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DIFFERENTIAL SCANNING CALORIMETRY
A tool to assess physical and chemical alterations in liposomes

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Abstract

This study was aimed to investigate the physicochemical changes induced in 200 nm extruded oligolamellar DPPC:DPPG (10:1) liposomes by freezing, followed by γ-irradiation, in the absence and presence of 5 mM stable cyclic nitroxide radicals, 2,2,6,6-tetramethylpiperidine-1-oxyl (Tempo) and 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (Tempol). The characterization is based on the use of differential scanning calorimetry (DSC) and was aimed to differentiate the contribution of freezing and γ-irradiation in the presence and absence of nitroxides. Liposomal preparations of DPPC/DPPG which have sub-, pre- and main-phase transitions in the temperature range ($0^{\circ}C<T_m<50^{\circ}C$) were used.

Our results show that: (1) freezing modified and induced fusion to MLV as well as fission to SUV, (2) freezing did not fully prevent the radiation-induced changes in the thermotropic characteristics of the liposomes, and (3) Tempo and Tempol did not prevent the changes in thermotropic behavior caused as a result of freezing of the liposomal dispersion. These results demonstrate that DSC is a powerful and sensitive tool in both physical and chemical studies of lipid assemblies.

Keywords: DSC, γ-irradiation, liposomes, liposome freezing, oxidative damage

Introduction

Differential scanning calorimetry (DSC) serves as a sensitive and powerful tool for physicochemical characterization of liposomes. The shape of the heat capacity curve contains information about the phase transition itself, which includes both chemical and physical aspects, such as the presence of impurities, the interaction of such impurities with the lipid bilayer, or size and curvature changes of the bilayer [1–3]. This is best exemplified by calorimetric studies using dipalmitoylphosphatidylcholine (DPPC), the lipid most extensively studied calorimetrically; liposomal preparations of this lipid have their sub-, pre-, and main-phase transition in an easily accessible temperature range ($0^{\circ}C<T_m<100^{\circ}C$)

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[1]. DSC was proven to be a powerful tool, especially regarding the thermodynamics of the varying first-order phase transitions (sub-, pre-, and main-phase transition) (rev. in [1]) and the nature of the phase transition with respect to having thermodynamically stable or metastable phases involved [4–8]. DSC is also a very powerful tool to assess modes of interaction of various analytes (low or high molecular weight) with membrane lipids [1].

In previous studies we demonstrated the physical changes caused to 200 nm DPPC:DPPG (10:1) unilamellar liposomes by: (i) γ-irradiation, (ii) freezing of the samples, and (iii) γ-irradiation of frozen samples in the presence of 10% trehalose [9–11]. Freezing was chosen in order to help clarify the mechanism of lipid irradiation damage, and the different roles played by primary radicals formed upon radiolysis of water and secondary organic radicals formed in the lipidic phase. The present study was aimed to further investigate: (1) the physical changes induced in frozen samples of 200 nm DPPC:DPPG (10:1) liposomes by γ-irradiation, without the presence of the cryoprotectant trehalose and (2) the effect of 5 mM nitroxides, 2,2,6,6-tetramethylpiperidine-1-oxyl (Tempo) and 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (Tempol), on those physical changes. Nitroxides, which have been widely used as biophysical probes [12–14], have demonstrated antioxidant properties in several recent studies [15–19]. Changes in the physical characteristics of the liposomes were assessed by following changes in liposome size distribution and thermotropic behavior. In this paper we will demonstrate that DSC can serve as one of the best approaches to assess alterations in physicochemical properties of liposome imposed by chemical degradation (such as oxidative damage), physical conditions such as freezing, or by the presence of nitroxides which protect liposomes against oxidative damage [19].

Experimental

Materials

Dipalmitoylphosphatidylcholine (DPPC, >99% pure) and dipalmitoylphosphatidylglycerol (DPPG, >98% pure) were gifts from Nattermann Phospholipid GmbH (Cologne, FRG). 2,2,6,6-tetramethylpiperidine-1-oxyl (Tempo) and 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (Tempol) were supplied by Aldrich Chemical (Milwaukee, WI, USA). All the chemicals were of analytical grade. Water was doubly distilled before use.

Preparation of liposomes

Liposomes were prepared by the 'thin film hydration' method. Mixtures of the lipids were dissolved in chloroform/methanol (1:1, v/v) in a round-bottom flask, and the organic solvent was removed under vacuum by rotary evaporation. The thin film obtained was dried for at least 3 h under reduced pressure, and then hydrated with 10 mM phosphate buffer, pH 7.4, in 0.13 M NaCl (phosphate buff-

ered saline, PBS), at 10°C above the temperature at the maximum of main phase transition (T_m) with or without the presence of 5 mM nitroxide. The liposome dispersions were extruded with an extrusion system (Sartorius, Göttingen, FRG) tone time hrough 0.6 μm and three times through 0.2 μm pore size polycarbonate filters, respectively (Nuclepore, Costar Corporation, Cambridge, MA, USA). The liposomes produced by this process are oligolamellar, having on the average 2-3 bilayers per vesicle [20]. The pH of the dispersion was measured before and after extrusion and was adjusted, if necessary. The final phospholipid concentration was 22 mM. Two-milliliter aliquots of the liposome dispersions were dispensed into 10 ml vials. Part of the liposomal-dispersion-containing vials were frozen at dry-ice temperature (194 K).

γ-*Irradiation*

The liposome samples were irradiated with a ^{60}Co source by Gammaster B.V. (Ede, The Netherlands) at ambient temperature or at 194 K, by 32 kGy at a dose rate of 3.4 kGy/h. The absorbed dose reported here is the minimum dose. Dosimetry was done using dosimeters consisting of red Perspex, and had a precision of 5% (as stated by Gammaster, see also Marino *et al.* 1984 [21]).

Differential scanning calorimetry

Tenfold-diluted liposome dispersions were degassed while stirring under reduced pressure, and placed in the sample cell, whereas the reference cell contained the liposome buffer (10 mM phosphate buffer, pH 7.4 in 0.13 M NaCI). Calorimetric scans from 25 to 60°C were performed in triplicate on an MC-2 DSC (Microcal, Northampton, MA, USA) at a scanning rate of 0.5°C min^{-1}. The phospholipid concentration of the sample in the cell was determined by phosphate measurement of the lower phase of a Bligh and Dyer extract of the dispersion [22].

Particle size determination

The Z-average particle size and polydispersity index (*p.d.*) at 25°C were determined by photon correlation spectroscopy (PCS) with a Malvern 4700 system using a 25 mW He-Ne laser (NEC, Tokyo, Japan) and the automeasure version 3.2 software (Malvern Ltd, Malvern, UK). The *p.d.* is a measure of the width of the particle size distribution and ranges from 0.0 for an entirely homogeneous size distribution, up to 1.0 for a completely heterogeneous one. For viscosity and refractive index the values of pure water were used

Electron paramagnetic resonance (EPR)

EPR spectrometry was employed to analyze and quantify the nitroxide free radicals, using a JES-RE3X ESR Spectrometer (JEOL, Tokyo, Japan). Samples were drawn by a micro-pipette into a gas-permeable Teflon capillary of 0.81 mm

inner diameter, 0.05 mm wall thickness, and 15 cm length (Zeus Industrial Products, Raritan, NJ, USA). Each capillary was folded twice, inserted into a 2.5 mm ID quartz tube open at both ends, and placed in the EPR cavity. EPR spectra were recorded with center field set at 336.1 mT, 100 kHz modulation frequency, 0.1 mT modulation amplitude, and non-saturating microwave power.

General

All studies were performed under an air atmosphere at ambient temperature.

Results

Changes in physical parameters of liposomes

The changes in the physical characteristics of the liposomes induced by γ-irradiation were assessed by following liposome size distribution and thermotropic behavior.

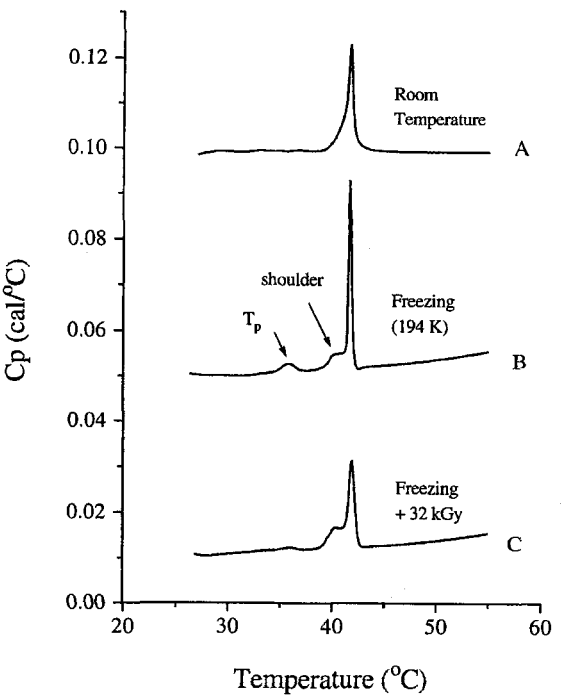

Fig. 1 The effect of freezing and irradiation on the thermotropic characteristics of DPPC:DPPG liposomes. DSC-scans of: (A) DPPC:DPPG 10:1 liposomal dispersions, (B) DPPC:DPPG 10:1 liposomal dispersions frozen at 194 K, and (C) DPPC:DPPG 10:1 liposomal dispersions frozen at 194 K and irradiated with a dose of 32 kGy

Size

The average size (and polydispersity) of extruded liposomes (see Materials and Methods) was 180 nm (*p.d.* ≈0.1) for 22 mM DPPC:DPPG 10:1 liposomes. Neither the presence of nitroxide, nor exposure to γ-irradiation had any effect on liposome size and polydispersity. Freezing, by cooling to 194 K, caused an increase in liposome size to 300–600 nm and polydispersity to 0.3–0.4.

Thermotropic behavior of liposomal bilayers

The DPPC:DPPG (10:1) liposomes demonstrated a main-phase transition at T_m=41.6±0.1°C and a pre-transition at T_p=36.2±0.9°C. The changes in their thermotropic characteristics as a result of γ-irradiation of non-frozen samples, in the presence and absence of nitroxides (partly studied in our previous work [9]) are summarized in Table 1. Freezing of these liposomes induced changes in the main-phase transition endotherm, introducing a 2–2.5°C wide 'shoulder' at $T<T_m$, and causing a significant increase of the hardly detectable T_p (charac-

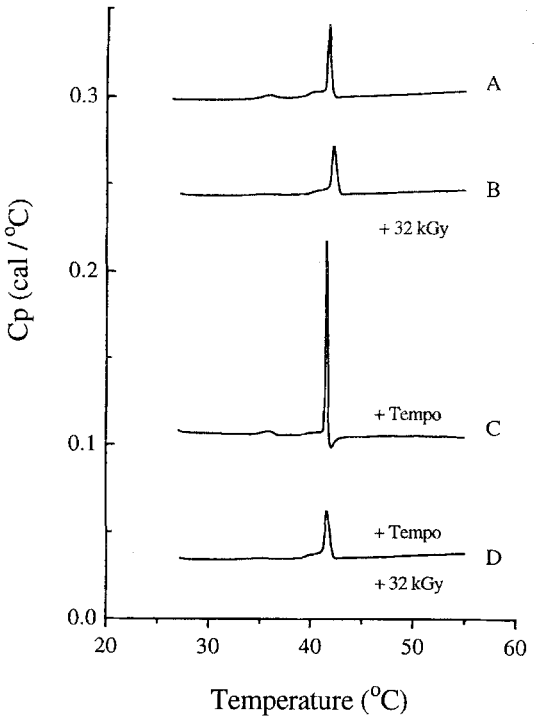

Fig. 2 The effect of freezing and Tempo on the thermotorpic characteristics of DPPC:DPPG liposomes. DSC-scans of DPPC:DPPG 10:1 liposomal dispersions, frozen at 194 K, before (A,C) and after (B,D) irradiation with a dose of 32 kGy, in the presence or absence of 5 mM Tempo

Table 1 Thermotropic characterization of irradiated and non-irradiated DPPC:DPPG (20 mM:2 mM) liposomes, in the presence and absence of Tempo and Tempol

Sample (room temperature)	Radiation dose	T_p/°C	T_m/°C	$\Delta T_{m1/2}$/°C	T_{on}/°C	ΔH_m/kcal mol^{-1}
DPPC:DPPG 10:1	0	36.17±0.9	41.62±0.05	0.36±0.12	40.06±0.10	7.88±0.66
DPPC:DPPG 10:1	32 kGy	ND	42.14±0.10	1.80±0.12	40.49±0.14	8.89±0.70
DPPC:DPPG 10:1 +5 mM Tempo	0	35.53±0.48	41.51±0.04	0.47±0.20	39.35±0.55	8.45±0.99
DPPC:DPPG 10:1 +5 mM Tempo	32 kGy	ND	41.46±0.06	2.09±0.15	38.50±0.07	8.77±0.51
DPPC:DPPG 10:1 +5 mM Tempol	0	35.81±0.18	41.58	0.46	39.88	8.13
DPPC:DPPG 10:1 +5 mM Tempol	32 kGy	ND	41.9	1.71	39.88	8.53

Table 2 Thermotropic characterization of irradiated and non-irradiated DPPC:DPPG (20 mM:2 mM) liposomes, cooled to 194 K, in the presence and absence of Tempo and Tempol

Sample (frozen at 194 K)	Radiation dose	T_m/°C	$\Delta T_{m1/2}$/°C	T_p/°C	ΔH_p/kcal mol^{-1}
DPPC:DPPG 10:1	0	41.67±0.01	0.33±0.01	35.65±0.15	0.84±0.42
DPPC:DPPG 10:1	32 kGy	41.93±0.02	0.67	36.26±0.26	0.4
DPPC:DPPG 10:1 +5 mM Tempo	0	41.52±0.01	0.24±0.01	35.72±0.27	1.71
DPPC:DPPG 10:1 +5 mM Tempo	32 kGy	41.58±0.04	0.5	35.16±0.05	0.64±0.19
DPPC:DPPG 10:1 +5 mM Tempol	0	41.66±0.04	0.29±0.04	35.84±0.13	1.11
DPPC:DPPG 10:1 +5 mM Tempol	32 kGy	41.63±0.01	0.39±0.04	34.80±0.07	0.8

teristic of DPPC MLV at 35°C), as compared with that of the non-frozen samples (Fig. 1). No chemical degradation of the phospholipids occurred as a result of freezing alone. γ-irradiation of frozen DPPC:DPPG (10:1) liposomes, alone or in the presence of 5 mM Tempo or Tempol, resulted in ≈50% decrease of the pre-transition's ΔH, and a broadening of the main-phase transition. The main-phase transition broadening is indicated by an increase of $\Delta T_{1/2}$ from 0.28±0.05°C to 0.51±0.09°C. No significant change in T_m or T_{on}, the temperature at the onset of main-phase transition, was found as a result of irradiation of frozen samples (Fig. 2, Table 2). The appearance of a shoulder at T≈39°C in the DSC scans of all frozen samples indicates the presence of small vesicles, formed as a result of the freezing and thawing of the liposomes [1, 3].

Degradation of nitroxides

To determine the residual fraction of the nitroxides upon irradiation, EPR spectra of irradiated and non-irradiated buffer solutions and liposomal dispersions containing Tempo and Tempol were compared. Irradiation of nitroxides in a frozen solution at 194 K in the absence and in the presence of liposomes practically did not reduce their concentration.

Discussion

The endotherms describing the pre- and main-phase transitions are good indicators of the quality of the liposomal lipid and of the presence of a bilayer interacting compound [1, 23]. Size changes are caused upon freezing of liposomes or upon formation of products inducing aggregation and/or fusion [23]. In the present study, size changes were seen as a result of freezing, both in irradiated and non-irradiated samples, in the presence or absence of nitroxide. This result indicates that although nitroxides inhibited radiation-induced chemical changes in liposomal lipids, they did not prevent freezing-induced size changes in the liposomes.

When DPPC:DPPG (10:1) liposomes were irradiated in a frozen state, their melting properties did change (although to a lesser extent). The main change appeared in a broadening of the phase transition temperature range ($\Delta T_{m1/2}$), while T_m, showed no significant change. The nitroxide did not affect the thermotropic behavior of either the irradiated or non-irradiated frozen liposomes (Fig. 1). Since the thermotropic characteristics are influenced by both the chemical composition of the liposomes and their physical state (especially vesicle size, curvature, and number of lamellae), these changes in the melting properties can be explained mostly by the physical changes caused during the freezing procedure, and not due to lipid or nitroxide degradation products. The increase in ΔH_p indicates formation of MLV [1], a finding correlated with the increase in liposome

size found upon our PCS measurements, probably due to freezing-induced fusion [2]. On the other hand, the shoulder at 39°C suggests that SUV are also formed, a fact undetected by PCS measurements. The presence of nitroxide did not affect ΔH_p, or prevent the decrease in ΔH_p upon γ-irradiation, from 1.36±0.31 to 0.65± 0.25 kcal mol^{-1}.

The change in $\Delta T_{m1/2}$ and T_{on} indicates a decrease in cooperativity of the transition, which can be caused by 'contamination' of the sample with even minor amounts of lipid degradation products, and/or degradation products of the nitroxides themselves. We could not show by HPLC a decrease in total phospholipid upon γ-irradiation [9]. This also demonstrates the sensitivity of DSC and its ability to monitor minor alterations in the liposomes. The degradation products of the nitroxides may have different, possibly greater, partition coefficients into the bilayer than the nitroxides themselves, thus having a greater effect

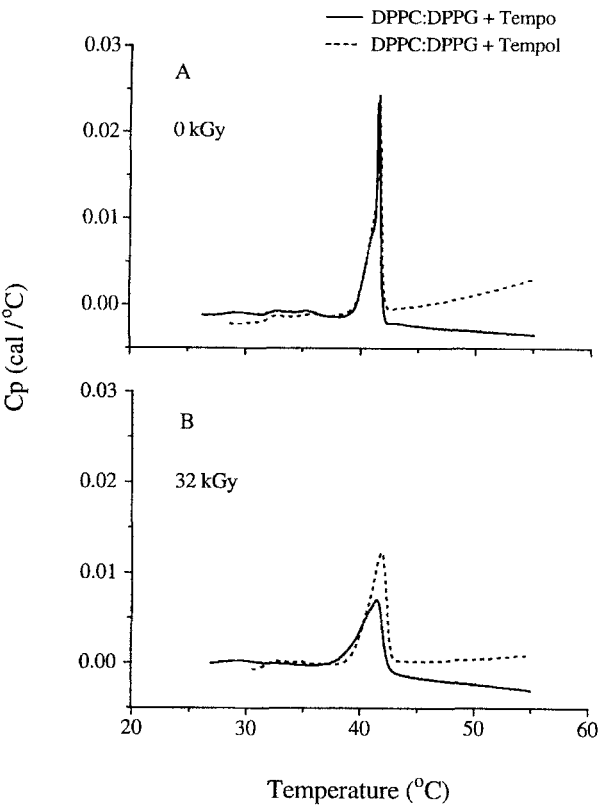

Fig. 3 Effect of Tempo and Tempol on the thermotropic characteristics of DPPC:DPPG liposomes. DSC-scans of DPPC:DPPG 10:1 liposomal dispersions before (A) and after (B) irradiation by 32 kGy at room temperature in the presence of 5 mM Tempo or Tempol

on the thermotropic behavior, and causing greater asymmetry in the main transition peaks (Fig. 3, based on results from our previous work [9]). These facts might indicate also that these degradation products are more soluble in the liquid-crystalline (fluidus) phase than in the gel (solidus) phase [1]. When comparing irradiated and non-irradiated nitroxide-containing samples, it can also be seen that in the non-irradiated samples Tempo and Tempol similarly affected the asymmetry in the main transition (Fig. 3). This similar effect was found despite the fact that Tempo has a higher partition coefficient into the bilayer. On the other hand, in the irradiated samples, Tempo induced greater asymmetry to the endotherm. Possibly the degradation products of Tempo are more soluble in the liquid-crystalline phase than those of Tempol. It is also possible that because Tempo is more hydrophobic and embedded deeper in the lipid bilayer, it has a greater impact on the cooperativity of the phase transition.

Conclusions

1. Freezing modified the vesicles' physical state, as demonstrated by size and DSC measurements. The DSC measurements demonstrate that formation of MLV as well as SUV were induced by freezing. The PCS size measurements, due to limited capability to observe low levels of small particles in a population of larger particles, is unable to show the formation of small particles. This result indicates that freezing causes fusion (leading to MLV formation [1, 2]) as well as fission of either the LUV or MLV to form smaller vesicles with larger curvature [3].

2. Freezing did not fully prevent the radiation-induced changes in the thermotropic characteristics of the liposomes.

3. Tempo and Tempol did not prevent the radiation-induced changes in $\Delta T_{m1/2}$ or the changes in thermotropic behavior caused as a result of freezing of the liposomal dispersion.

List of abbreviations

DPPC	dipalmitoylphosphatidylcholine
DPPG	dipalmitoylphosphatidylglycerol
DSC	differential scanning calorimetry
DSC terminology:	
T_m	main thermal transition temperature, the temperature at the maximum of the main-phase transition
T_p	pre-transition temperature
ΔH_m	enthalpy change associated with the main-phase transition
ΔH_p	enthalpy change associated with the pre-transition

$\Delta T_{m1/2}$	ΔT at half the height of the main-phase transition
T_{on}	the temperature at the onset of main-phase transition
EPR	electron paramagnetic resonance
LPO	lipid peroxidation
PCS	photon correlation spectroscopy
Tempo	2,2,6,6-tetramethylpiperidine-1-oxyl
Tempol	4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl

* * *

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