

Ultrasonic Spectroscopy and Differential Scanning Calorimetry of Liposomal-Encapsulated Nisin

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The thermal stability of phosphatidylcholine (PC) liposomes (colloidal dispersions of bilayer-forming polar lipids in aqueous solvents) in the presence and absence of the antimicrobial polypeptide nisin was evaluated using differential scanning calorimetry (DSC) and low-intensity ultrasonic spectroscopy (US). PC liposome mixtures with varying acyl chain lengths (C16:0 and C18:0) were formed in buffer with or without entrapped nisin. Gel-to-liquid crystalline phase transition temperatures (T_M) of liposomes determined from DSC thermograms were in excellent agreement with those determined by ultrasonic velocity and attenuation coefficient measurements recorded at 5 MHz. The dipalmitoylphosphatidylcholine (DPPC) T_M measured by DSC was ~ 41.3 and ~ 40.7 °C when measured by ultrasonic spectroscopy. The T_M of distearoylphosphatidylcholine (DSPC) and DPPC/DSPC 1:1 liposomes was 54.3 and 54.9 °C and ~ 44.8 and ~ 47.3 °C when measured by DSC and US, respectively. The thermotropic stability generally increased upon addition of nisin. Analysis of the stepwise decrease in ultrasonic velocity with temperature indicated an increased compressibility corresponding to a loss of structure upon heating.

KEYWORDS: Liposomes; vesicles; ultrasound; calorimetry; entrapment; spectroscopy

INTRODUCTION

Liposomes, spherical bilayer vesicles formed by dispersion of polar lipids in aqueous solvents, have been widely studied for their ability to act as drug delivery vehicles by shielding reactive or sensitive compounds prior to release (1–4). Liposome entrapment has been shown to stabilize encapsulated bioactive materials against a range of environmental and chemical changes, including enzymatic and chemical modification, as well as protecting against extreme pH, temperature, and high ion concentrations. Liposomes have been especially useful to researchers in studies of physiological processes as models of biological membranes in both eukaryotes and prokaryotes. Industrial applications include encapsulation of pharmaceuticals and therapeutics, cosmetics, and anticancer and gene therapy drugs. In the food industry, liposomes have been used to deliver food flavors and nutrients (5, 6). More recently, liposomes have been investigated for their ability to incorporate food antimicrobials, such as the polypeptide antimicrobial nisin, that could

aid in the protection of food products against growth of spoilage and pathogenic microorganisms (7–10).

The functional properties of liposomes depend on their size, composition, and stability in food systems (11). Liposomes are typically spherical in shape and may consist of single or multiple bilayers composed from amphiphilic molecules such as polar lipids (12, 13). Their sizes can vary widely, anywhere from ~ 40 – 50 nm up to ~ 1 – 3 μm , depending on the molecular properties of the lipid molecules, environmental conditions, and method of formulation (14–19). Of particular importance for food applications is their ability to withstand thermal stresses that may be encountered during processing, packaging, or storage. While a large number of analytical techniques have been used to characterize the structural and functional properties of liposomes and investigate their responses to various stimulants and stressors, differential scanning calorimetry (DSC) has proven to be one of the most powerful methods to analyze macroscopic physicochemical and thermodynamic properties of liposomes (20–23). The technique has been widely used to describe liposomes of all types and has led to the creation of common databases such as LIPIDAT that contains comprehensive information on lipid mesomorphic and polymorphic transitions and miscibility (24–27).

Ultrasonic spectroscopy (US) has been introduced as a new analytical technique to characterize dispersed systems such as

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emulsions of interest to food scientists. While there exist published reports on the use of the technology to measure the properties of some food systems, it is not widely utilized by food scientists (28, 29). Low-intensity ultrasonic waves that propagate through a system containing dispersed particles are scattered, diffracted, and refracted (30). As a consequence, ultrasonic velocity and attenuation change depending on composition, physical state, microstructure, and molecular relaxation phenomena. Furthermore, US is capable of detecting temperature-dependent phase transitions similar to information obtained in a differential scanning calorimeter because the ultrasonic velocity and attenuation of a material in the solid state differ from that in the liquid state (31, 32). For example, McClements and coauthors studied the ultrasonic attenuation and velocity of hexadecane-in-water emulsions (28). Authors reported distinct changes in compressibility of the lipid droplets when the temperature was increased from 0 to 25 °C and calculated a phase transition temperature T_M of 18 °C (28). US has been utilized to determine properties of liposomes composed of phospholipids with varying degrees of unsaturation in the acyl chains and with varying sterol contents (33). Authors reported that the ultrasonic velocity increased with an increased number of double bonds in the acyl chains and sterol content and was likely a function of the packing of the polar lipids within the vesicle (33).

In this study, we investigated the thermal stability of liposomes formed from synthetic phospholipids containing either an aqueous buffer or a low concentration of nisin using both DSC and high-sensitivity US. The two analytical methods were compared for accuracy and sensitivity in determining the gel-liquid crystalline phase transition temperature (T_M) of liposomes. Finally, the influence of entrapment of nisin on the phase transition temperature of liposomes was examined. Possible explanations of observed changes in thermodynamic properties of liposomes upon entrapment of nisin are offered.

MATERIALS AND METHODS

Phospholipids and Antimicrobial. Dipalmitoylphosphatidylcholine (DPPC) and distearoylphosphatidylcholine (DSPC) were purchased from Avanti Polar Lipids (Alabaster, AL) in chloroform and stored at -20 °C until used. Nisin (CAS 1414-45-5; 2.5% w/w in sodium chloride and denatured milk solids) was obtained from Sigma-Aldrich (St. Louis, MO) and stored in a desiccator at 5 °C until used.

Liposome Formation. Multilamellar vesicle (MLV) liposomes of DPPC, DSPC, and a 1:1 mixture of DSPC and DPPC were formed according to the method of Pinnaduwa and Bruce (34). Lipids dispersed in chloroform were dried under N_2 to disperse lipids over the walls of glass reaction tubes. In the case of DSPC-DPPC liposomes, the two chloroform-dispersed phospholipids were mixed at a 1:1 ratio, vigorously shaken to ensure a homogeneous mix of the two species, and then dried under N_2 . Following chloroform evaporation, phospholipids were rehydrated in 0.1 × PBS buffer (0.017 M KH_2PO_4 , 0.05 M Na_2HPO_4 , and 1.5 M NaCl at pH 7.4) (Biowhittaker, Rockland, ME) to a concentration of 3 mg/mL and mixed vigorously by vortexing to suspend lipids in the aqueous buffer. For liposomes containing antimicrobial, nisin was solubilized in 0.1 × PBS buffer at 1 mg/mL, which was used to rehydrate the phospholipids to obtain a final nisin concentration of 0.25 mg/mL. Following MLV formation, liposomes were frozen in liquid nitrogen for ~10 s and gently thawed in water (25 °C) for ~10 s and 70 °C water for 15 s. The process was repeated four times. Liposomes were held at 70 °C for 1 h to guarantee that phospholipids were above their respective gel-liquid crystalline phase transition temperature (T_M). Liposomes were finally sonicated for 20 min in reaction tubes using an ultrasonic water bath according to the method of Were et al. (7).

DSC. Thermal analysis of liposomes was performed using a Micro-Differential Scanning Calorimeter (VP-DSC, MicroCal, Northampton,

MA). Liposomes and 0.1 × PBS were degassed with stirring under vacuum at 25 °C for 20 min using a Thermovac (MicroCal) to prevent bubble formation or attachment of bubbles to the wall of the measurement cell during the temperature scans. DSC reference and sample cells were loaded with ~0.5 mL of reference buffer (0.1 × PBS; Biowhittaker) and liposome suspension, respectively. Samples were equilibrated to 25 °C for 10 min and then heated from 25 to 75 °C at a heating rate of 0.5 °C/min. All sample scans were repeated a minimum of six times. Upon completion of scans, the DSC sample cell was thoroughly cleaned using 80 mL of 1% Dawn Ultra Concentrated detergent (Procter & Gamble, Cincinnati, OH) (warmed) and 60 mL of absolute ethanol (Sigma Chemical Co.); 100 mL volumes of Millipore water were used before, between, and after both the detergent and the ethanol to rinse the cell. The reference cell was cleaned with 100 mL of Millipore water only.

US. US analysis of MLVs was carried out using a high-resolution ultrasonic spectrometer (HR-US 102, Ultrasonic Scientific, Dublin, Ireland). Liposomes and 0.1 × PBS were degassed with stirring under vacuum at 25 °C for 20 min, and 1 mL of sample and reference buffer (0.1 × PBS; Biowhittaker) were loaded into the sample and reference cell. The temperature of both sample and reference was equilibrated to 25 °C for 10 min. After equilibration, ultrasonic peak profiles were determined to select an optimum resonance frequency at 5 MHz for subsequent ultrasonic velocity and attenuation measurements. Samples were then heated from 25 to 75 °C using a heating rate of 0.3 °C/min, and the ultrasonic velocity and attenuation were recorded. All US experiments were run in duplicate. Upon completion of scans, sample and reference cells were thoroughly cleaned using 1% Dawn solution (Procter & Gamble) and 1 N HCl (Sigma-Aldrich), followed by extensive rinsing with high-performance liquid chromatography grade water (Fisher Scientific, Fairlawn, NJ).

Data Analysis. PeakFit v. 4.1.2 (Seasolve Software Inc., Framingham, MA) was used to determine phase transitions from the peaks of plots of the ultrasonic attenuation (α) as a function of temperature and the peaks of plots of the temperature derivative of the ultrasonic velocity ($dv_{1/2}/dT$) vs temperature. Statistically significant differences in phase transition temperatures were determined with Student's *t*-test ($p < 0.05$) using SAS v. 8.0 (SAS Institute, Cary, NC).

RESULTS

Figure 1 shows the heat capacity c_p of the sample liposomes as a function of temperature both without (**Figure 1A**) and with entrapped antimicrobial (**Figure 1B**). DPPC MLVs demonstrated a phase transition of 41.2 °C; the addition of 1 mg/mL nisin increased the T_M to 41.3 °C (**Table 1**). For DSPC, the phase transition observed with the differential scanning calorimeter was 54.3 °C and did not change upon inclusion of nisin (54.3 °C). In the case of the DSPC-DPPC, liposomes with no entrapped antimicrobial had a phase transition at 44.8 °C but this shifted to 46.5 °C when nisin was encapsulated in the liposomes (**Table 1** and **Figure 1**).

Figure 2 shows the ultrasonic attenuation coefficient (α) as a function of temperature measured at a frequency of 5 MHz for liposomes with and without entrapped nisin. As the temperature increased, the baseline of the ultrasonic attenuation generally decreased. During the transition of the lipids from the gel to the liquid crystalline form, the attenuation almost tripled to reach a maximum at the midpoint temperature of the transition. Calculated phase transition temperatures of MLVs of DPPC, DSPC, and DPPC-DSPC without entrapped nisin were 40.6, 54.6, and 47.4 °C, respectively (**Table 1** and **Figure 2A**). For liposomes with entrapped antimicrobial, attenuation analysis revealed a slight increase in the T_M of vesicles with DPPC, DSPC, and DPPC-DSPC of 41.8, 55.2, and 48.1 °C, respectively (**Figure 2B**).

Figure 3 shows the temperature dependence of the ultrasonic velocity increment (i.e., the velocity measured in the samples

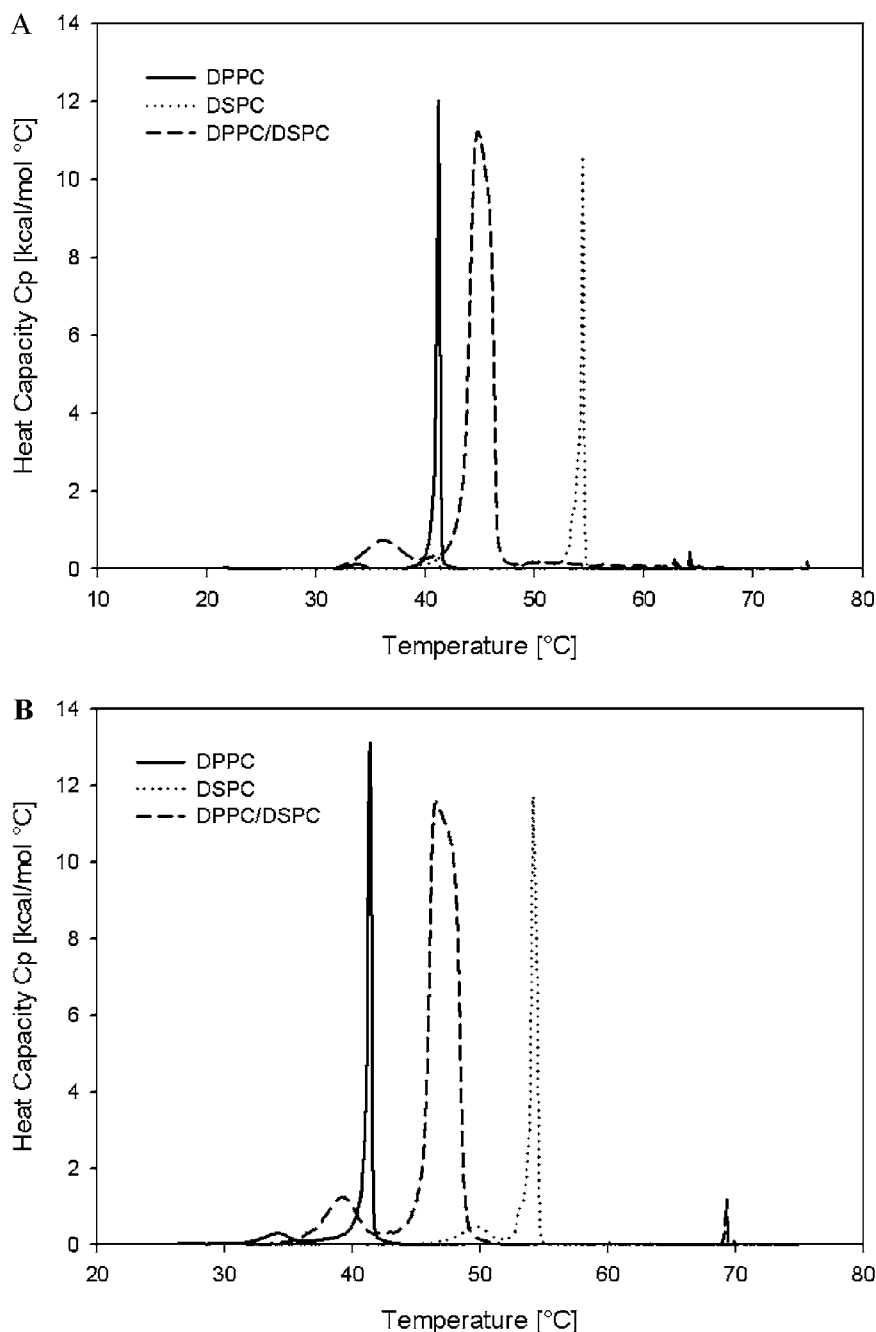


Figure 1. Heat capacity c_p in kcal/mol °C as a function of temperature of DPPC, DSPC, and DPPC: DSPC 1:1 liposomes (A) without nisin (B) containing 0.025 mg/mL nisin. Plots represent duplicate replications. Scan rate, 0.5 °C/min; sample volume, 0.5 mL; reference buffer, 0.1× PBS (0.5 mL); and liposomes composed of 3.0 mg/mL phospholipid.

minus the velocity measured in a reference cell containing 0.1× PBS buffer). The ultrasonic velocity increment generally decreased with increasing temperature. Prior to the phase transition and after the phase transition, the decrease in velocity with temperature was nearly linear; however, the slope prior to the transition was larger than the slope after the transition. During the transition of all lipids regardless of whether nisin was present or absent, the temperature-dependent ultrasonic velocity decrease had an approximately sigmoidal shape. Transition temperatures based on measurements of the ultrasonic velocity were determined from plots of the temperature derivative of the ultrasonic velocity differential as a function of temperature (Figure 4). Phase transition temperatures were 39.0, 54.3, and 44.6 °C for DSPC, DPPC, and DPPC–DSPC vesicles without nisin, respectively (Table 1). Liposomes

composed of DPPC with entrapped nisin had a T_M of 40.7 °C while liposomes composed of DSPC and DPPC–DSPC 1:1 with entrapped nisin had a T_M equal to 54.4 and 44.3 °C, respectively.

DISCUSSION

This study demonstrates the utility of US for the analysis of phase transitions induced by temperature in the presence and absence of entrapped antimicrobial polypeptides such as nisin. Statistical analysis of T_M values for the different analytical methods indicated excellent agreement between the ultrasonic velocity and the DSC measurements (Table 1). With the exception of the DPPC–nisin liposomes, transition temperatures obtained with the two methods were not statistically different. However, transition temperatures obtained from ultrasonic

Table 1. Comparative Analysis of Phase Transition Temperatures T_M of Liposomes (3.0 mg/mL) with or without Entrapped Nisin (1 mg/mL) Calculated from Results of DSC and US^a

method ^b	without nisin			with nisin		
	DPPC	DSPC	DPPC–DSPC	DPPC	DSPC	DPPC–DSPC
DSC	41.22 a	54.33 d	44.79 g	41.34 j	54.32 m	46.54 p
US- α^b	38.99 b	54.28 e	44.63 h	40.71 k	54.36 n	46.25 q
US- Δv_{12}^b	40.64 c	54.58 f	47.44 i	41.80 l	55.15 o	48.07 r

^a Values with differing superscript letters across rows, but not columns, are statistically different ($p < 0.05$). Values are means of duplicate replications.

^b Abbreviations: US- α , ultrasonic attenuation; US- Δv_{12} , ultrasonic velocity.

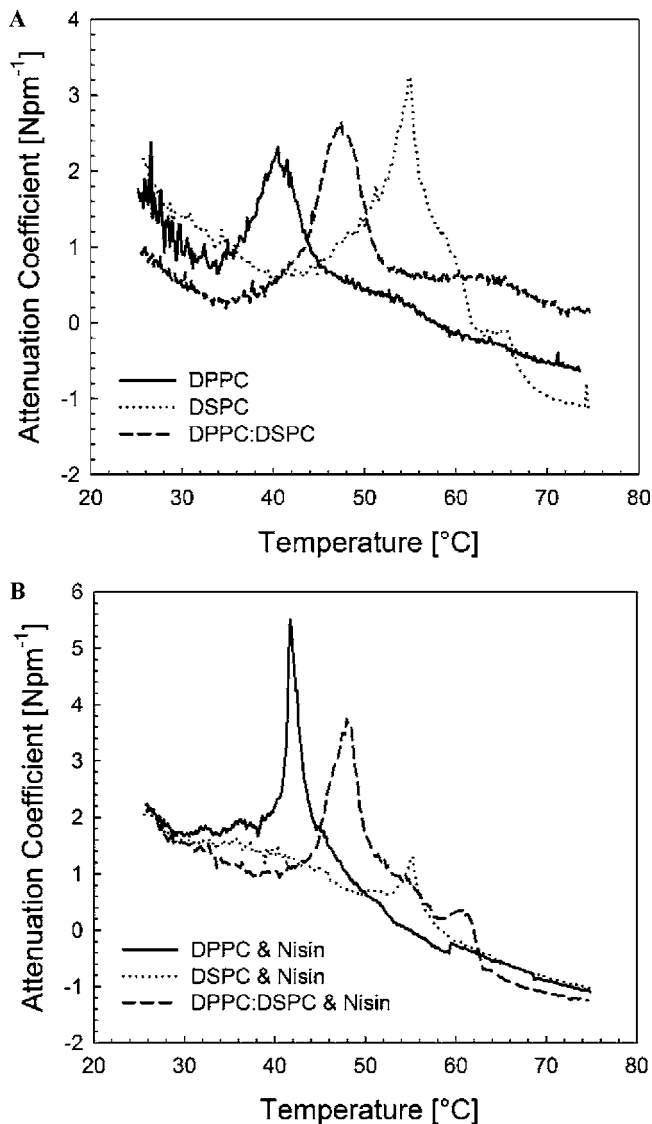


Figure 2. Ultrasonic attenuation coefficient α in Npm^{-1} measured at 5 MHz as a function of temperature of DPPC, DSPC, and DPPC:DSPC 1:1 liposomes (A) without nisin (B) containing 0.025 mg/mL nisin. Plots represent duplicate replications. Scan rate, 0.3 $^{\circ}\text{C}/\text{min}$; sample volume, 0.5 mL; reference buffer, 0.1 \times PBS (0.5 mL); and liposomes composed of 3.0 mg/mL phospholipid.

attenuation measurements, with the exception of DSPC–nisin samples, were consistently higher and statistically different in comparison to the other two methods ($p < 0.05$).

US has been shown to be capable of detecting phase transition in a variety of dispersed systems with high accuracy. For

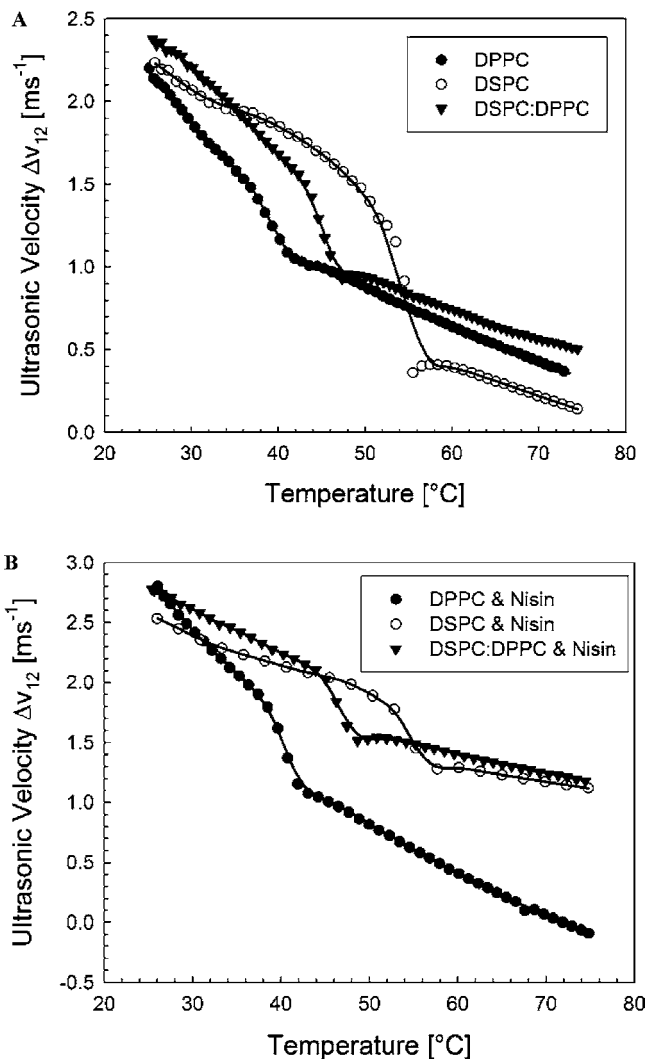


Figure 3. Ultrasonic velocity differential Δv_{12} in ms^{-1} between sample and reference cell measured at 5 MHz as a function of temperature of DPPC, DSPC, and DPPC:DSPC 1:1 liposomes (A) without nisin (B) containing 0.025 mg/mL nisin. Plots represent duplicate replications. Scan rate, 0.3 $^{\circ}\text{C}/\text{min}$; sample volume, 0.5 mL; reference buffer, 0.1 \times PBS (0.5 mL); and liposomes composed of 3.0 mg/mL phospholipid.

example, McClements and coauthors demonstrated that ultrasonic attenuation and velocity measurements can be used to follow the crystallization and melting of lipids in emulsion droplets (28) based on the fact that ultrasonic attenuation and velocity of solid and liquid phases differ greatly. The ultrasonic velocity is particularly sensitive to phase transitions that involve formation of crystalline or quasi-crystalline structures because these phase changes are accompanied by large changes in adiabatic compressibility (35).

Generally, propagation of ultrasonic waves through a system that contains dispersed particles gives rise to reflected compression, thermal, and shear waves as well as transmitted compression, thermal, and shear waves in the dispersed particle. The overall attenuation (α) by ultrasound in a sample can be divided into a molecular relaxation component (α_{MR}) and a scattering component (α_{S}):

$$\alpha = \alpha_{\text{MR}} + \alpha_{\text{S}} \quad (1)$$

Because the scattering contribution of the overall attenuation primarily depends on the size and concentration of dispersed particles, it is the molecular relaxation component that is most

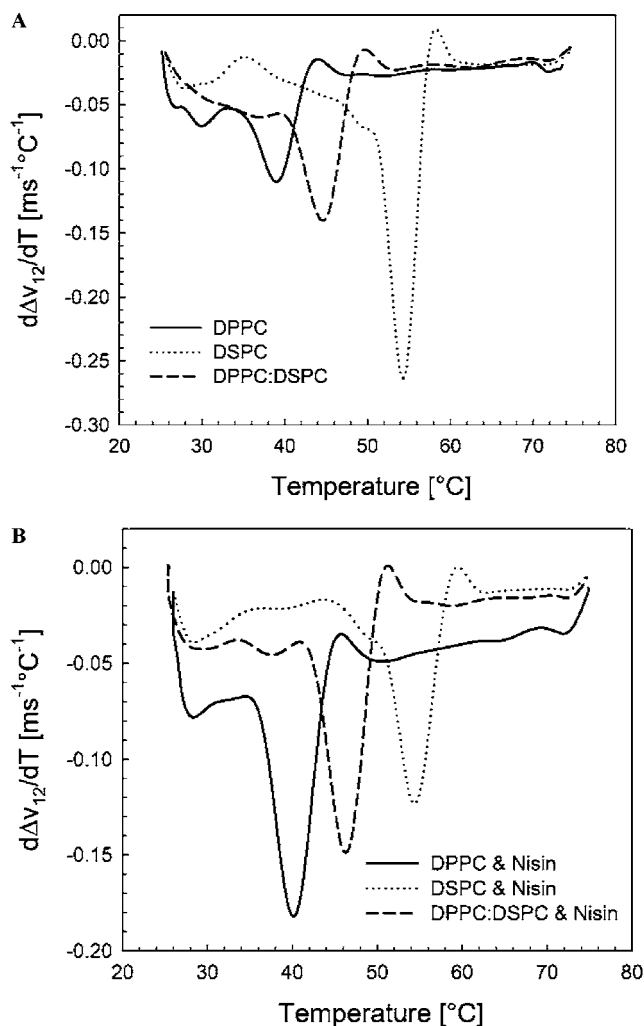


Figure 4. Temperature derivative of ultrasonic velocity differential $d\Delta v_{12}/dT$ in $\text{ms}^{-1}\text{C}^{-1}$ between sample and reference cell measured at 5 MHz as a function of temperature of DPPC, DSPC, and DPPC:DSPC 1:1 liposomes (A) without nisin (B) containing 0.025 mg/mL nisin. Plots represent duplicate replications. Scan rate, 0.3 °C/min; sample volume, 0.5 mL; reference buffer, 0.1× PBS (0.5 mL); and liposomes composed of 3.0 mg/mL phospholipid.

sensitive to phase changes. Assuming a single relaxation mechanism, the attenuation due to molecular relaxation can be described as:

$$\alpha_{\text{MR}} = \frac{A_{\text{T}}}{1/f^2 + (2\pi\tau)^2} + Bf^2 \quad (2)$$

where f is the frequency and τ is a characteristic relaxation time. A and B are system specific constants. A , for example, depends on the number of groups involved in the relaxation process and the “strength” of the relaxation process (36) while B is the attenuation due to classical adsorption mechanisms (viscous and thermal conductivity losses). The ultrasonic energy “feeds” into the transition between the two states, and as a result, the attenuation greatly increases during a phase transition and decreases after the system has reached a stable state.

Thus, at a constant angular frequency, the measurement will be highly sensitive to changes in compressibility and densities of the dispersed phase fraction. In the case of liposomes, both are altered as the polar lipids transition from the gel to the fluid phase. Harkness and White found that because of the changes

in density and compressibility, the ultrasonic attenuation of LUV (large unilamellar vesicle) suspensions greatly increased in the vicinity of the T_{M} while the ultrasonic velocity decreased (37). Finally, Reef and coauthors in an ultrasonic study of the role of deuterium chain substitution in the phase transition of DPPC–DPPG LUVs pointed out that ultrasonic attenuation studies were much more specific than DSC studies, i.e., depending on the frequency of the ultrasonic wave, and the interaction of ultrasound occurred primarily with the hydrophobic side chains and the measurements were sensitive to localized structural reorganizations of small domains of molecules (38). This may explain some of the observed differences between T_{M} values determined from ultrasonic attenuation vs transition temperatures from DSC and ultrasonic velocity measurements. Clearly, more measurements at different ultrasonic frequencies are required to clarify this point.

For DPPC, all three analytical methods reported a lower T_{M} than the average value of 41.3 °C reported by Koynova and Caffrey in their review of records from LIPIDAT (39). The $T_{\text{M,DSC}}$ of 41.2 °C and $T_{\text{M},\Delta v_{12}}$ of 39.0 °C were ca. 0.2 and 6.3% lower, respectively. In the case of DSPC, all methods showed very good agreement (<0.3 °C difference) with previously reported data listed in LIPIDAT (39). Liposomes consisting of a 1:1 ratio of DPPC and DSPC both without and with entrapped nisin had single phase transitions of 44.79 and 46.54 °C as measured by DSC, respectively. The presence of single peaks rather than two individual peaks indicates a strong competitive interaction between the two phospholipid species, a phenomenon that also was noted by other authors (40–42). Because these two particular PC species undergo phase transitions that are approximately 13 °C apart, it is unlikely that the single peak transition represents an unresolved or overlapping two peak transition. Rather, it may be the result of miscibility of both phospholipids in both solid (gel) and liquid (liquid crystal) phases. Thus, the system consists of liposomes with bilayers that are composed of a mixture of the two lipids instead of a mixture of single species liposomes.

The increase in T_{M} with the entrapment of nisin in all liposomes led us to conclude that nisin at low concentrations acts to stabilize PC vesicles. Generally, this is in agreement with low rates of leakage of nisin from liposomes that was reported earlier and is in contrast to the inclusion of more complex antimicrobial polypeptides, such as lysozyme, that were found to permeabilize the bilayer structure resulting in a gradual loss of the encapsulated material (7). Currently, measurement of surface charge of nisin-free and nisin-containing liposomes is underway in our laboratories to gain a better understanding of the specific interaction of nisin with vesicles composed of DPPC, DSPC, or DPPC:DSPC.

DSC analysis of DPPC with nisin reported a T_{M} of 41.34 °C, representing an increase in T_{M} of 0.14 °C, possibly due to stabilization of liposomes as a consequence of the nisin insertion (Table 1). This small upshift in T_{M} and consequent stabilization was also observed with DSPC and DSPC–DPPC 1:1 liposomes, although to a lesser extent (Table 1). Wiener et al. (43) observed similar increases in T_{M} of DPPC–DPPG 1:1 liposomes containing a matrix protein of vesicular stomatitis virus, potentially a result of association of the protein with liposomes restricting the mobility of lipid acyl chains thereby increasing the thermal energy requirements for lipid chain expansion. While antimicrobials such as nisin typically function to permeabilize and/or cause pore formation in cytoplasmic membranes of their target microorganisms, their insertion at low concentrations may result

in increases in membrane stability through the stabilization of inner layer concave inverted lipids (44, 45).

Membrane lipid–polypeptide interactions may also help explain the specific inability of nisin to destabilize PC liposomes. Lohner et al. (46) in DSC analysis of human defensin HNP-2 demonstrated that the polypeptide interacted primarily with bacterial mimetic membranes composed of phosphatidylglycerol (PG). On the other hand, erythrocyte–mimetic PC membranes were not thermotropically altered by HNP-2 (46). Nisin is an antimicrobial that is bacterially synthesized as a defense mechanism against competing microflora. Its cationic nature and interaction with the bacterial cell wall peptidoglycan-associated receptor lipid II and anionic membrane lipids such as PG provide nisin with a greater specificity to attack prokaryotic rather than eukaryotic species where PC permeates membranes (47–49). While some have reported differing effects of nisin and other lytic peptides on model membranes as a function of phospholipid composition, we have been unsuccessful in our searches throughout the literature to locate other studies that have reported either similar or contradictory findings using DSC analysis to study the effects of nisin entrapment on thermotropic properties of phospholipids (50–52). Hence, these findings lead us to conclude that the peptide nisin can act to stabilize PC-based liposomes at a low peptide concentration that is observable via an increase in the T_M .

In conclusion, this study relates the measurement of T_M values of liposomes composed of the synthetic phospholipids DSPC and DPPC, both with and without encapsulated nisin via DSC and low-intensity US analysis. Our results indicate excellent agreement between DSC, a widely accepted method for measurement of liposomal thermotropic properties, and US. Our results suggest that US measurements represent a viable alternative to the measurement of T_M values of dispersions of polar lipids by DSC. In addition, attenuation measurements as a function of frequency may yield additional insights into the structure of liposomes that cannot be obtained by DSC. Upon incorporation of nisin into liposomes via encapsulation, the observed increases in sample T_M lead us to conclude that nisin may act to stabilize liposomes at very low concentrations via a possible lowering of curvature stresses. Furthermore, the interaction of nisin with those lipids not commonly found in prokaryotic membranes (e.g., phosphatidylcholine) is likely to produce an alternate effect than that of permeabilization, in this case a stabilizing effect. Finally, companies planning to incorporate polypeptide-based antimicrobials in polar lipid dispersions for use in food products need to carefully evaluate the effect of the encapsulated material on the thermal stability of the dispersion, i.e., stabilization vs permeabilization.

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