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MORPHOLOGICAL AND THERMAL PROPERTIES OF VESICULAR PHOSPHOLIPID GELS STUDIED BY DSC, RHEOMETRY AND ELECTRON MICROSCOPY

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Abstract

Semisolid phospholipid preparations have been well known for several years and are still investigated as drug carrier systems, e.g. for potential cancer therapy. They may be applied parenterally as semisolid vesicular phospholipid gels suitable as implants for sustained drug release or as liposomal preparations after redisperging the stable storage form. Due to enhanced stability, mixtures of hydrated phospholipids and cholesterol are more suitable than natural unsaturated phospholipids. In order to describe characteristics of vesicular phospholipid gels, only a few techniques may be useful. Especially the structure of the semisolid preparation is not yet completely understood. We tried to get some more information about these systems by using a combination of freeze-fracture electron microscopy, differential scanning calorimetry and rheometry to elucidate, on the one hand, the inner structure or homogeneity and, on the other, the thermotropic phase transition of the three-dimensional lipid network and the temperature dependency of the fluidity/viscosity of the samples.

Using freeze-fracture electron microscopy we found coexisting phospholipid domains of lamellar sheets and vesicular structures. With the help of differential scanning calorimetry the reasons for the different phase behaviour were elucidated. Rheometric measurements show increased intermediate viscosity at the thermotropic phase transition of the lipid bilayers, possibly induced by interacting membrane defects.

Keywords: DSC, freeze-fracture electron microscopy, rheometry, vesicular phospholipid gel

Introduction

Drug carrier systems containing phospholipid (PL) are investigated mainly for their suitability with less side effects of already known drugs [1] especially as anticancer drug formulations [2–5], which have to fullfill particular toxicological and technological requirements such as sterility or stability. The demand for those systems is very high and vesicular phospholipid gels (VPG) fulfill these requirements to a wide extent in contrast to other investigated liposomal dispersions for parenteral application.

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VPG today are used as a suitable storage form of liposomes to minimize the decrease of encapsulation efficiency during storage or as implants with controlled release properties. Furthermore, the needed drug amount for high encapsulation can be reduced by 'passive-loading'of the VPG under suitable conditions [6]. Up to date, VPG have been developed as carriers of drugs for cancer treatment, such as gemcitabine, vincristine or carboplatine [7, 8].

However, there are still some open questions about their morphology and the chemical and structural alterations during storage, depending on the storage conditions [9, 10]. Furthermore, the alterations of these semisolid systems under thermal stress (simulating sterilization procedures) are of high interest.

The best known VPG systems consist of soy or egg lecithin which show insufficient chemical stability due to their unsaturated fatty acids. We used now synthetic phospholipids and fully hydrated egg phosphatidylcholin of defined high purity (at least 98% phosphatidylcholin) to optimize the homogeneity, stability and morphology of the VPG.

Generally only a few techniques are suitable to get a better insight into the supermolecular structures and alterations of these semisolid preparations. In order to get some more information about VPG concerning stability aspects or parenteral implantability we used differential scanning calorimetry (DSC) and freeze-fracture electron microscopy (FFEM) of VPG prepared by high pressure homogenization.

Furthermore, we compared the data of phase transition temperatures obtained by DSC with rheological measurements in the oscillation mode (OSC) which were temperature-controlled as well. This method was used to correlate the thermotropic behaviour, e.g. phase transition temperature, with the superstructural effects.

Experimental

Material and methods

Several species of highly purified phospholipid were used for preparation of VPG: *L*-α-dipalmitoyl-sn-glycero-3-phosphocholin (DPPC, Sygena Ltd., CH-Liestal), *L*-α-dimyristoyl-sn-glycero-3-phosphocholin (DMPC, Sygena Ltd., CH-Liestal) and hydrated egg phosphatidylcholin (HEPC, Lipoid GmbH, D-Ludwigshafen). Mixtures with cholesterol that was crystallized from methanol (Merck KgA, D-Darmstadt) in a molar ratio of 5.5:4.5 (PL:cholesterol) were prepared of HEPC. The gels were prepared without gelifying agent using TRIS/maleic acid buffer (20 mmol) pH 6.5 and lipid. The buffer contained 10% (mass/mass) glycerol and the total lipid content of each sample was equivalent to 40% (mass/mass). After at least two hours of magnetic stirring and swelling at room temperature the already semisolid mass of approximately 40 g was ten times high pressure homogenized at 70 MPa in a discontinuously working apparatus (APV Gaulin Micron Lab 40, D-Lübeck) [11, 12].

When cholesterol was used, phospholipid and cholesterol were first dissolved in organic medium, then the solvent was removed under vacuum yielding a homogeneous lipid film. After adding buffer, glycerol and glassbeads the sample was stirred.

When using saturated or synthetic phospholipids like DMPC and DPPC the homogenizer was thermostated 40°C (DMPC) and 60°C (DPPC and HEPC) and the samples were tempered up to these temperatures as well before homogenization.

Rheometry was performed in the oscillation mode with a plate/plate geometry of 25 mm in diameter and a slit of 0.5 mm (Rheostress 100, Haake, D-Karlsruhe). Performing stress-sweeps, the shear rate was determined first individually depending on the phospholipid species. Frequency was 1 Hz. For each measurement, a sample volume of 0.2 mL was necessary to fill the slit. Before starting the measurement, a recovery time of 3 min was applied after the slit size was reached. The recovery time was unavoidable in order to relax the prestressed sample. Cooling and heating was controlled between 0 and 70°C by a Peltier element (TC 81, Haake, D-Karlsruhe). A solvent trap was used to avoid the loss of water during the measuring time. Due to an optimized heating rate of 1°C min^{-1}, this effect was minimized.

For DSC an instrument was used which works with the heat-flow principle and which was cooled by nitrogen (PL-DSC Model 12000, PL Thermal Sciences Ltd., UK-Epsom). The heating rate was 5 K min^{-1}. A sample volume between 5 to 10 mg was weighted and sealed in aluminium pans. At least 4 to 7 separate samples of each VPG were measured. The instrument was calibrated with a sapphire. The temperature was calibrated with naphthalin and CCl_4 with melting points at 80.28 and –22.8°C, respectively. Calibration was performed every 24 h after burning out the oven up to 500°C.

For calculating the phase transition point both the onset and the peak maximum were observed. The parameters were determined manually or automatically by the software. The onset is less dependent on sample parameters like mass or homogeneity which may be an advantage in some cases.

Samples for freeze-fracture electron microscopy were fixed under liquid nitrogen using the sandwich technique on fine meshed gold grids between two copper layers [13, 14]. They were shock frozen in liquid ethane at 90 K and transferred in a BAF 301 (Balzers AG, FL-Balzers) at 173 K. Then they were fractured and covered first with platinum/coal under an angle of the oven position of 45°. Immediately in a second step the fractured sample was covered only with coal at 90°. After removing the rest of lipid from the replicas by washing them with water/ethanol and chloroform, micrographs were obtained using a transmission electron microscope (Zeiss 912, Leo Elekronenoptik, D-Oberkochen).

Results and discussion

DSC

VPG volumes between 5 to 10 mg were sealed in aluminium pans. Synthetic PL, saturated natural PL with or without cholesterol were investigated. Depending on the type of PL different temperature ramps were used and the measurements were performed using a heating rate of 5 K min^{-1}.

Comparing literature data with our experiments, it is necessary to be aware of the different principles of measurement. Literature data determined of phospholipid

(PL) dispersions were measured using an energy compensating principle with an adiabatic instrument. Here, the maximum point of phase transition temperature T_m marks the point of maximal rate of reaction. Using an instrument based on the heat-flow principle, T_m marks the point where the reaction is terminated. Moreover, we had to use a dynamic instrument since it is not possible to pour the VPG in the very small sample cell of an adiabatic instrument without destroying the gel stucture and without producing air bubbles in the cell. Furthermore, only diluted PL dispersions are measured in an extremely sensitive adiabatic apparatus and the PL content of the VPG samples would be too high.

In Fig. 1, each two independent runs of lyophilized (lyo) DMPC and VPG of the same compound were compared. Surprisingly, despite of excess of buffer (60% buffer corresponds to a molar lipid/buffer ratio of 1:65), the decrease of the phase transition temperature from 57°C for unhydrated to fully hydrated DMPC was less than expected for DMPC vesicles with 24°C [15]. We found an onset of heat flow increase at 27.3 and a main peak at 31.6 followed by a high-temperature shoulder about 34.9°C (Table 1).

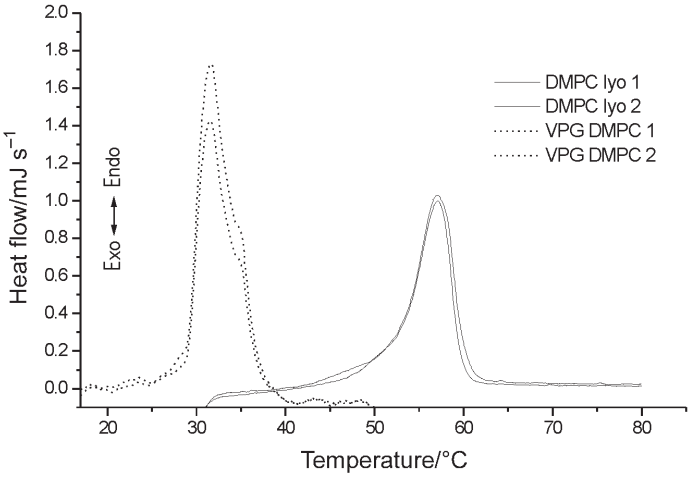

Fig. 1 Shift of phase transitions of dry PL DMPC (lyo) and VPG DMPC

In contrast to DMPC, phase transition temperature of DPPC was characterized by pre- and main transition. Phase changes started at 35 and resulted in a shoulder at 42°C (pretransition). The main transition was characterized by an onset at 42°C that corresponded exactly to peak maximum values of PL dispersions in literature. The peak maximum of VPG was found at 45°C. A shift of about 3°C in VPG compared to the peak maximum value for DPPC in vesicular form was remarkable.

VPG of HEPC showed one peak with a maximum at 51.7 and the onset at 48°C. The value of vesicular dispersions in literature is 48.6°C [15]. In this case again a shift of nearly 3°C was the difference between peak maxima of vesicular dispersion and semisolid VPG.

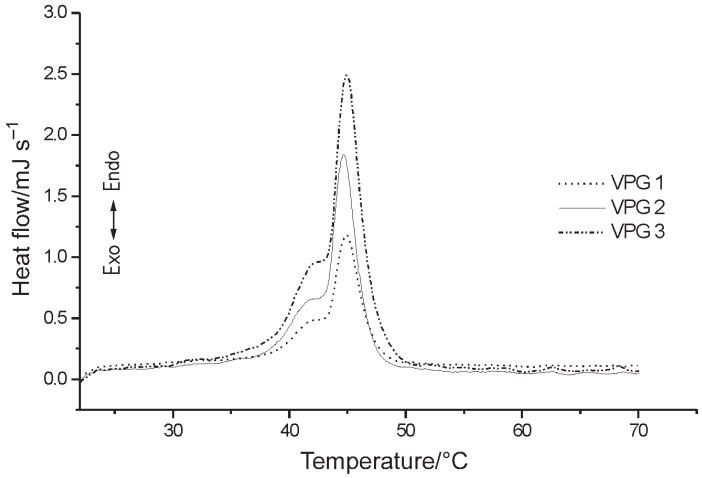

Fig. 2 Three samples of different mass of VPG DPPC showing broad pre- and main phase transitions

Samples containing 45 mol% cholesterol did not show any phase transition. This phenomenon is well known [16–18]. This effect of disturbing the gel phase and condensing the liquid crystal phase becomes obvious at a cholesterol content of 20 mol% and results in a flatening of the peak maximum until it disappears at 45 mol% cholesterol.

Table 1 T_m, s. d. and $\bar{x}$ calculated from peak maxima and onsets of the main peak (°C)

Sample	$\bar{x}$S/$\bar{x}$ peak$_{max}$	σ_{n-1}	$\bar{x}$ onset	Ramp/°C
DMPC (lyo), *n*=2	57.3	–	52.1	30–80
DMPC	(34.9 S) 31.6	0.21 (0.16 S)	27.3	10–50
DPPC	(44.8 S) 42.0	0.04 (0.09 S)	41.5	20–70
DMPC/DPPC (1:1 mol/mol)	32.6	0.09	29.3	10–60
HEPC	51.7	0.23	49.0	20–60
HEPC/Chol. (5.5:4.5 mol/mol)	–	–	–	20–200

n=4; S=Shoulder

Rheometry

The complex viscosity η^* which describes the total strengths *vs*. the dynamic shear stress, was measured over a suitable temperature range. η^* is composed of η' and η'' which are values for the viscous part and the elastic part of a semisolid system. Elasticity may be used as value for the part of energy that is stored by a system during structural changes by influence of shear stress. Depending on time these changes may be reversible under suit-

able conditions and consumption of the stored energy. Viscosity represents the part of energy which contributes to a structural change in the sample and which is lost then. Thus, an increase of the total strengths corresponds to an increase of elasticity. A decrease gives a hint for an increase of viscosity that means a destruction of the original structure, in case of the VPG the three-dimensional vesicular structure.

In the case of VPG of DPPC, η^* decreased constantly with increasing temperature until a first minimum was reached. At this point it increased again to a maximum from where it fell down to a final value (Fig. 3).

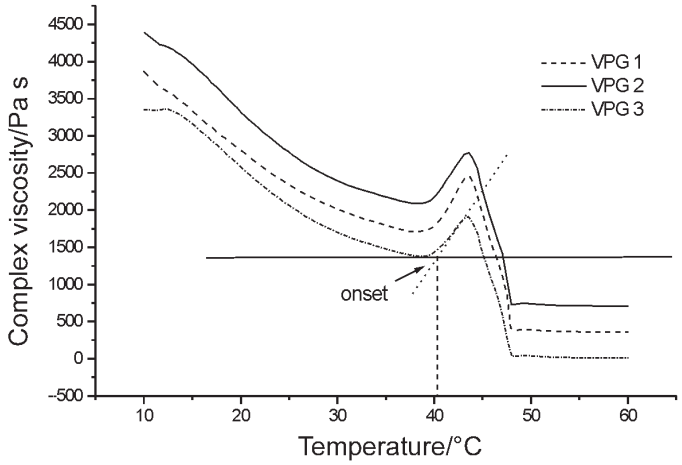

Fig. 3 Determination of phase transition temperature by following the complex viscosity of VPG DPPC

Obviously the viscoelastic character in the gel state decreased due to increased fluidity in the membrane bilayers and in the VPG structure. At the onset of phase transition, membrane defect formation probably led to stronger interaction between membranes and to an increase of viscosity in the temperature range of phase transition, which coincided very well with the data obtained by DSC in case of DPPC. For the other lipids, T_m values obtained by rheometry were 3–5°C lower compared to DSC indicating that superstructural alterations upon mechanical stress were more sensitive to temperature changes than fluidization of the membrane bilayers.

In contrast to DPPC, VPG of DMPC showed a different behaviour which could be observed only within 48 h after preparation. Regarding the gel state range below 15°C, no changes were visible. Two peak maxima were found, the first one near the main transition temperature of 24°C and a second one between 30 and 31°C (Fig. 4). Rheometry was able to define two domaines here. These two maxima could not be observed by DSC, only a weak shoulder at 35°C in the left hand tailing was remarkable (Fig. 1). In this case rheometry offered a method to indicate defect structures and PL domains very well.

Several hours later phase separation took place. A part of the VPG became fluid and opalescent at the bottom of the vials, the other one kept the white and creamy semisolid consistence on the top of the vials.

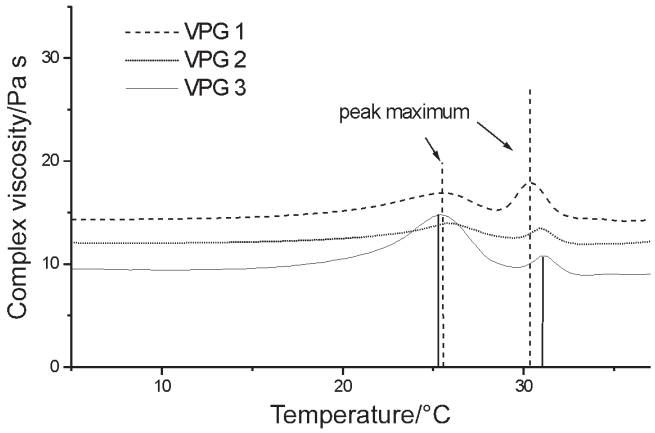

Fig. 4 Determination of phase transition temperatures of VPG DMPC via peak maximum

Rheology allowed in case of VPG of DMPC a view to quite fast inner changes concerning different structural domaines that took place a short time after the preparation. While the preparation seemed to be obviously intact before phase separation took place, physical changes were already detectable by rheometry.

Saturated natural PL did not show such extraordinary behaviour. Similar to DPPC, the fluidity increased in the gel state and after the first minimum a phase transition occurred with only one detectable peak. Using 45 mol% cholesterol, no phase changes were visible and only a baseline was recorded.

Table 2 Rheometrically determined T_m, s. d. and $\bar{x}$ of peak maxima and onsets (°C)

Sample	$\bar{x}$ $peak_{max}$ (S)	σ_{n-1} (S)	$\bar{x}$ onset	Ramp/°C
DMPC	25.7 (31.1)	0.43 (0.26)	22.1	0–40
DPPC	43.5	0.08	40.3	20–60
DMPC/DPPC (1:1 mol/mol)	27.9 (32.6)	0.16 (0.32)	23.3	0–70
HEPC	47.9	0.95	45.4	20–60
HEPC/Chol (5.5:4.5 mol/mol)	–	–	–	20–80

n=7; S=Shoulder

Determination of T_m by peak onset resulted in some lower values, by peak maximum in higher (Table 2) values in comparison to the DSC values in literature. A general shift to higher values (peak maximum) characterised the VPG prepared from pure synthetic lipids regarding the main transitions. We assume that depending on the kind of lipid the hydration of the lipid molecules is not homogeneous in the sample. Moreover

the sample volume was relatively large (0.2 mL), requiring an extending heating time, which contributed to a broadening of the transition, too.

Freeze-fracture EM

Cryo images of VPG made of natural unsaturated PL showed areas with vesicular morphology (Fig. 5). In contrast VPG made of DPPC, DMPC or mixtures thereof show vesicular structures coexisting with membrane fractures like broad lamellar lipid layers (Figs 6–7).

Vesicular areas show small vesicles of quite similar sizes. Samples containing DMPC in mixture with DPPC were characterised by domaines of ripple phases beside areas of vesicular character. The ripple phase is predicted to consist of periodic

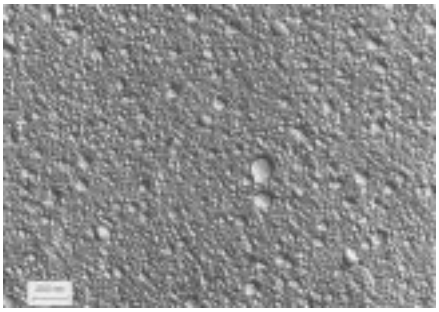

Fig. 5 Very small and uniform vesicles in the inner sheets of VPG made of E 80 40%, a natural PL that contained at least 80% egg phosphatidylcholin

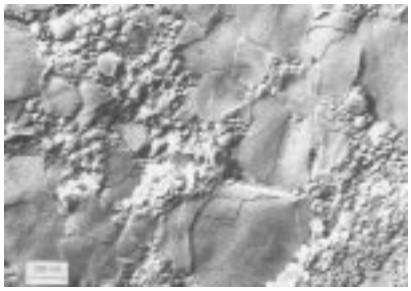

Fig. 6 Coexisting lamellar phases and vesicular structures in VPG of 40% DPPC

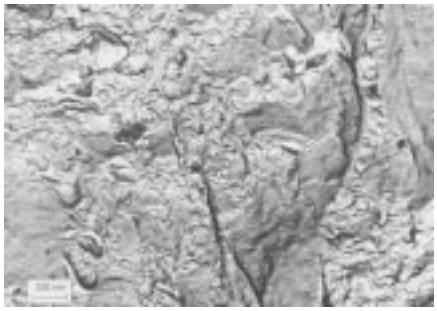

Fig. 7 Lamellar sheets seem to dominate the inner structure of VPG of DMPC/DPPC

arrangements of gel and fluid domains, chain melting may be discussed as intermedium state between gel state and liquid crystalline state [18–20]. Both types of domaines which are clearly separated in pure DPPC VPG seem to fleed into each other in mixture with DMPC. It is not clear whether different domains of lipid/lipid interactions of VPG may exist for seconds or be stable for a long time comparing to model membranes in lipid dispersions [21]. At present those questions are investigated especially via theoretical approaches, as well, e.g. Monte-Carlo simulations, which describe the nearest neighbours' interactions in lipid bilayers.

FFEM images confirmed the assumption that hydration did not take place regularly in the whole mass. Even if the molar lipid/buffer ratio of 40% (mass/mass) VPG was about 1:65 (MW phospholipid 780.0 g; water 18.0 g). Phase behaviour depending on the extent of hydration and ionic environment was already found for liquid phospholipid preparations [22–25].

Conclusions

In contrast to VPG made from natural phospholipids (PL), VPG of synthetic PL prepared by the same method are incompletely hydrated as observed in freeze-fracture images and DSC. Consequently different phases coexist in these VPG. The crystalline lamellar structure dominates in certain areas beside the vesicular morphology. We suggest that for hydrated phospholipids it is only possible to prepare VPG with high lipid concentration if cholesterol is added to a high amount. Otherwise hydration is not complete at the chosen lipid/buffer ratio. A surprising finding was that DMPC of VPG were physically not stable 48 h after preparation since phase separation took place.

The shift to higher phase transition temperatures as determined directly by DSC and indirectly by rheometry is well known from incompletely hydrated lipids. Regarding the peak maxima, T_m was shifted in a range between 3–5°C. Shoulders may indicate pretransitions or coexisting different domaines such as fully and less hydrated areas. This interpretation corresponds to the freeze-fracture images. However, the shift of T_m (DSC) may be interpreted as well by the different principles of measurement in our study and in the literature. Concerning the experiments of the current study, T_m marked the end of phase transition while other instruments determine the maximum reaction rate as T_m. The shift may additionally be due to the high PL content of semisolid preparations and to a worse energy transport in the sample, compared with vesicular PL dispersions.

We found that oscillation rheometry is a valuable alternative and supporting method to determine defect structures and special features of semisolid liposomal preparations in addition to DSC. The temperature-dependent changes of the viscoelastic properties in the VPG are very sensitive to alterations in their superstructural arrangement.

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