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Size, Stability, and Entrapment Efficiency of Phospholipid Nanocapsules Containing Polypeptide Antimicrobials

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The effect of lipid composition [phosphatidylcholine (PC), phosphatidylglycerol (PG), and cholesterol] on size, stability, and entrapment efficiency of polypeptide antimicrobials in liposomal nanocapsules was investigated. PC, PC/cholesterol (70:30), and PC/PG/cholesterol (50:20:30) liposomes had entrapment efficiencies with calcein of 71, 57, and 54% with particle sizes of 85, 133, and 145 nm, respectively. Co-encapsulation of calcein and nisin resulted in entrapment efficiencies of 63, 54, and 59% with particle sizes of 144, 223, and 167 nm for PC, PC/cholesterol (70:30), and PC/PG/cholesterol (50:20:30), respectively. Co-encapsulation of calcein and lysozyme yielded entrapment efficiencies of 61, 60, and 61% with particle sizes of 161, 162, and 174 nm, respectively. The highest concentration of antimicrobials was encapsulated in 100% PC liposomes. Nisin induced more calcein release compared to lysozyme. Results demonstrate that production and optimization of stable nanoparticulate aqueous dispersions of polypeptide antimicrobials for microbiological stabilization of food products depend on selection of suitable lipid−antimicrobial combinations.

KEYWORDS: Liposome; nisin; lysozyme; antimicrobial; encapsulation

INTRODUCTION

Small unilamellar liposomes are artificial bilayer structures with sizes in the nanometer range that can be utilized as controlled-delivery systems. The ability of liposomes to trap water-soluble substances has been employed in various pharmaceutical and cosmetic applications to protect and control the release of active compounds (*1*−*3*). However, the use of liposomes to encapsulate antimicrobials to improve the microbiological stability and safety of foods has only recently received the attention of investigators (*2*, *3*). Antimicrobials are additives that, when added to foods, inhibit or prevent the growth of pathogens and spoilage organisms, thereby improving shelf life and safety of the product (*4*).

A particularly promising class of food antimicrobials is naturally derived polypeptides such as nisin and lysozyme. Nisin is a 3.5 kDa positively charged antimicrobial produced from *Lactococcus lactis* strains. The polypeptide is effective at inhibiting the growth of Gram-positive bacteria such as *Listeria monocytogenes* (*2*, *3*). The antimicrobial activity of nisin against bacteria has been suggested to be a direct result of the electrostatic interaction of the positively charged carboxyl-terminal end of nisin with negatively charged bacterial membrane lipids (*23*). Incorporation and binding of nisin in the bacterial membrane destabilize the overall membrane structure and lead to pore formation. Subsequent leakage of ions causes catastrophic changes in transmembrane potential and internal pH that are ultimately lethal to bacteria (*25*). Nisin exists in two related forms, nisin A and nisin Z. The two forms differ at amino acid position 27; that is, nisin A includes histidine, whereas nisin Z contains asparagine at this position (*5*). The commercially available form of nisin A is sold as Nisaplin or Chrisin for various food applications and contains 2.5% pure nisin (*6*). More recently, commercial nisin Z (Novasin) has been recognized as GRAS (generally recognized as safe) (*7*). The enzyme lysozyme is a naturally occurring antimicrobial that can be derived from eggs, plants, bacteria, and animal secretions (*8*). The molecular weight of chicken lysozyme is 14.3 kDa (*9*). Lysozyme is commercially used to inhibit the growth of *Clostridium tyrobutyricum* in cheese (*7*).

The antimicrobial efficacy of both lysozyme and nisin may be reduced because of (a) chemical and physical changes during food process operations and (b) undesirable interactions with food components, necessitating the addition of large quantities (*4*). Encapsulation of antimicrobials in liposomes may offer a potential solution to protect antimicrobials and enhance their efficacy and stability in food applications. For example, liposome encapsulation of nisin Z has been shown to result in enhanced activity and stability, inhibiting the growth of *Listeria innocua* during cheese ripening (*2*, *3*). Another advantage of

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liposomal delivery systems is the ability to release components on demand (*10*). The addition of trigger compounds that disrupt the vesicular bilayer structure can force the release of the encapsulated matter (*10*, *11*).

The functional properties of liposomal carriers to encapsulate antimicrobials depend on the interaction of antimicrobials with both the liposome membrane and the bacterial cell membrane. Addition of positively charged nisin Z to anionic phospholipid [phosphatidylglycerol (PG)] containing liposomes may result in association due to attractive electrostatic interactions, whereas addition to neutral liposomes may result in association due to hydrophobic interactions (*12*). Addition of cholesterol or stearic acid to PC-based liposomes may reduce liposome permeability (*13*−*15*). Cholesterol interacts with fatty acids of liposomes via hydrogen bonding, increasing the cohesiveness and mechanical strength of the vesicular membrane (*15*). Knowledge of liposome characteristics is required to develop liposome formulations that have optimal entrapment efficiencies and allow the controlled release of antimicrobials.

At present, food encapsulation studies with nisin have focused on the use of pure nisin Z and not the commercially available form that contains 2.5% nisin nor on the use of lysozyme. The objectives of this research were to encapsulate a commercial nisin extract and lysozyme in PC-, PG-, and cholesterol-containing liposomes and study the influence of lipid composition on their physicochemical properties, that is i.e., entrapment efficiency, mean average size, and stability.

MATERIALS AND METHODS

Materials. Lipids [soybean phosphatidylcholine (PC), 1,2-dioleoyl-*sn*-glycerol-3-phospho-*rac*-1-glycerol (PG), and cholesterol] were obtained from Avanti Polar Lipids (Alabaster, AL). Calcein, Triton X-100, EGTA (a metal chelator), nisin, and lysozyme were purchased from Sigma Chemical Co. (St. Louis, MO). Nisin used in this study contained 2.5% pure nisin and closely resembles commercially available formulations used in foods (according to the manufacturer, the formulation contains 75% NaCl and 22.5% denatured milk solids as fillers). All nisin concentrations are based on the weight of the nisin formulation, not pure nisin. Millipore nylon filters (0.2 μm) were obtained from Fisher Scientific (Fair Lawn, NJ). Phosphate-buffered saline (PBS) 10× (0.017 M KH_2PO_4, 0.05 M Na_2HPO_4, 1.5 M NaCl, pH 7.4) was purchased from Bio Whittaker (Walkersville, MD), bicinchoninic acid (BCA) reagents were obtained from Pierce Biotechnology (Rockford, IL), and high-purity chloroform was purchased from Burdick and Jackson (Muskegon, MI).

Liposome Production. Liposomes were manufactured using a modified method of Pinnaduwage and Bruce (*16*). PC was used as the major component in all liposome preparations. Lipid stocks (3 mL, 10 mM) dissolved in chloroform were dried under a stream of nitrogen with different mole percentage combinations [PC (100%), PC/cholesterol (70:30), and PC/PG/cholesterol (50:20:30)]. To remove any traces of solvent, the lipids were subsequently dried overnight under vacuum. The structures of lipids used are shown in **Figure 1**. Dried lipid combinations were then hydrated with 0.1× PBS containing 1 mM EGTA and 50 mM of the fluorescent probe calcein with or without 3.33 mg/mL nisin or lysozyme. Solutions were ultrasonicated four times for 5 min at 45 min intervals using a sonicator bath (Laboratory Supplies Co. Inc., Hicksville, NY) to initiate encapsulation and liposome formation. Vesicles were collected from the supernatant after centrifugation (Sorvall RT 6000 centrifuge, 2500*g*, 10 min). Intact vesicles with entrapped calcein and/or antimicrobials were separated from unencapsulated calcein and antimicrobials by size exclusion chromatography (SEC) on a Bio-Gel A0.5M column (200−400 mesh).

Parts of the liposome solutions were filter-sterilized using 0.2 μm nylon filters for subsequent protein content, entrapment efficiency, leakage, and particle size analysis. Filter sterilization ensured that interfering particulates in the liposome preparations were removed and did not affect the dynamic light scattering (DLS) analysis. Filtration also eliminated microbiological contaminants that may have been introduced during the preparation steps. Solution sterility was required for subsequent antimicrobial activity assays.

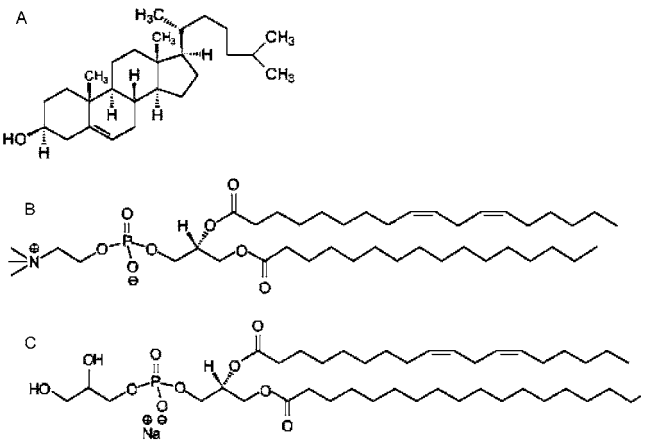

Figure 1. Structures of lipids used to produce liposomes: (**A**) cholesterol; (**B**) 1,2-dioleoyl-*sn*-glycerol-3-phospho-*rac*-1-glycerol (PG); (**C**) phosphatidylcholine (PC).

Liposomal Entrapment Efficiency and Leakage. Entrapment efficiency and antimicrobial-induced leakage of liposomes were determined fluorometrically at excitation and emission wavelengths of 490 and 515.4 nm, respectively, using a fluorescence spectrophotometer (Wallac Victor, Perkin-Elmer Life Sciences, Gaithersburg, MD). The fluorescence assay is based on the self-quenching of calcein at high concentrations; that is, at high concentrations of calcein the fluorescence intensity is low. Calcein release was activated by the addition of 0.1% Triton X-100, which solubilizes the liposome membranes, thus inducing release of the entrapped contents. Once calcein is released from the liposomes, the fluorescence intensity increases due to dilution of calcein. The entrapment efficiency was taken as the fluorescence quench, which was calculated using the formula

$$\%\ \text{quench} = \left(1 - \frac{F}{F_t}\right) \times 100 \qquad (1)$$

where F is the fluorescence prior to the addition of 0.1% Triton X-100 and F_t is the fluorescence after the addition of Triton X-100. The leakage of calcein from liposomes was calculated as

$$\%\ \text{leakage} = \frac{F - F_0}{F_t - F_0} \times 100 \qquad (2)$$

where F is the fluorescence after the addition of antimicrobial, F_0 is the fluorescence of liposomes, and F_t is the fluorescence after Triton X-100 addition.

Protein Content. The concentration of nisin or lysozyme entrapped in the liposomes was determined using the BCA assay. In this assay the BCA reagent (Pierce Biotechnology) was added to dilutions containing the sample protein. After incubation at 37 °C for 30 min, the absorbance at 562 nm was measured using a UV−visible spectrophotometer. Bovine serum albumin containing 2 mg/mL protein was diluted and used as the protein standard.

Particle Size Analysis. The particle size (average mean diameter) of liposomes composed of different lipid compositions was measured using a DLS technique (BI 200SM, Brookhaven Instruments Corp., Holtsville, NY). Undiluted liposome samples (1.5 mL) were analyzed prior to and after filtration through 0.2 μm nylon filters to determine the impact of filter sterilization on particle size and distribution. A wavelength of 632 nm at a scattering angle of 90° at 25 °C was used

to determine the effective diameter, d_{65}. d_{65} is defined as

$$d_{65} = \frac{\sum_i n_i d_i^6 P_i}{\sum_i n_i d_i^5 P_i} \quad (3)$$

where n_i is the number of particles in each size class with size d_i and P_i is a factor that accounts for angular scattering effects for particles larger than $\lambda/20$. For each liposome preparation, DLS measurements were performed immediately after the liposomes were prepared. Change in effective diameter was monitored periodically for 2 weeks for liposomes stored at 4 °C.

Microcalorimety of Sonicated Lysozyme. To determine possible denaturation of lysozyme in liposomes, a differential scanning microcalorimeter technique was used (VP-DSC, MicroCal, Northampton, MA). Sonicated lysozyme liposomes and native lysozyme were subjected to a temperature scan from 15 to 90 °C using a scanning speed of 1.5 °C/min. Native and sonicated lysozyme samples and buffer solutions were degassed prior to loading in sample and reference cells. Sample cells were cleaned with a series of rinses using 2% pepsin, 10 mM EDTA, and 5% Contrad 70 solutions followed by rinsing with double-distilled and demineralized water between runs. The buffer background was subtracted from the protein supernatant fraction prior to determination of the midpoint transition temperature.

Replications. All experiments were conducted with duplicate samples, and all measurements were conducted in triplicates.

RESULTS AND DISCUSSION

Encapsulation Efficiency and Protein Content of Liposomes. Stable liposomes containing either calcein, nisin, calcein *and* nisin, or calcein *and* lysozyme were produced successfully, whereas production of liposomes containing lysozyme without calcein was unsuccessful. Liposomes eluted in the void volume of a Bio-Gel A0.5M SEC column. Calcein fluorescence of liposomes indicated quenching of >54% for all liposome preparations (**Figure 2**). PC, PC/cholesterol (70:30), and PC/PG?cholesterol (50:20:30) had entrapment efficiencies with calcein of 71, 57, and 54%, respectively. Co-encapsulation with nisin and calcein resulted in entrapment percentages of 63, 54, and 59 for the PC, PC/cholesterol (70:30), and PC/PG/cholesterol (50:20:30), respectively, whereas co-encapsulation of lysozyme and calcein indicated percentage entrapments of 61, 60, and 61 for the PC, PC/cholesterol (70:30), and PC/PGG/cholesterol (50:20:30), respectively. On the basis of the statistical analysis with ANOVA (Proc GLM), the results of the fluorescence measurements were not significantly different ($P < 0.05$), an indication that the different liposomal preparations resulted in similar encapsulation efficiencies.

The protein content of intact liposome preparations was measured to determine the total amount of antimicrobial entrapped (**Figure 3**). With equivalent concentrations of nisin or lysozyme added to liposome formulations containing different ratios of PC, PG, or cholesterol, the lowest concentration of nisin or lysozyme was incorporated in PC/PG/cholesterol (50:20:30) liposomes, whereas the highest concentration of antimicrobials was incorporated in the 100% PC liposomes. Liposomes containing nisin without calcein had a protein content of 0.23−0.38 mg/mL depending on liposome composition. Co-encapsulated nisin/calcein vesicles had a protein concentration of 0.28−0.39 mg/mL. When lysozyme and calcein were co-encapsulated in PC liposomes, the protein content was higher than in PC liposomes containing nisin and/or calcein. Because PC monolayers do not carry a surface charge at pH 7.4 (the buffer solution pH), electrostatic interactions with nisin or lysozyme can be excluded (*14*). Thus, the increased insertion

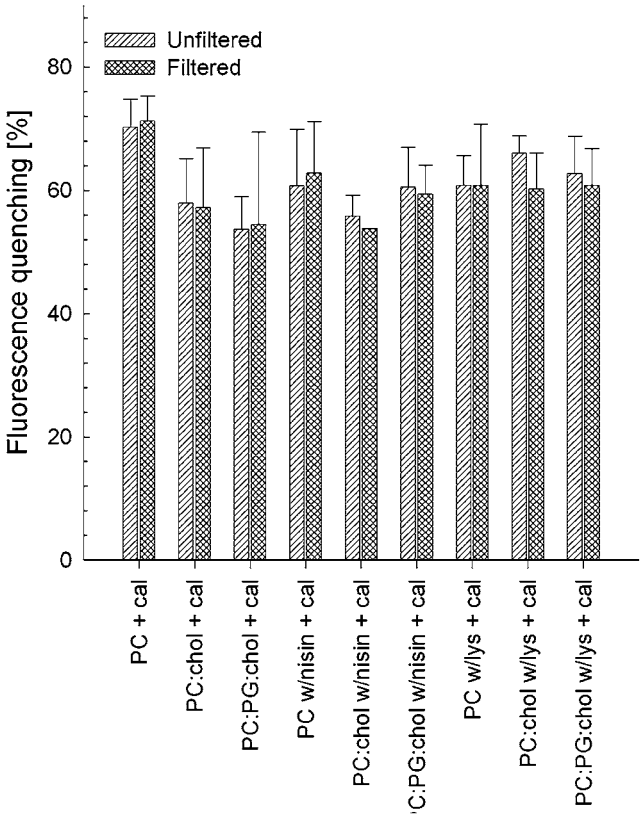

Figure 2. Entrapment efficiency of phosphatidylcholine-based liposomes made from different lipids as measured by fluoroscence quenching of calcein: (slashed bars) unfiltered liposomes; (cross-hatched bars) filter-sterilized liposomes. Cal, calcein; lys, lysozyme; PC, 100% phosphatidylcholine liposomes; PC:chol, phosphatidylcholine/cholesterol (70:30) liposomes; PC:PG:chol, phosphatidylcholine/phosphatidylglycerol/cholesterol (50:20:30) liposomes; w/, with. Each point represents the mean ± standard deviation from two different experiments.

of lysozyme in PC liposomes may be due to hydrophobic interactions and association with PC bilayer structures (*17*). The decreased antimicrobial concentration in cholesterol-containing liposomes is in agreement with results reported in earlier studies in which increased cholesterol concentration decreased the amount of encapsulated nisin Z (*17*). It has been suggested that introduction of cholesterol affects the bilayer curvature (*13*). Cholesterol introduction reduced polypeptide affinity, thus reducing the concentration of antimicrobials that can be incorporated (*17*). In conclusion, protein content measurement indicated that nisin, co-encapsulated nisin and calcein, and co-encapsulated lysozyme and calcein formed stable liposomes for all lipid formulations used; however, the stability depended on the lipid composition. The determination of the exact location and distribution of antimicrobials in the liposomes (e.g., inside the liposomal core or incorporated in the vesicle membrane) will require additional investigations.

To ensure accurate particle size determination and microbiological sterility, samples of liposome preparations were filter-sterilized. When the protein contents of filter-sterilized and unfiltered liposomes were compared, protein loss due to filter sterilization was <10% for all liposome preparations (**Figure 3**), indicating that filter sterilization is a suitable method to remove foreign particles such as microorganisms from liposome dispersions while maintaining their integrity. Microbiological assays conducted in a concurrent study confirmed that solutions were sterile (*18*).

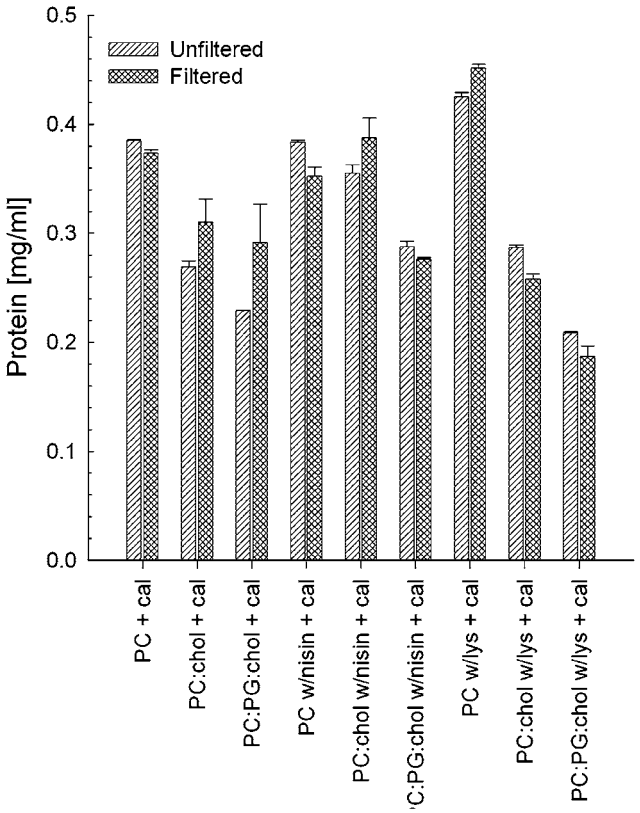

Figure 3. Protein content (percent) of phosphatidylcholine-based liposomes made from different lipid compositions: (slashed bars) unfiltered liposomes; (cross-hatched bars) filter-sterilized liposomes. Cal, calcein; lys, lysozyme; PC, 100% phosphatidylcholine liposomes; PC:chol, phosphatidylcholine/cholesterol (70:30) liposomes; PC:PG:chol, phosphatidylcholine/phosphatidylglycerol/cholesterol (50:20:30) liposomes; w/, with. Each point represents the mean ± standard deviation from two liposome preparations.

Although nisin could be entrapped in liposomes, no stable liposomes containing only lysozyme could be produced. The protein concentration in fractions collected when lysozyme without calcein was encapsulated was below the detection limit. This may have been due to lysozyme sedimentation during the centrifugation step prior to separation on the SEC column. Sedimentation was likely a result of lysozyme denaturation followed by loss of solubility during the various sonication cycles. Sonication has previously been shown to result in precipitation of lysozyme and loss of activity (*19*). A microcalorimetry experiment was conducted in which sonicated lysozyme liposomes and native lysozyme were subjected to a temperature scan from 15 to 90 °C using a scanning speed of 1.5 °C/min. Although the native lysozyme preparation showed a first-order transition at 76.47 °C, which is in close agreement to the denaturation temperature of 77.36 °C reported in the literature (*20*), no transition peak above 50 °C was detected for sonicated lysozyme in PC liposomes. Successful encapsulation of lysozyme in the presence of calcein could have been due to a stable complex formed between calcein and lysozyme. Calcein forms complexes with trace metals, for example, iron, and a similar complex with lysozyme could have been formed when both were co-encapsulated in the liposomes (*21*). Complex formation between calcein and lysozyme may or may not affect the stability of liposomes. Clearly further investigation will be needed to verify potential complex formation and its effect on bilayer stability. On the basis of these results, food manufacturers planning to encapsulate lysozyme either may be required to use an adjunct or may alternatively have to use a homogenization process instead of high-intensity ultrasonication to prevent denaturation and loss of lysozyme activity.

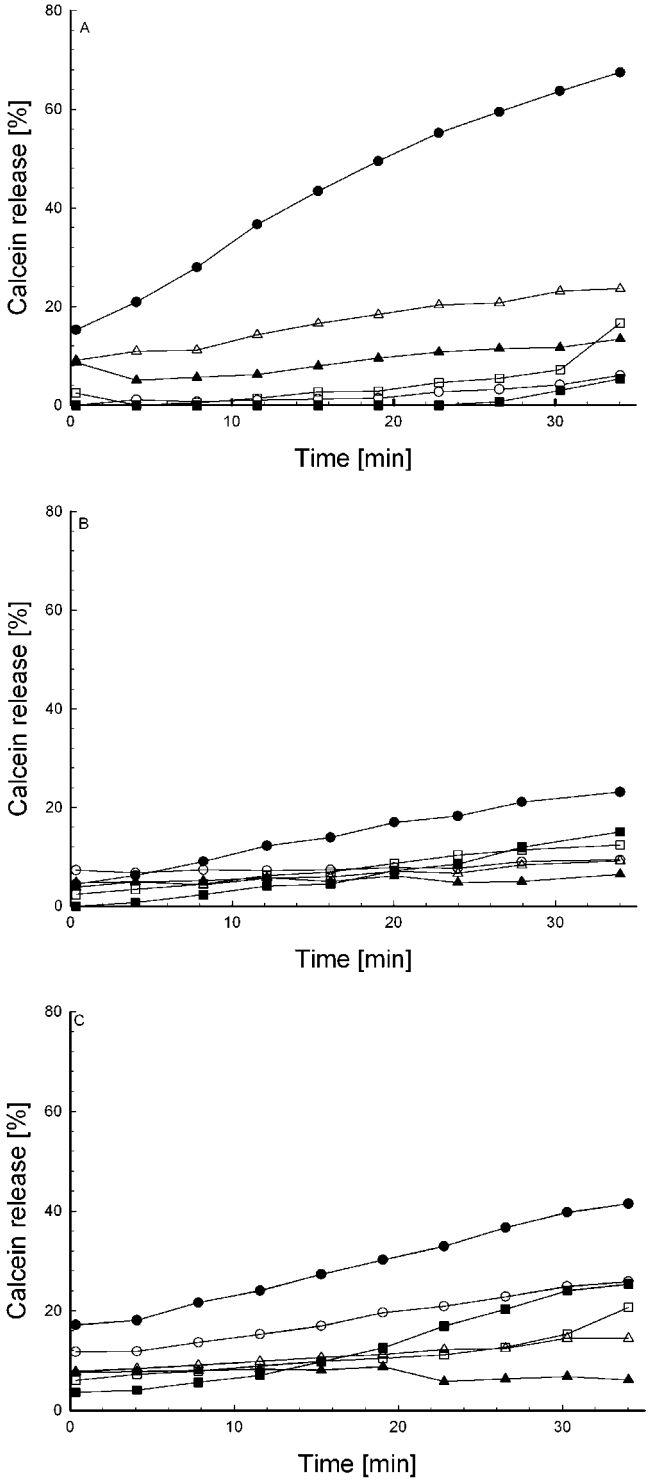

Figure 4. Antimicrobial-induced leakage of phosphatidylcholine-based liposomes: (**A**) nisin, lysozyme, and nisin/lysozyme mixture induced leakage of phosphatidylcholine liposomes; (**B**) nisin, lysozyme, and nisin/lysozyme mixture induced leakage of phosphatidylcholine/cholesterol (70:30) liposomes; (**C**) nisin, lysozyme, and nisin/lysozyme mixture induced leakage of phosphatidylcholine/phosphatidylglycerol/cholesterol (50:20:30) liposomes; (○) 150 μM nisin; (●) 375 μM nisin; (△) 150 μM lysozyme; (▲) 375 μm lysozyme; (□) 150 μM mixture; (■) 375 μM mixture.

Effect of Lipid Composition on Stability of Liposomes. The ability to entrap antimicrobials in liposomes may be compromised by the fact that antimicrobials may negatively

Table 1. Effective Diameter (Nanometers) of Phosphatidylcholine-Based Liposomes with Calcein and Antimicrobials as a Function of Time

	PC		PC/cholesterol (7:3)		PC/PG/cholesterol (5:2:3)	
day	unfiltered	filtered[a]	unfiltered	filtered[a]	unfiltered	filtered[a]
			Calcein			
1	96.4 (0.26)[b]	84.9 (0.25)	152.8 (0.17)	133.3 (0.26)	155.3 (0.26)	145.3 (0.24)
3	116.1 (0.20)	98.8 (0.25)	155.9 (0.27)	142.0 (0.26)	155.8 (0.26)	141.1 (0.22)
5	114.5 (0.23)	103.3 (0.24)	160.9 (0.26)	142.3 (0.25)	162.0 (0.23)	147.4 (0.24)
7	109.3 (0.26)	101.6 (0.24)	153.7 (0.25)	142.3 (0.23)	155.9 (0.24)	142.8 (0.24)
11	106.8 (0.16)	90.3 (0.23)	141.5 (0.26)	140.2 (0.23)	158.7 (0.18)	140.2 (0.23)
13	105.0 (0.23)	85.3 (0.23)	148.5 (0.22)	136.8 (0.26)	155.2 (0.27)	146.5 (0.25)
15	101.8 (0.24)	96.4 (0.26)	152.3 (0.15)	139.6 (0.26)	202.6 0.25)	146.4 (0.24)
			Nisin			
1	131.3 (0.25)	124.4 (0.26)	177.5 (0.23)	169.0 (0.26)	181.9 (0.26)	171.6 (0.26)
5	143.7 (0.27)	126.9 (0.23)	174.0 (0.25)	170.2 (0.24)	175.9 (0.28)	165.5 (0.27)
9	139.9 (0.26)	123.0 (0.23)	174.7 (0.27)	165.5 (0.24)	177.3 (0.26)	173.7 (0.25)
11	150.1 (0.12)	124.5 (0.24)	167.4 (0.25)	166.9 (0.23)	176.6 (0.27)	171.0 (0.24)
15	136.1 (0.28)	111.5 (0.25)	160.0	158.7 (0.25)	165.1 (0.26)	160.0 (0.24)
			Nisin + Calcein			
1	151.4 (0.24)	149.0 (0.25)	165.7 (0.24)	139.9 (0.23)	160.3 (0.22)	151.7 (0.22)
4	164.0 (0.23)	162.8 (0.24)	167.8 (0.24)	144.3 (0.23)	168.0 (0.24)	162.9 (0.24)
5	164.0 (0.23)	158.7 (0.26)	171.7 (0.17)	143.5 (0.22)	159.5 (0.25)	155.5 (0.24)
7	158.7 (0.24)	155.9 (0.24)	166.6 (0.27)	139.1 (0.21)	158.0 (0.26)	150.9 (0.24)
11	153.2 (0.25)	148.6 (0.26)	162.7 (0.18)	138.8 (0.22)	151.6 (0.27)	148.5 (0.23)
15	156.5 (0.19)	154.4 (0.26)	160.6 (0.21)	142.8 (0.22)	157.1 (0.20)	157.9 (0.24)
			Lysozyme + Calcein			
1	140.9 (0.21)		234.0 (0.29)	204.7 (0.24)	208.5 (0.28)	173.5 (0.26)
3	137.8 (0.25)		208.2 (0.22)	194.6 (0.22)	204.1 (0.27)	161.7 (0.26)
5	138.5 (0.25)		215.5 (0.26)	187.0 (0.23)	198.0 (0.24)	156.3 (0.26)
7	145.5 (0.18)		215.2 (0.21)	186.6 (0.23)	191.9 (0.29)	153.9 (0.25)
11	134.2 (0.23)		203.3 (0.18)	183.9 (0.23)	186.9 (0.28)	154.9 (0.23)
15	129.2 (0.24)		204.8 (0.25)	177.9 (0.23)	185.2 (0.29)	148.3 (0.26)

[a] Filter-sterilized using 0.2 μm nylon membranes. PC, phosphatidylcholine; PG, phosphatidylglycerol. [b] Numbers in parentheses are polydispersity values indicating how homogeneous the liposome solutions were.

interact with liposome membranes and disrupt the bilayer structure. Therefore, antimicrobial-induced leakage was investigated. The percentage of calcein release was monitored over a period of 35 min. A comparison of PC and PC/cholesterol (70:30) liposomes showed that the addition of cholesterol reduced leakage for both nisin- and lysozyme-containing liposomes as indicated by the smaller calcein release (**Figure 4**). Incorporation of cholesterol has previously been reported to decrease nisin-induced leakage in PC liposomes (*22*). This has been attributed to cholesterol being able to reduce membrane permeability and increase rigidity of the PC liposomal bilayer structure (*19*, *22*). The percentage calcein release upon addition of 375 μM nisin was highest for PC liposomes and lowest for PC/cholesterol (70:30) liposomes, whereas at 150 μM nisin addition, the percentage release was highest for PG-containing liposomes. These results correspond to studies that indicated a concentration-dependent effect of nisin-induced leakage of PC and PG liposomes (*22*).

The electrostatic interaction of nisin with negatively charged membrane phospholipids such as PG has been recognized to be more pronounced than the interaction with neutral lipids such as PC (*23*). Generally, the surface charge of manufactured liposomes was either negative or zero at pH 7.4, depending on the phospholipid type and concentration. Because nisin is positively charged at neutral pH, the electrostatic interaction with negatively charged PG-containing liposomes was attractive and may have led to the formation of unstable pores due to binding to the charged phospholipid headgroups of PG. This mechanism was also suggested by Bonev and coauthors (*23*), and similar nisin-induced calcein release kinetics have been reported by El Jastimi et al. (*22*).

At the same molar concentration of nisin and lysozyme, nisin generally induced more calcein release compared to lysozyme. The nisin/lysozyme mixture caused the smallest release for PC liposomes. Upon addition of 375 μM nisin, the rate of the destabilization kinetics of liposomes was higher than upon addition of either lysozyme or a nisin/lysozyme mixture (**Figure 4**). The increased leakage in the presence of nisin compared to lysozyme may be attributed to the molecular weight of antimicrobials. The smaller molecular weight and size of nisin compared to lysozyme may possibly ease insertion into the liposome membranes, causing more leakage of the fluorescent probe from the vesicles.

Effect of Lipid Composition, Filter Sterilization, and Storage Time on Effective Particle Diameter. DLS revealed homogeneous liposome populations in the filter-sterilized preparations. Two populations of liposome sizes were detected in unfiltered PC and PC/cholesterol (70:30) liposomes with calcein. After filtration, a unimodal size population was observed (data not shown). A similar trend was observed when antimicrobials were encapsulated. Liposome sizes varied from 85 to 239 nm depending on lipid composition, encapsulated matter, and incubation time of liposomes (**Table 1**). The effective diameter of liposomes was more affected by lipid composition of liposomes than by filter sterilization or incubation time of liposomes. This indicated that liposomes were physically stable over the 2-week period investigated. When the same compound was encapsulated, the smallest liposomes were obtained with PC liposomes. The presence of cholesterol generally increased liposome size. When calcein was encapsulated, the effective diameter of PC/cholesterol (70:30) liposomes was slightly smaller than that of PC/PG/cholesterol (50:20:30) liposomes (**Table 1**). The percentage increase in size of filter-sterilized calcein-containing liposomes after 2 weeks storage was highest for PC liposomes (14%) compared to PC/cholesterol (70:30)

(5%) and PC/PG/cholesterol (50:20:30) (0.8%) (**Figure 4A**). The polydispersity of all liposome preparation was <0.3.

Co-encapsulation of antimicrobials with calcein increased the effective diameter of liposomes. The proteins may alter the packing of phospholipids in the liposomal membrane and promote curvature changes (*2*). When nisin and calcein were co-encapsulated in liposomes, the PC/cholesterol (70:30) liposomes were larger than liposomes manufactured from PC/PG/cholesterol (50:20:30). Increase in liposome size upon nisin encapsulation is in contrast to results reported by Laridi et al. (*17*), who showed a reduction in size of nisin Z-containing proliposomes compared to empty vesicles. The authors used commercial proliposomes made from higher melting point phospholipids (*17*). Proliposomes are dry free-flowing granules that form a liposomal dispersion when added to water (*17*). Laridi et al. attributed the reduction in size to a reorganization of the lipid bilayer, leading to much smaller vesicles. It should be noted, though, that liposome composition and nisin variant differed from the current study. The increase in liposome size when lysozyme and calcein were co-encapsulated in PC liposomes could be due to the creation of a more swollen membrane structure. An increase in size when the anionic PG was part of the liposome composition could partly be due to attraction between the charged groups of liposomes and antimicrobials. Liposome size thus was dependent on the lipid composition and the encapsulated compound. During the 2-week storage of liposomes at 4 °C, there were slight fluctuations in effective diameter (**Table 1**), which were, however, not statistically significant.

Conclusions. The protein concentration and fluorescence quench results indicate antimicrobials were efficiently incorporated in PC-based liposomes. The antimicrobial loading was decreased by the addition of cholesterol and PG. Although application of nisin and lysozyme affected liposome stability as indicated in the calcein leakage studies, intact encapsulated liposomes were physically stable for 2 weeks. It should be noted that although the highest loading was obtained with 100% PC liposomes, this also resulted in higher leakage. Addition of cholesterol decreased leakage of PC liposomes while maintaining a high concentration of antimicrobials.

The concentration of compounds that can be entrapped is a function of lipid composition and may be attributed to electrostatic and hydrophobic interactions between antimicrobials and phospholipids. To relate liposomal properties such as encapsulation efficiency, total antimicrobial content, stability, and size to membrane composition, a mechanistic model will be needed that incorporates molecular interactions. Driessen and coauthors developed an early model based on the electrostatic interactions of nisin with liposome phospholipids that described subsequent nonselective pore formation (*24*). In the future, isothermal titration calorimetry experiments may yield additional insights that could be used to expand this model.

The results demonstrate the feasibility of producing stable antimicrobial-containing liposome capsules to inhibit the growth of pathogens and improve the stability and microbiological safety of food products.

ABBREVIATIONS USED

BCA, bicinchoninic acid; BSA, bovine serum albumin; DLS, dynamic light scattering; EGTA, ethylene glycol bis(2-aminoethyl)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PG, phosphatidylglycerol; SEC, size exclusion chromatography.

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