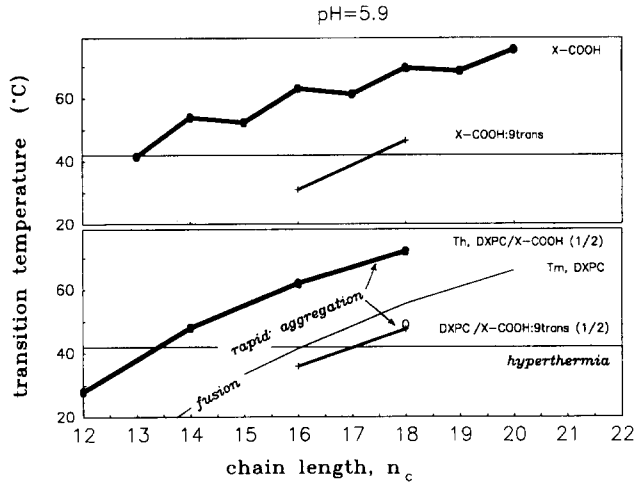

Fig. 3. Chain melting phase transition temperature of the dry fatty acids (upper panel) and of the fully hydrated phosphatidylcholines (DXPC) or DXPC mixed in a molar 1:2 ratio with the appropriate fatty acids. Upon the transition into a nonlamellar phase ($T = T_h$), massive membrane aggregation and fusion occurs; near and at the gel-to-fluid lamellar phase transition of the phosphatidylcholine component another fusion maximum is observed (cf. Fig. 2). Horizontal line gives the temperature of therapeutically interesting hyperthermia. The optimum for the thermolabile fusogenic liposomes is achieved when the rapid aggregation and fusion curves are made to coincide. Data for pure fatty acids stem from the literature and the results for the mixed-systems from the turbidity measurements and DSC.

3.3. Chain-length dependence

In order to maximize the chances for the success we have based our search for thermolabile fusogenic vesicles suitable for the in vivo applications on the principles of rational membrane design. Fig. 3 gives the rationale and the experimental basis for this quest and simultaneously illustrates the effect of hydrocarbon chainlength on the colloidal and phase properties of the mixed PC/FA systems.

In the upper panel of Fig. 3 the chain-melting phase transition temperatures of pure fatty acids are given; in the lower figure half, the corresponding data for the pure phosphatidylcholines (thin curve), for the homologous PC/saturated-FA-mixtures (1:2; thick curve with dots) and for the DPPC/alkenoic-FA 1:2-mixtures (line with crosses) are plotted. Corresponding asymmetric DPPC/MA and DPPC/SA mixtures have a chain-melting phase transition at 54°C and 66°C, respectively (not shown).

In general, the free fatty acids have a stronger impact on the thermodynamic properties of the PC/FA (1:2) complexes than the esterified fatty acids. Substitution of free palmitic acid with 9-*trans*-octadecenoic acid (C18:1*t*, elaidic acid; ELA-COOH: T_m = 51°C) in the DPPC/FA-complexes, for example, shifts the chain-melting phase transition temperature from approx. 62°C to 45°C (cf. Fig. 3). Substitution of palmitic acid with 11-*trans*-octadecenoic acid (C18:1*t*, *trans*-vaccenic acid: T_m = 39°C) brings this transition temperature value from 62°C to 46°C at pH 5.9 (open disk in Fig. 3). (Scanning calorimetry in the acetate buffer (pH 4.9) gives 41°C for the latter PC/FA complex.) Fully hydrated DPPC/oleic acid (1:2) complexes melt near 40°C. Palmitic acid replacement with the 9-*cis*-hexadecenoic acid (C16:1*t*, palmitoleic acid: T_m = 0.5°C) in the DPPC/FA-complexes lowers the T_m value to approx. 36°C. In contrast to this, an extension of the phospholipid chains by two methylene groups (DPPC → DSPC) rises the phase transition temperature only by a few degrees, the T_h value then being approx. 65°C in the case of DSPC/PA (1:2), for example.

Of all the PC/FA mixtures explored in this work, the 1:2 molar mixture of DPPC/ELA-COOH comes the closest to our desires described in the previous paragraphs. This lipid mixture was, therefore, used for the majority of further investigations reported in this work.

3.4. pH-Dependence of the properties of PC / FA-mixed system

The stoichiometric 1:2 mixture of DPPC and ELA-COOH in calorimetry shows a moderately broad, high-enthalpy phase transition near ≈ 41°C in the neutral pH-region (Fig. 4). Decreasing the bulk pH-value shifts this temperature upward. At pH 5.3, the chain-melting phase transition of such lipid mixture reaches its maximum temperature value, T_m = 48°C, and is also quite broad. In addition to the main peak, a small endothermic peak is also often observed around 42°C, the transition temperature of the pure DPPC component.

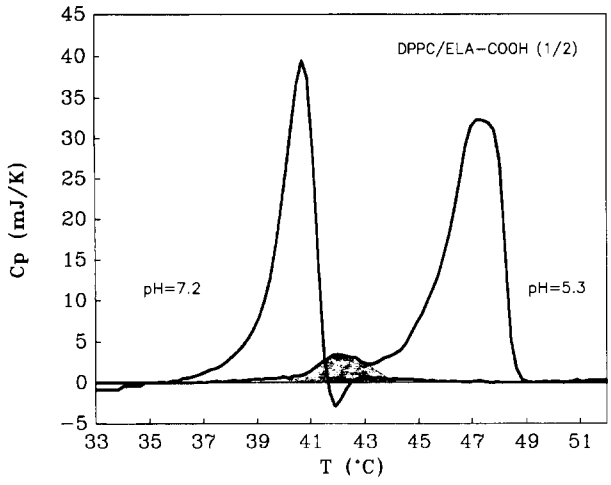

Fig. 4. Differential scanning calorimeter tracings of a DPPC/ELA-COOH (1:2) mixture in the suspensions with different proton concentrations. Transition at pH 7.2 is from a lamellar gel into a fluid lamellar phase ($L_{\beta'} \rightarrow L_{\alpha}$); phase change at pH 5.3 is from a lamellar gel into the inverted hexagonal phase. The small $L_{\beta'} \rightarrow L_{\alpha}$ 'pretransition' only involves DPPC. All these phase assignments are based on the results of independent X-ray diffraction measurements [23].

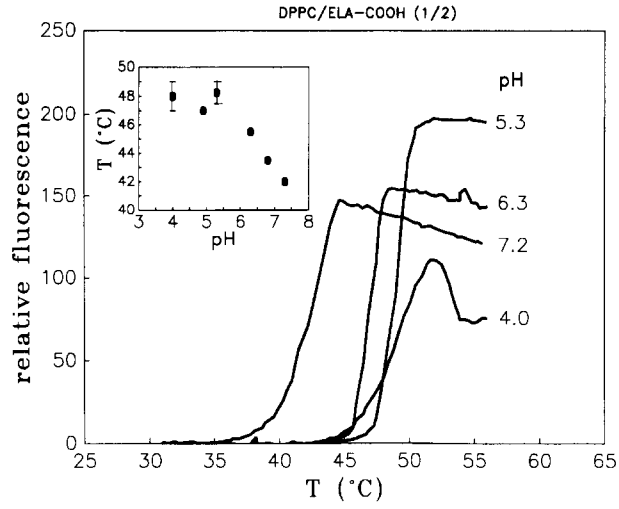

Fig. 6. Temperature dependence of the fluorescence intensity of R18 in DPPC/ELA-COOH (1:2) mixed vesicles as a function of the bulk pH value, measured in the second and subsequent heating scans. (Label concentration: 5 mol%; scanning rate 1 degree/min.) Inset: phase transition temperature as a function of the bulk pH value.

Lipophilic fluorescence labels as detectors of the phase behaviour of the DPPC/ELA-COOH (1:2) mixtures give similar results (Figs. 5 and 6). The temperature dependence of the fluorescence intensity during the first heating scan reveals the transition temperature of such labelled acidic vesicles to be approx. 47°C. Another, less prominent phase transition is also observed at 38°C. This latter phase transition disappears upon sample cooling and is not seen upon immediate re-heating (Fig. 5). At pH 7.2, however, the hydrocarbon chains of DPPC/ELA-COOH mixtures melt at ≈ 41°C already. DPPC mixed with the unsaturated fatty acids, in this respect, behaves very much like a DPPC/PA mixture (cf. Fig. 1).

Progressive fatty acid protonation not only increases the chain-melting phase transition temperature; it also lowers the colloidal stability of the entire lipid suspension. Acidification of the mixed lipid suspension made of PC/FA (1:2) thus always induces a massive vesicle aggregation, which is also a prerequisite for the successful intervesicle fusion. When studied by means of the energy-transfer fluorescence assay based on the DPPE-NBD and DPPE-Rh labels, mixtures of DPPC and palmitic acid at pH 4.8 reveal such an effect above T_h, this is, above approx. 62°C. If studied only for a short period of time, the same suspensions show no anomaly at 42°C, however. The situation is qualitatively similar at pH 7: no significant aggregation or fusion is observed for the DPPC/PA mixtures near 42°C but a minor increase is found above 62°C. Above pH 8 no changes are detected in such a temperature range, however. With the DPPC/ELA-COOH (1:2) mixed lipid vesicles the corresponding fusogenic temperature region is detected between 40°C and 50°C (cf. Fig. 7, bottom).

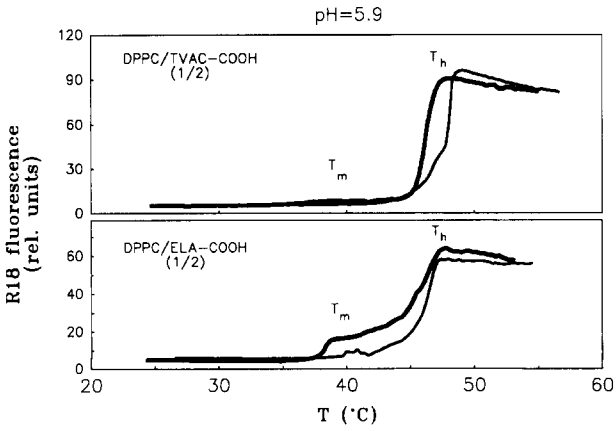

Fig. 5. Phase transition measurements with R18-labelled (5 mol%) DPPC/ELA-COOH (1:2) and DPPC/TVAC-COOH (1:2) mixed vesicles. The results of the first up- (thick line) and down-scan (thin line) are shown (scanning rate: 1 degree/min).

By a suitable calibration procedure we have also determined the absolute short-term fusion efficacy of the DPPC/ELA-COOH (1:2) mixed vesicles. At pH 4.9 and 37°C, the absolute success of the fusion process during a period of 5 min was found to be only 5%, despite the fact that vesicle-vesicle aggregation under such conditions was high. Membrane fusion efficacy has increased to 12%, however, at $T = 42$°C. At 47°C a rather high value of 30% was measured (Fig. 8).

Conversely, the vesicle propensity for fusion is always significantly lower at pH 7.2 than at pH ≤ 6. At 37°C and pH 7.2, for example, only 1% of vesicles fuses within 5 min. At 42°C, which is the chain-melting phase transition temperature of the mixed DPPC/ELA-COOH (1:2) vesicles in the neutral pH region (cf. Fig. 4), the relative fusion efficacy gets somewhat higher. This notwithstanding, its

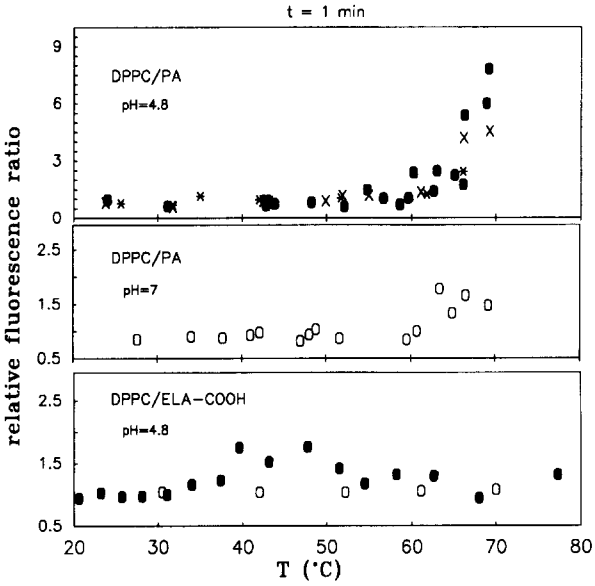

Fig. 7. Relative fusion efficacy of the DPPC-fatty acid (1:2) mixtures as a function of temperature after 1 min incubation at various protons concentrations. The ordinate gives the ratio between the resonance energy transfer efficiency of the Rh-PE and NBD-PE labelled vesicles prior and after the induction of fusion. Open symbols always correspond to pH 7; closed symbols always stem from the measurements done at low pH. Symbols in the top panel give the results of measurements with the vesicles containing 2.5/0.5 mol% of Rh-PE/NBD-PE (●), 1.5/0.5 mol% (×) and 0.5/0.5 mol% (*) of Rh-PE/NBD-PE.

absolute value remains low. Typically it is only 3% after 5 min. This clearly demonstrates that only a combination of the high proton concentration and the elevated temperature can promote effectively the fusion between DPPC/ELA-COOH (1:2) mixed vesicles. The situation in vivo is much better, however, as will be shown in our next publication (Zellmer, S. and Cevc, G., unpublished data).

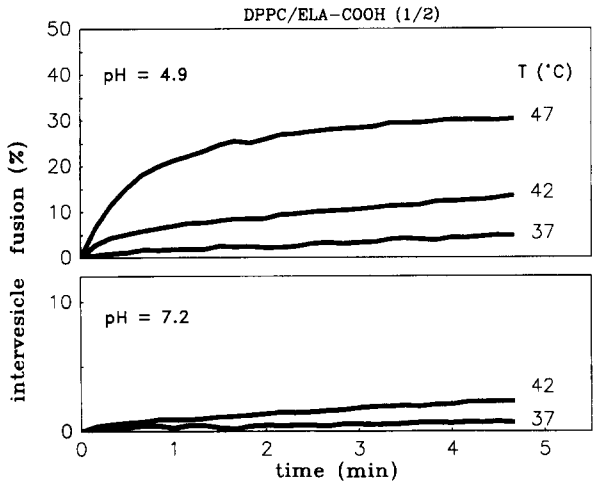

Fig. 8. Absolute efficacy of fusion between DPPC/ELA-COOH (1:2) mixed vesicles as a function of temperature and the bulk pH value, as measued by means of the R18 dilution assay. Note different scales in both panels.

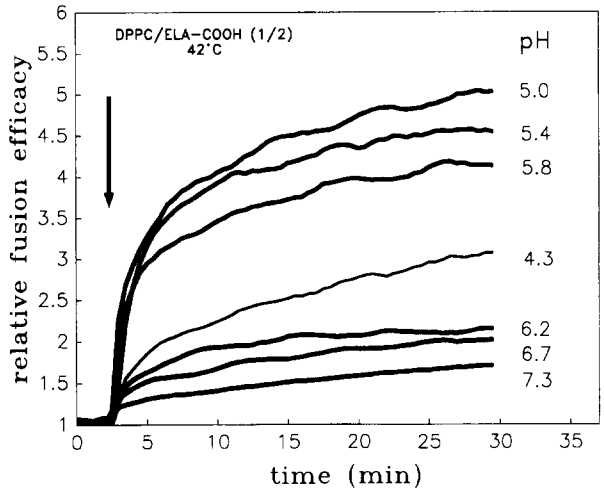

Fig. 9. Fusion of DPPC/ELA-COOH (1:2) mixed vesicles as a function of the bulk pH value at 42°C, as measured by R18 dilution assay. Arrow indicates the time at which the R18-labeled liposomes were diluted with a tenfold amount of the unlabelled vesicles in order to start fusion. The curve pertaining to pH 4.3 (thin curve) is quantitatively unreliable, owing to the extensive intervesicle aggregation at such pH (see also legend to Fig. 2).

3.5. Time dependence of the membrane fusion

Fusion between the mixed PC/FA vesicles is easier to detect after long assay times. If the bulk pH value is much lower than 7 the majority of DPPC/ELA-COOH (1:2) vesicles at 42°C, according to the fluorimetric fusion assay has fused within one hour measuring time (Fig. 9). Below pH 5.5 the interbilayer fusion is then sufficiently fast to ensure nearly complete vesicle unification within just a few minutes [3]. On the contrary, at neutral pH only marginal fusion is detected even at $t = 60$ min.

In order to get a deeper insight into the kinetics of the temperature-induced bilayer fusion we have analyzed the data of Fig. 9 numerically. The characteristic half-time of fusion ($\tau_{1/2}$) and the corresponding fusion efficacy (FE) were deduced from Fig. 9 by fitting the data with a single-exponential function. The corresponding results are shown in Fig. 10. They show that for the experimental temperature of 42°C the largest changes in the fusion rate occur in the pH-range between 7.2 and 5.8.

The efficacy of membrane fusion reveals a similar pH-dependence; the value of this parameter increases with decreasing bulk pH value until it reaches a constant value at pH 5.3 and below.

In order to corroborate all these conclusions we have also studied the effects of temperature and of the bulk pH on the average vesicle size by means of the dynamic light

[3] Exact fusion measurements at pH ≤ 5 are, therefore, almost impossible. Large vesicle aggregates, which float near the surface of the vesicle suspension, are just one source of tremendous experimental difficulties. This can not be prevented even by vigorous stirring.

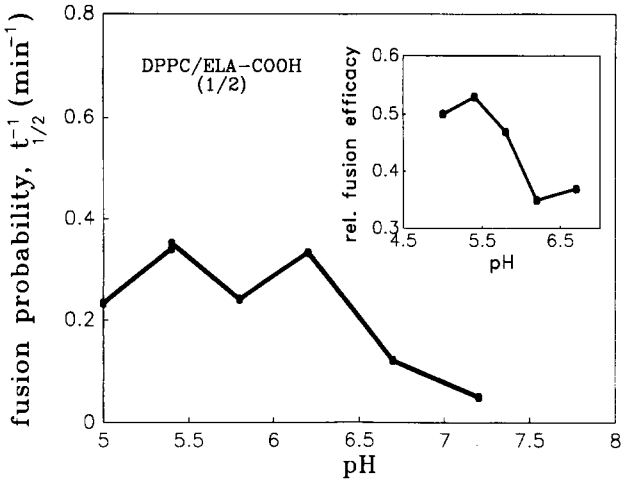

Fig. 10. Fusion probability (i.e. inverse half-time of fusion: $\tau_{1/2}^{-1}$) relative fusion efficacy (as defined in the text) for DPPC/ELA-COOH (1:2) mixed vesicles as a function of the bulk pH value. All values were obtained by analyzing the data given in Fig. 9 within the framework of a single exponential approximation (see the text).

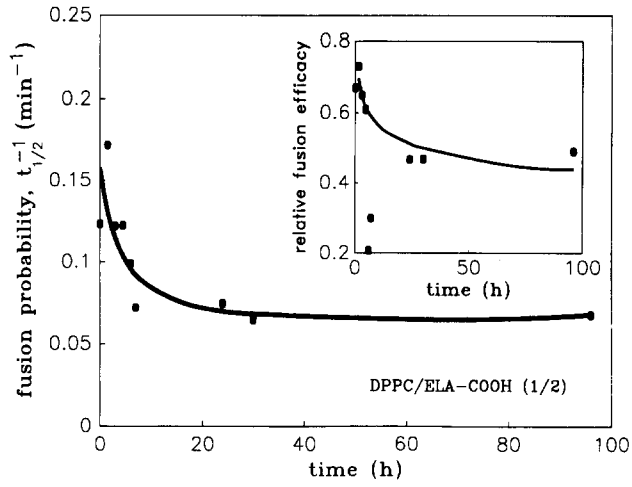

Fig. 12. Fusion probability as a function of time after the sonication of a mixed lipid suspension consisting of DPPC/ELA-COOH (1:2). Fusion was induced by keeping the bulk pH value at 5.8 and then setting $T = 42$°C. It was measured by means of the R18 dilution assay.

scattering. In a typical experiment the incubation of lipid vesicle suspension at 42°C for 5 min (pH ≃ 7.4) has only resulted in a minor vesicle-size increase (Fig. 11). Vesicle aggregation and fusion upon the incubation at 37°C were also not measurable. It was necessary to keep the temperature at 42°C and to follow the process of fusion for at least 10 min if one was to measure significantly positive results and estimate the increase of vesicle-size reliably.

3.6. Defects effects

Another important determinant of the efficiency of membrane fusion is the concentration of intramembrane defects. In order to prove this, we have followed the

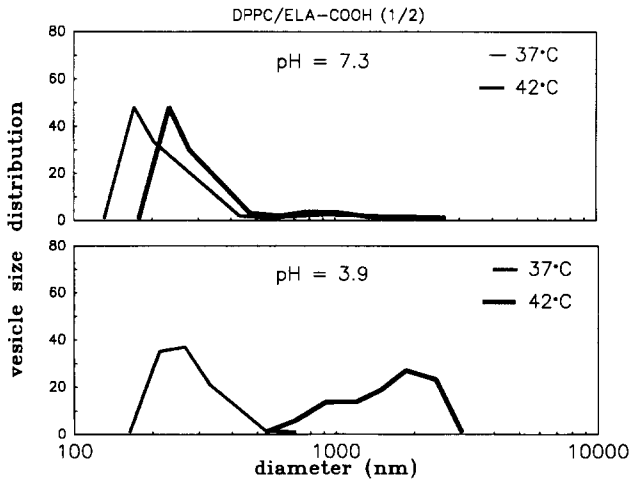

Fig. 11. Increase of the diameter of DPPC/ELA-COOH (1:2) mixed vesicles after 10 min incubation at 37°C and 42°C for two different bulk pH values (3.9 and 4.9). All vesicle diameters were measured at pH 7 and at $T = 25$°C, where there is little or not danger of the interference with (non-lamellar) lipid aggregates.

efficacy of membrane fusion at 42°C and pH 5.8 as a function of vesicle age. Specifically, we have studied the fusion of DPPC/ELA-COOH (1:2) vesicles at different time intervals following the lipid vesicle preparation by means of ultrasonication. Total investigation time was 100 h. The characteristic decay time of the vesicle fusion rate and fusion efficacy was then determined by the numerical data analysis within the framework of a single-exponential approximation. The fusion-decay and defect annihilation time was calculated to be 7 h. The relative fusion efficacy, for example, was measured to decrease by approx. 40%, to 62% of the initial value, over a period of 100 h (Fig. 12) [4]. During the same time the rate of fusion has decreased by a factor of 2.4, also in an exponential manner. It is noteworthy that the first ('low temperature') fusion maximum (cf. Fig. 2) has also decreased dramatically upon aging of the vesicle suspension.

4. Discussion

Diacylphosphatidylcholines and their fatty acid homologues mixed in a stoichiometric 1:2 molar ratio exhibit a sharp phase transition from a lamellar gel ($L_{\beta'}$) into an inverted hexagonal, fluid (H_{II}) phase [18,19]. Such a transition occurs in the temperature region intermediate to the chain-melting phase transition temperatures of both pure components (cf. Fig. 3). The value of this transition temperature is, of course, also a function of acyl chain length, specific values being 48°C for the DMPC/MA (1:2), 62°C for the DPPC/PA (1:2), and 74°C for the DSPC/SA (1:2) mixtures [3]. In addition to this, azeotropism has been

[4] The apparent discrepancy between data points 5 and 6 and the calculated curve are due to the fitting problems.

reported for the DPPC/PA and DSPC/SA mixtures close to the 1:2 molar ration [24]. This is slow, however, and thus did not affect the results of our study.

The number and the position of double bonds in the individual lipid chains influences the chain-melting phase transition temperature as well. While the transition temperature of DPPC/PA (1:2) mixed vesicles is 62°C at pH 3.5 [3], the same saturated phosphatidylcholine in a mixture with two mono-unsaturated C18-fatty acids containing a double bond in the 9-*trans* configuration (elaidic acid; ELA-COOH) undergoes the corresponding phase transition at 48°C and pH 3.8 (cf. Figs. 4 and 5). Similar conclusion also holds for the DPPC-oleic acid mixtures. (The double bonds are here in the 9-*cis* conformation of Ol-COOH.) For the DPPC/Ol-COOH (1:2) complex one gets $T_m \approx$ 40°C, however.

Both fatty acids just mentioned (C18) exceed the palmitic acid (C16) by two methylene groups. In spite of this, the transition temperature of the DPPC/octadecenoyl-fatty acid mixtures is always lower than the T_m value of the DPPC/PA (1:2) complexes. Double bonds in the fatty acid chains thus have a strong impact on the gel-phase stability [25] and shift the chain-melting phase transition temperature downward significantly.

Figs. 1 and 4 show, moreover, that the chain-melting phase transition temperature of the stoichiometric phosphatidylcholine-fatty acid mixtures is strongly affected by the bulk pH-value. Upon fatty acid protonation, the charge density, hydration, as well as the lateral pressure in the plane of the mixed lipid bilayer all decrease [3]. This increases the chain-packing density in the bilayer and, consequently, shifts the chain-melting phase transition temperature upward (cf. Fig. 1 and Ref. [3]). The chain-melting phase transition temperature of PC/FA mixtures is therefore very sensitive to the bulk pH value. This is true, in particular, in the pH-region between 5.8 and $pK = 7.3$, where the ionization state of the mixed PC/fatty acid (1:2) bilayers varies most. At higher pH-values this dependence is less obvious, possibly due to the high water-solubility of the fully ionized fatty acid salts, which deprive the bilayers of some of their single-chain component.

pK-value of the membrane-bound fatty acid was reported to be between 7.2 [3] and 8 [26]. It is thus higher for the PC/FA complexes than it is for the simple COOH-groups or dissolved fatty acids ($4.8 \leq pK \leq 5$). (This discrepancy is probably due to the hydrogen bonding between the carboxylic group of the fatty acids and the phosphate group of the phospholipid molecules [27].) Proton concentration variations in the concentration range between 10 μM (pH 5) and 1 mM (pH 3), therefore, do not affect the chain melting phase transition temperature of DPPC/ELA-COOH (1:2) mixtures appreciably.

In a detailed previous study [3] we have determined the chain-melting phase transition temperatures of several phosphatidylcholine/fatty acid (1:2) mixtures. We have shown that all such mixtures revert from a lamellar gel-into a nonlamellar fluid-phase. This transformation is associated with massive interbilayer fusion. By referring to the results of ^{31}P-NMR spectroscopy [18] we have, furthermore, argued that near the chain-melting phase transition temperature of the individual phospholipid components minor bilayer re-organization is likely. According to the X-ray diffractograms measured in this temperature range, this does not involve non-bilayer phases, however [27].

In agreement with this, our vesicle turbidity measurements have revealed a small, but significant, increase in the sample absorbance [3] at the chain-melting phase transition temperature of the corresponding phospholipid component (at approx. 23°C, 42°C and 55°C for the DMPC, DPPC and DSPC mixed with their fatty-acid homologues, respectively). Fig. 2B demonstrates that the fusion between PC/FA membranes also shows the corresponding temperature dependence: fusion-efficiency is first observed to increase upon heating near the chain-melting phase transition temperature of the pure phosphatidylcholine component. We infer that this is due to fusion events initiated by the critical fluctuations in the lipid bilayers. We envisage the latter to arise at or near the domain boundaries between the segregated phospholipid and the mixed phospholipid/fatty acid complexes. These fluctuations, consequently, are suggested to act as 'fusogenic excitations' [3,16].

It is noteworthy, that our membrane fusion data are not biased by the existence of a H_{II} phase in the high temperature region, unless they were measured above the lamellar-to-non-bilayer phase transition temperature. Vesicles that were kept at $T \ll T_h$ during the induction of fusion as well as during the measurement have never been in a non-lamellar phase and only experience stresses and changes that eventually could bring them into such a phase. After having been properly aged, such vesicles and their descendents are also sufficiently stabilized to represent an essentially steady-state system.

Previously studied homologues diacylphosphatidylcholine/saturated fatty acid (1:2) mixtures fuse extensively only at the temperatures which are physiologically uninteresting. Fig. 2 demonstrates this: the relative fusion efficacy of such mixed lipid systems has a narrow maximum at the chain-melting phase transition temperature of the pure phosphatidylcholine component and a step-like, overwhelming fusion region at $T = T_h$, associated with the transition into the inverted hexagonal non-lamellar phase. The latter increase is really quite trivial since any transition into the non-bilayer state goes hand in hand with extensive membrane fusion. It is also not really useful for the practical applications, since it is accompanied by a complete loss of the vesicle contents at $T = T_h$ (P. Risse and G. Cevc, unpublished data).

We have, therefore, attempted to find a compromise between these divergent requirements. On the one hand, we have tried to maintain the chain-melting phase transi-

tion temperature of the phospholipid component near 42 · · · 43°C, which is the therapeutically interesting hyperthermia region. On the other hand, we have tried to bring the chain-melting phase transition and the T_h value of the stoichiometric phospholipid/fatty acid mixture (T_h) close – but not too close – to this temperature $T_m \lesssim T_h$. This should rise the number of critical fluctuations in the bilayers near 42 · · · 43°C and simultaneously increase the probability for membrane aggregation but also prevent excessive agent loss from the vesicle interior.

The solution to this problem was to lower the chain-melting phase transition temperature of the complex, and thus the T_h value, close to the phase transition temperature of the DPPC component at T_m(DPPC) = 41.75°C. Such a proximity of $T_h \equiv T_m$(DPPC/ELA-COOH) and T_m(DPPC) values should increase, respectively, the probability of intervesicle aggregation (and thus the propensity for the close membrane approach) and the likelihood for the formation of 'fusogenic excitations'. Both are necessary for the successful membrane fusion.

We have achieved this goal by substituting the palmitic acid molecules (C16:0; T_m = 62°C) by the elaidic acid molecules (C18:1*t*; T_m = 46°C). In the lipid mixtures containing DPPC and the latter type of fatty acids in the appropriate 1:2 molar ratio massive bilayer fusion was then, indeed, observed near 42°C; this is identical to T_m(DPPC) ≈ T_m(ELA-COOH). The proviso for this was that the bulk pH value was low enough to allow extensive vesicle aggregation (cf. Figs. 2, 3).

Analysis of membrane fusion data from Fig. 9 reveals that the speed as well as the efficacy of the fusion between DPPC/ELA-COOH (1:2) vesicles at 42°C increases by approx. a factor of 5 to 2, respectively, upon decreasing the bulk pH value from 7 to 5 (Fig. 10). The chain-melting phase transition temperature of such vesicles is simultaneously shifted upward, owing to the fatty acid protonation (Fig. 4). The fact that the fusion between DPPC/ELA-COOH membranes takes place at 42°C in the entire investigated pH-region, moreover, demonstrates that the chain-melting of phosphatidylcholine domains is responsible for the 'fusogenic excitations'. This also provides a circumstantial evidence for the catalytic role of the chain melting phase transition in the process of intervesicle fusion. In other words: in order to promote the fusion between lipid membranes it is not necessary to push the whole system into a non-bilayer state. Vesicles incubation close to their lamellar-to-nonlamellar phase transition temperature suffices for this as it increases the bilayer tendency to come into close contact (high aggregation constant values, C_a), provided that the defects in the membranes catalyze membrane-membrane unification (high fusion constant values, C_f). Fig. 13 illustrates this schematically.

Local defects are likely to arise or accumulate near the phase boundaries [15]. The latter can, but need not, be induced by the vicinity of a phase transition temperature. This, theoretically well known [15], fact is indirectly confirmed by the experiments of Massari and Colonna [29]. These authors have demonstrated that the demixing of lipids that differ in their chain lengths widely gives rise to the formation of local defects. These then act as nucleation centres for the inter-membrane fusion.

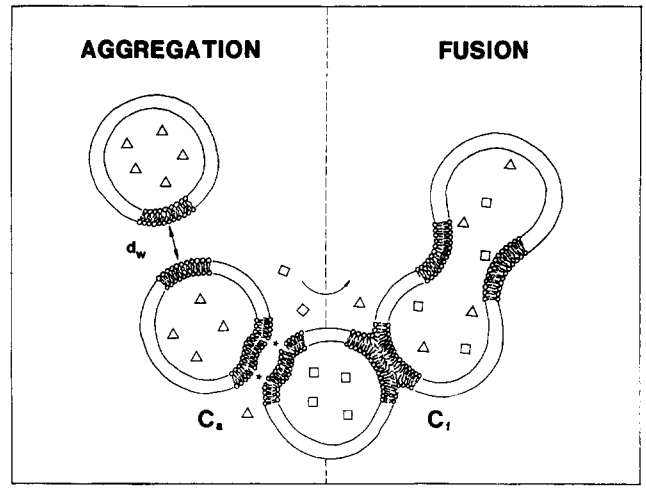

Fig. 13. Schematic representation of membrane fusion illustrating the influence of membrane proximity and defect density. In the system studied in this work, non-lamellar phases are not necessarily involved in the membrane fusion process; local membrane disorder, this is, high local defect density is fully sufficient.

At constant temperature, local defects can be induced mechanically. Such defects are created, for example, during vesicle preparation or fragmentation by ultrasound. Defect density in the lipid bilayers, therefore, decreases as a function of time after the termination of ultrasonication. In our opinion, this is the reason why the rate and the efficacy of fusion between liposomes are both influenced by vesicle age (Fig. 12), the latter being inversely proportional to the defect density. (The reason for this is the defect annealing process.) After 24 h of aging at room temperature, most of the local defects in the DPPC/ELA-COOH (1:2) mixed vesicles are normally annealed. In agreement with this, our fusion measurements done with 1-day-old vesicles revealed a nearly constant fusion rate and efficiency values (Fig. 12).

Thermolabile fusogenic liposomes, in this respect, show a behaviour very similar to that of the simple phospholipid vesicles, made of pure DPPC, for example, fusion rate being a good monitor of the defect density in the bilayers. Indeed, according to Lawaczeck et al. [30], the number of defects in such vesicles decreases with vesicle age and temperature. The first – and the fastest – step in the process of membrane defect annealing is completed for the pure DPPC vesicles within 1 h. This estimate pertains to an incubation temperature 3°C higher than the chain-melting phase transition temperature. Higher incubation temperatures speed up this annealing process; at 50°C it is completed within 10 min. Within 24 h, the majority of residual defects is thus eliminated [31].

In contrast to Lawaczeck and his colleagues, who have worked with vesicles in the fluid-lamellar phase, our DPPC/ELA-COOH (1:2) suspensions were kept at room temperature during the annealing process. This is approx. 20°C below the chain-melting phase transition temperature and might explain why the defect annealing time in our system is 7-times longer than that of the pure DPPC bilayers in the fluid phase.

Calibration of the membrane fusion assay was not fully successful in our hands: 100% fusion was never achieved (Fig. 8). Even the bulk pH value of 4.9 and incubation temperatures of 47°C (the chain-melting phase transition temperature at this bulk pH value) have not increased the overall fusion efficacy over 30%. One possible explanation for this is 'semi-fusion'. During such an incomplete fusion only 50% of the membrane-associated probes, this is, only probes from the outer bilayer leaflet, are diluted into the 'receptor' membrane. We find this explanation to be very unlikely in light of the observation that the average vesicle size during fusion increases by a factor of 10 (Fig. 11) [5]. Moreover, the tension in lipid bilayers that would be produced by a semi-fusion process would reduce the vesicle stability considerably. This would make it impossible to observe semi-fused vesicles after a long measuring time.

An alternative explanation for the low fusion efficacy is the non-homogeneity of the fluorophore distribution in the bilayers. By sonicating vesicle suspensions after the membrane fusion assay we have attempted to improve the uniformity of membrane probe distribution between all bilayers. We have hoped that the fluorescence intensity of sonicated suspensions would mimic perfect fusion process. Fluorescence intensity prior and after the sonication remained nearly the same, however. This suggests that enforced fusion process was, indeed, complete even prior to the sample sonication.

Our observations are in good agreement with the recent findings of Wilschut and co-workers [32]. The R18 fusion assay in their experience gives a fusion efficacy value which is significantly lower than that obtained in the pyrene-PC test. The distribution of the membrane-bound marker R18, moreover, is reported not to be homogeneous in the studied membranes. All this affects the relative accuracy of R18 fusion assay; definitive data calibration is thus difficult, if not impossible.

Different bilayer fusion assays have been described in the literature (for a review, see [7]). Many of them rely on the detection of vesicle contents mixing. Such fusion assays are of little use for the investigations of DPPC/ELA-COOH (1:2) fusion: the high permeability of such mixed lipid membranes at $T_m \simeq T_h$, as determined by the calcein leakage measurements [33], is the reason for this. (A detailed report on this will be given separately.) Such assays are also inappropriate for studying membrane fusion, however, as they bring the danger of misinterpretation. Vesicle leakage during the fusion process, for example, precludes an efficient mixing vesicle contents. Such mixing therefore cannot be used as a hallmark of membrane fusion. In other words: an absence of mixing between the aqueous compartments of vesicles does not signal the lack of *bilayer* fusion.

In summary, we have shown that properly designed thermolabile complex lipid vesicles are strongly fusogenic near the chain-melting phase transition temperature of the phospholipid component (T_m(PC)) if the bilayer-to-nonbilayer transition temperature (T_h) of the mixture is not too far above this temperature. We have presented the data on the pH-dependence of this fusogenicity, for one particularly interesting mixed lipid system; stoichiometric 1:2 dipalmitoylphosphatidylcholine/elaidic acid mixtures were used as an example for this. The fusion optimum for all such phosphatidylcholine/fatty acid mixed membranes is below pH 6 and near and above $T = 42$°C. This latter temperature coincides with the T_m(DPPC) = 41.5°C and is just below $T_h = 46$°C.

The proximity of the transition into a non-lamellar phase is a sufficient but not the necessary condition for the onset of membrane fusion. In our experience, the existence of nonlamellar membrane domains is not required for the induction of membrane fusion, notwithstanding the fact that at $T = T_h$ all adjacent membranes will fuse with each other. (If such non-bilayer domains were necessarily involved in the process of fusion, the efficiency as well as the rate of fusion would both strongly depend on the similarity of T_m- and T_h-values.) Comparison of our data measured with DPPC/PA and with DPPC/El-COOH (1:2) mixtures proves this assumption to be wrong, however. The chief effect of the vicinity of a bilayer-to-nonbilayer phase transition temperature is thus to affect the rate of membrane aggregation and fusion. The fusion efficacy, however, crucially depends on the local density of membrane defects. The latter must be sufficiently high for a successful membrane fusion. Membrane unification may thus be concluded to be a rather local and 'messy' process, which probably involves reorganization of only a small number of the lipid molecules at a time.

Our data elucidate the membrane fusion process in artificial lipid bilayers and, moreover, provide a basis for the future design of the fusogenic drug carriers for the therapeutic applications in vivo.

Acknowledgements

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[5] The 10-fold vesicle size increase is largely a consequence of the temperature dependent membrane fusion. This can be seen from the fact that the vesicles incubated at pH 3.9 and 37°C do not change in size much and thus probably do not undergo fusion.

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