Integrated Light-Scattering Spectroscopy, A Sensitive Probe for Peptide-Vesicle Binding: Application to the Membrane-Bound Colicin E1 Channel Peptide

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ABSTRACT Integrated light-scattering (ILS) spectroscopy was used to monitor the binding of the colicin E1 channel peptide to POPC:POPG large unilamellar vesicles (LUV; 60:40, mol:mol) at acidic pH (3.5). Binding conditions were chosen such that nearly all of the channel peptide was bound to the vesicles with little free peptide remaining in solution. The increase in vesicle size upon the insertion of the channel peptide was measured by performing a discrete inversion technique on data obtained from an ILS spectrometer. Vesicle size number distributions were determined for five different systems having peptide/vesicle ratios of approximately 0, 77, 154, 206, and 257. The experiment was repeated four times (twice at two different vesicle concentrations) to determine reproducibility. The relative changes in vesicle radius upon peptide binding to the membrane vesicles was remarkably reproducible even though these changes represented only a few nanometers. A comparison of vesicle size number distributions in the absence of bound peptide was made between ILS and dynamic light scattering (DLS) data and showed similar results. However, DLS was incapable of detecting the small changes due to peptide-induced vesicle swelling. The membrane-bound volume of the colicin E1 channel peptide was $\sim 177 \pm 22$ nm^3. These data indicate that in the absence of a membrane potential (closed channel state) the colicin E1 channel peptide inserts into the membrane resulting in a significant displacement of the lipid bilayer as evidenced from the dose-dependent increase in the vesicle radius. These results indicate that ILS spectroscopy is a sensitive sizing technique that is capable of detecting relatively small changes in membrane vesicles and may have a wide application in the determination of peptide binding to membrane vesicles.

INTRODUCTION

Light-scattering techniques (both dynamic light scattering (DLS) and integrated light scattering (ILS)) provide a method for obtaining size distribution information on particles typically between 0.01 and 2 μm (Selser and Yeh, 1976; Goll and Stock, 1977; Goll et al., 1982; Ruf et al., 1989; McCracken and Sammons, 1987; Rouch and Chen, 1987; Burchard, 1988; Helmstedt, 1988; Hallett et al.; 1989, 1991; Sheu, 1992; Finsy et al., 1992) and have been applied to a wide variety of problems in biophysics and biophysical chemistry. In this particular study, the purpose was to examine the hypothesis that membrane-soluble peptides displace lipid molecules and lead to an overall expansion in the membrane surface area. Furthermore, the intent of this study was to determine whether the techniques of ILS and DLS are sufficiently sensitive to monitor the relatively small changes in radius that should occur when a membrane-soluble peptide becomes incorporated into the vesicle lipid bilayer. It was expected that upon binding of the colicin E1 channel peptide, the vesicle radius would increase in direct proportion to the number of peptides embedded. This study, then, involves the application of a novel technique to obtain the membrane-bound volume (size) of a membrane-active protein. Colicin E1 is a 57-kDa bacteriocin protein that exhibits its lethality on sensitive coliform bacteria by forming an ion channel in the host cell's cytoplasmic membrane, which is sufficiently conductive to depolarize this membrane and cause cell death (Gould and Cramer, 1977; Cleveland et al., 1983). Active channel-forming peptides can be formed by mild proteolysis of whole colicin E1 with a variety of proteases, including thermolysin. The thermolytic channel peptide is a 178-residue protein, which has been proposed to form a monomeric four-helix bundle channel in membrane bilayers (Merrill and Cramer, 1990; Zhang and Cramer, 1992; Palmer and Merrill, 1994). However, despite a large array of information obtained by a variety of techniques there is still considerable controversy concerning the membrane-bound topology of the closed state of this channel peptide. The closed state can be formed by the addition of the channel peptide to LUV consisting of at least 20 mol % acidic phospholipid (A. R. Merrill and L. R. Palmer, unpublished observations). The active channel state must be formed by imposing a *trans*-negative membrane potential across the membrane bilayer, which presumably induces structural rearrangements within the membrane-bound peptide that result in the active channel lumen being formed (Slatin et al., 1986; Peterson and Cramer, 1987; Merrill and Cramer, 1990; Cramer et al., 1990).

The current study reports on the use of ILS and DLS spectroscopies to measure vesicle swelling (relative size increases) upon titration of a model vesicle system (POPC:POPG LUV) with the colicin E1 channel peptide. The

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vesicles were composed of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC), and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylglycerol (POPG) and produced by the method of extrusion (Hope et al., 1985). Five different peptide-to-vesicle ratios were studied. Although the original suspensions produced by extrusion were isotonic, it was necessary to create a slightly hypotonic environment (intravesicular water concentration < extravesicular water concentration) for the light-scattering studies. This slight pressure gradient across the membrane was needed to "round up" the vesicles and ensure a spherical shape.

THEORY

To obtain the radius distribution function, $G(r)$, of the vesicles, it is necessary to successfully invert the angle dependent scattered intensity, $I(Q)$, according to the relation,

$$I(Q) = \int_0^\infty I(Q, r)_{TH}\, G(r)\, dr. \tag{1}$$

where $I(Q, r)_{TH}$ is the theoretical scattered intensity for vesicles of radius r, and Q is the magnitude of the scattering vector given by

$$Q = \frac{4\pi n_0}{\lambda_0} \sin\frac{\theta}{2} \tag{2}$$

where n_0 is the solvent refractive index, λ_0 is the wavelength of the light in vacuo, and θ is the scattering angle. The inversion (Eq. 1) is ill conditioned, but a discrete non-negative least-squares inversion method involving a range and spacing of predetermined trial radii has been developed (Strawbridge and Hallett, 1994). The trial radii, between r_{min} and r_{max}, are distributed geometrically, i.e.,

$$r_n = r_{min}\left[\left(\frac{r_{max}}{r_{min}}\right)^{1/m}\right]^{n-1} \tag{3}$$

where m is the total number of radii. Operationally, the program minimizes var where

$$\text{var} = \left[I(Q) - \sum_{n=1}^{m} a_n\, I(Q, r_n)_{TH}\right]^2 \tag{4}$$

where a_n is constrained to be positive and represents the amplitudes of a histogram. The size distribution program, written in FORTRAN, uses a non-negative least-squares fitting routine to calculate number distributions for hollow spheres using Mie theory (Bohren and Huffman, 1983) for $I(Q, r)_{TH}$. The program outputs a number distribution in the form of a histogram, the fitted form of $I(Q)$, and information on the moments of the distribution.

The scattering factors appropriate for vesicles were generated using Mie theory. It should be noted that a Rayleigh-Gans-Debye scattering factor would work as well as Mie in the size regime associated with these vesicles. However, in producing the data analysis program the objective was to create a package that would be useful for the broadest range of particle sizes and not be limited only to the smaller particles where the Rayleigh criterion is satisfied.

The scattered intensity from a hollow sphere system for vertically-polarized incident light is given by Bohren and Huffman, (1983)

$$I(Q)_{TH} = \frac{I_0}{kr^2}\,|S_1|^2 \tag{5}$$

where k is the wave number, r is the distance between the scattering particle and the point of the intensity measurement, and

$$S_1(\cos\theta) = \sum_{n=1}^{\infty} \frac{2n+1}{n(n+1)}\,(a_n\pi_n + b_n\tau_n). \tag{6}$$

The angle-dependent functions are

$$\pi_n = \frac{P_n^1(\cos\theta)}{\sin\theta} \quad \text{and} \quad \tau_n = \frac{dP_n^1(\cos\theta)}{d\theta}$$

where $P_n^1(\cos\theta)$ is the associated Legendre polynomial and the scattering coefficients a_n and b_n for a coated sphere are given by

$$a_n = \frac{\Psi_n(y)[\mathcal{A}] - m_2\Psi_n'(y)[\mathcal{A}]}{\xi_n(y)[\mathcal{A}] - m_2\xi_n'(y)[\mathcal{A}]} \tag{7}$$

$$b_n = \frac{m_2\Psi_n(y)[\mathcal{B}] - \Psi_n'(y)[\mathcal{B}]}{m_2\xi_n(y)[\mathcal{B}] - \xi_n'(y)[\mathcal{B}]} \tag{8}$$

where

$$\mathcal{A} = \Psi_n'(m_2 y) - A_n X_n'(m_2 y) \quad \mathcal{B} = \Psi_n'(m_2 y) - B_n X_n'(m_2 y)$$

where

$$A_n = \frac{m_2\Psi_n(m_2x)\Psi_n'(m_1x) - m_1\Psi_n'(m_2x)\Psi_n(m_1x)}{m_2X_n(m_2x)\Psi_n'(m_1x) - m_1X_n'(m_2x)\Psi_n(m_1x)}$$

$$B_n = \frac{m_2\Psi_n(m_1x)\Psi_n'(m_2x) - m_1\Psi_n(m_2x)\Psi_n'(m_1x)}{m_2X_n'(m_2x)\Psi_n(m_1x) - m_1X_n'(m_1x)X_n(m_2x)}$$

The functions

$$\Psi_n(z) = z\,j_n(z), \qquad X_n(z) = -z\,y_n(z), \qquad \xi_n(z) = z\,h_n^{(1)}(z)$$

are Riccati-Bessel functions with

$$m_1 = \frac{n_1}{n_0}, \qquad m_2 = \frac{n_2}{n_0}, \qquad x = kr_1, \qquad y = kr_2$$

and the variables n_0, n_1, and n_2 are the refractive indices of the medium, lumen, and coat, and r_1 and r_2 are the inner and outer radii, respectively.

MATERIALS AND METHODS

The fiber optic-based ILS spectrometer, described elsewhere in detail (Strawbridge et al., 1991), was used to generate $I(Q)$ versus Q data on LUV. The light source was a continuous wave, tunable, argon-ion Lexel (Model no. 95 Lexel Laser, Inc., Fremont, CA) laser set to a wavelength of 488 nm. The temperature reservoir was maintained at 23°C for all experiments, and the data collection time for each experiment was ~40 min. In calculating

the scattering factors, the vesicles were modeled as hollow spheres with a membrane thickness of 4 nm and refractive indices of $n_0 = 1.33$, $n_1 = 1.33$, and $n_2 = 1.44$.

The theory and operation of the DLS spectrometer are now commonplace in the literature. The detailed methods for applying DLS methods to the size analysis of the vesicle system used in this study have been previously described by Hallett et al. (1989, 1991). Briefly, a DLS experiment was performed on the same sample of peptide-free vesicles used in the ILS experiments. These vesicles were diluted 10-fold with the vesicle preparation buffer to a phospholipid concentration of 0.025 mg/ml. After a 15-min equilibration of this sample at 25°C, six 10-min runs on the DLS instrument were performed. The data from these consecutive runs were then combined and the data analyses were performed on a microcomputer using the FORTRAN programming language. The analysis incorporated Rayleigh-Gans-Debye scattering factors for coated spheres (Hallett et al., 1991) and yielded number distributions in the form of a histogram.

LUV containing POPC:POPG (60:40; mol:mol) were prepared on site by the method of extrusion (Hope et al., 1985). The POPC and POPG were purchased from Avanti Polar Lipids (Birmingham, AL). In brief, stock solutions of POPC and POPG (in chloroform) were mixed and dried under N_2, followed by further drying in vacuo for 1 h. The resulting lipid pellet was hydrated in 100 mM NaCl, 10 mM dimethylglutarate (DMG) buffer, pH 3.5 (vesicle preparation buffer), with vigorous mixing on a vortex mixer. It was imperative that all vessels were immaculately clean and that all buffers were filtered (0.1 μm pore size, Millipore Corp., Boston, MA) to reduce background scatter caused by dust particles. This lipid dispersion was then extruded under high pressure (130 psi) 10 times, through two 0.1-μm polycarbonate filters. The concentration of the lipid solution was determined by the micro-Bartlett assay (Bartlett, 1958), after charring with $Mg(NO_3)_2$ to degrade the phospholipids (Ames and Dubin, 1960).

Colicin E1 was purified from *Escherichia coli* IT3661 cells harboring the plasmid, pSKE1⁻ as described elsewhere (Song et al., 1991), and the channel peptide (MW, 22864) was isolated by thermolytic digestion of whole colicin E1 as described earlier (Merrill et al., 1990). Cytotoxicity and channel-forming ability were assayed and characterized as previously discussed (Merrill et al., 1993). A pair of experiments was performed at two different vesicle concentrations. For the first experiment, the channel peptide was added to the vesicles at a lipid concentration of 0.25 mg/ml and peptide concentrations of 0.0075 mg/ml, 0.015 mg/ml, 0.020 mg/ml, and 0.025 mg/ml, respectively. The second experiment was performed at double these concentrations. The total volume was made up to 2 ml with 100 mM NaCl and 10 mM DMG buffer, pH 3.5 (vesicle preparation buffer). This mixture was then equilibrated at 23°C for 15 min before making the scattering measurements to allow for complete binding of the peptides to the vesicles. Recent concerns regarding the shape of the vesicles after extrusion (Mui et al., 1993) were addressed by diluting slightly the vesicle medium with 0.1 ml of water producing a mildly hypotonic environment. It was determined during this study that it was essential to add water to the vesicles without peptide, but the effect was negligible for vesicles with peptide. The slightly hypotonic environment induces ellipsoidal vesicles to "round up." In the case of the proteoliposomes, this small shift was not observed. The peptide may have already encouraged rounding and thus a slightly hypotonic environment would have a null effect on vesicle shape.

RESULTS AND DISCUSSION

Samples of freshly prepared POPC:POPG vesicles were analyzed by both DLS and ILS techniques before the addition of the peptide. Fig. 1 compares size distributions obtained on similar samples by the two techniques. It is evident from this figure that the distributions compare favorably. The mean radius obtained by DLS (53.2 nm) is slightly larger than that obtained by ILS (47.5 nm). This is consistent with other measurements made by the authors on latex particle suspensions (Strawbridge and Hallett, 1994). The slightly larger

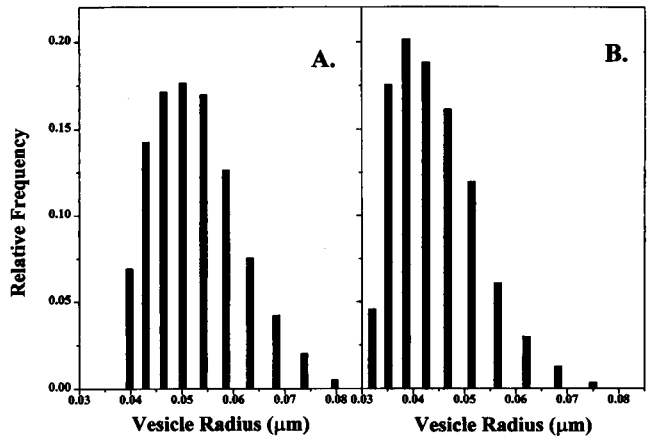

FIGURE 1 Histograms showing the size number distributions obtained from POPC:POPG vesicles in the absence of peptide using (*A*) DLS spectroscopy and (*B*) ILS spectroscopy. See Fig. 2 for ILS experimental conditions and data analysis. DLS experimental conditions were as indicated in Materials and Methods.

DLS size is thought to arise for several reasons. 1) The DLS measures a hydrodynamic size, which is slightly larger than the actual particle size. 2) The DLS analysis routine incorporates a Rayleigh-Gans-Debye scattering factor in a two-step analysis procedure, whereas the ILS method utilizes a Mie scattering factor. The two scattering factors are virtually identical for 50 nm radius particles, but begin to show slight differences for particles at the high end of the size distributions observed. 3) The absolute sizes determined by ILS depend on the accuracy of a calibration run involving known size particles (48 nm radius latex spheres). The maximum error in absolute size expected due to calibration effects is ~10% or ~5 nm for 50-nm vesicles (Strawbridge and Hallett, 1994). However, the error in fitting ILS data due to noise on the signal is only ~1% or 0.5 nm. DLS does not require calibration. 4) Finally, recently published work by Harada and Asakura (1994) indicates that DLS can overestimate particle size as a result of suppression of diffusive motion by radiation pressure. The present observations support their interpretation. Thus, while the ILS technique may be more prone to absolute error than DLS, it is extremely sensitive to relative size changes.

The data collection times for the ILS technique were 40 min, whereas DLS required 4–6 h to obtain data of sufficient quality to enable the extraction of size distributions. The long data collection times for DLS prohibit its usefulness for examining time-sensitive samples. Although in principle it should be possible to measure the small changes in vesicle radius resulting from peptide incorporation into the membrane, in practice it is sometimes not possible to keep both the hardware and the sample constant over the long data collection times needed to observe small size changes. According to our experience, it has been noted that, to have confidence in the size distributions obtained by DLS, data need to be collected in eight or more half-hour runs. These runs were first analyzed separately by moments analysis to

ensure that all runs yielded equivalent moments (sometimes the presence of dust, for example, can destroy a run). If a majority of the runs were equivalent in terms of their moments and the quality of fit, then the equivalent group was averaged before performing the discrete inversion and recovering the distribution. When this procedure was followed with the peptide-loaded vesicles a situation was rarely observed where the majority of the runs were deemed to be equivalent. While the specific causes of this effect are not known, it is our belief that it is primarily due to changes in the sample (perhaps aggregation) that occurred over a few hours. Because of this difficulty, the following sections deal only with the ILS results.

The $I(Q)$ versus Q plot (Fig. 2) show the data for one individual experiment and the fit obtained by the ILS fitting program. These data indicate that the binding of the channel peptide at acidic pH (under conditions where the peptide is known to exhibit total binding to the vesicle surface) leads to an increase in the size of the vesicles. This size increase was proportional to the amount of added protein, indicating that the protein does indeed embed into the nonpolar phase of the membrane bilayer under conditions known to form the closed state of the channel (Zhang and Cramer, 1992; Palmer and Merrill, 1994).

The resulting histograms that give rise to the $I(Q)$ versus Q fits are shown in Fig. 3. It can be seen from these histograms that there is a progressive shift in the size distributions of the vesicles to larger radii upon titration of the suspension with channel peptide. A quantitative comparison of distribution means for all four experiments is shown in Table 1 as well as the average value for all four experiments. The mean radius of the vesicles without added peptide was 46.2 ± 1.0 nm, and the vesicle size increased to 55.6 ± 1.3 nm when 257 peptides for every vesicle were present. It is clear from Table 1 that the absolute value of the mean varies slightly from sample to sample. Experiments 1 and 2 were performed on the same batch of extruded vesicles but a day apart; experiments 3 and 4 were performed similarly. The data representing the radii determined for the vesicles without peptide (see Table 1) indicate that the variance is not only a function of the reproducibility of the extrusion technique but perhaps shows a slight swelling of the vesicles over a 24-h period. Nonetheless, the accuracy and resolution of these ILS data overall were remarkable and allowed for the determination of the membrane-bound volume per peptide in this model system.

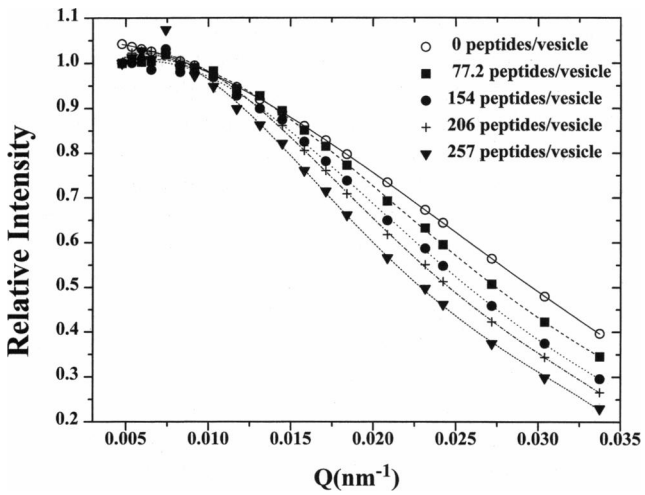

FIGURE 2 An ILS plot of the relative intensity ($I(Q)$) against Q for POPC:POPG (60:40, mol:mol) vesicles with no peptide (○) or in the presence of peptide at various peptide/vesicle ratios: 77 (■), 154 (●), 206 (+), and 257 (▼). The lines represent the fit determined by the ILS size distribution program after a run of 40 min. The vesicles were prepared in 100 mM NaCl, 10 mM DMG buffer, pH 3.5 by the extrusion technique. A small amount of water (100 μl) was added to the vesicle solution (2 ml) to make the external medium slightly hypotonic and cause rounding up. The measurements were made after equilibration of this mixture for 15 min at 23°C. In the case of the proteoliposome samples, the channel peptide was added to the vesicles and the mixtures were similarly rounded up and equilibrated before making the measurements.

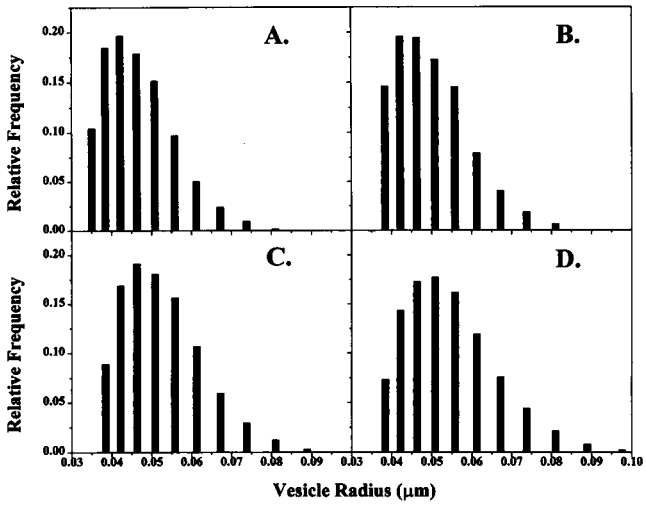

FIGURE 3 A set of histograms showing the size number distributions obtained from POPC:POPG membrane vesicles at various peptide/vesicle ratios: (*A*) 77:1, (*B*) 154:1, (*C*) 206:1, and (*D*) 257:1. Conditions as described in Fig. 2.

Fig. 4 shows the resulting ILS histograms obtained from two consecutive experiments on the same system (206 peptides added per vesicle). Two separate aliquots from the same extrusion were identically prepared and the results indicate a high degree of confidence in system preparation and analysis.

To calculate the number of protein molecules per vesicle, the mean radius obtained from averaging all four experiments for the vesicles without peptide was first used to determine the surface area of the vesicle. The surface area together with the area of the phospholipid headgroup (~0.65 nm^2), assuming that there is 20% more lipid found on the outer surface of the vesicle, yielded the number of phospholipids per vesicle. There is very little agreement in the literature on the size of the PC headgroup (Nagle, 1993). Nagle (1993) quotes values in the literature ranging from 0.576 to 0.709 nm^2 and reports a value of 0.621 nm^2 using nuclear magnetic resonance. The area of the PG headgroup

TABLE 1 Mean LUV radii as determined by integrated light scattering spectroscopy

Peptides Added Per Vesicle	Mean VesicleRadius, nm(SD)	POPC:POPG LUV vesicle radius (0.25 mg/ml) Experiment 1 (nm)	POPC:POPG LUV vesicle radius (0.25 mg/ml) Experiment 2 (nm)	POPC:POPG LUV vesicle radius (0.50 mg/ml) Experiment 3 (nm)	POPC:POPG LUV vesicle radius (0.50 mg/ml) Experiment 4 (nm)
0	46.2 (1.0)	46.0	47.5	45.1	46.0
77.2	48.7 (1.3)	48.1	50.5	47.6	48.4
154	51.1 (0.9)	50.2	52.4	50.9	51.0
206	53.3 (0.5)	52.5	53.7	53.4	53.4
257	55.6 (1.3)	53.8	55.9	55.8	56.9

ILS data were collected with conditions as described in Fig. 2. The mean vesicle radii were obtained from the averages of the individual experiments (1–4) indicated ± SD.

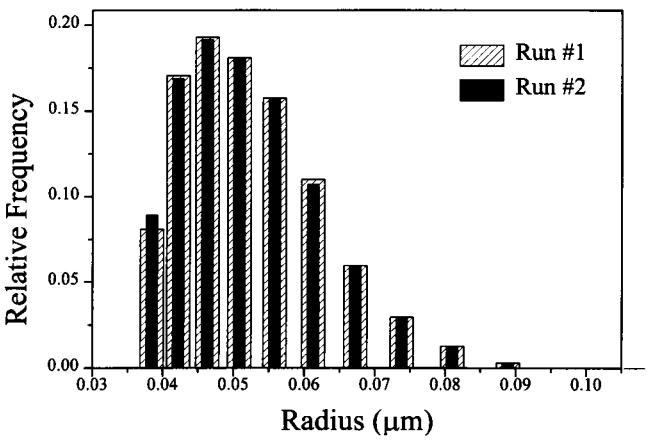

FIGURE 4 Histograms obtained by ILS spectroscopy for two separate experiments on the same batch of extruded POPC:POPG LUV with a 206:1 peptide/vesicle ratio. Conditions as described in Fig. 2.

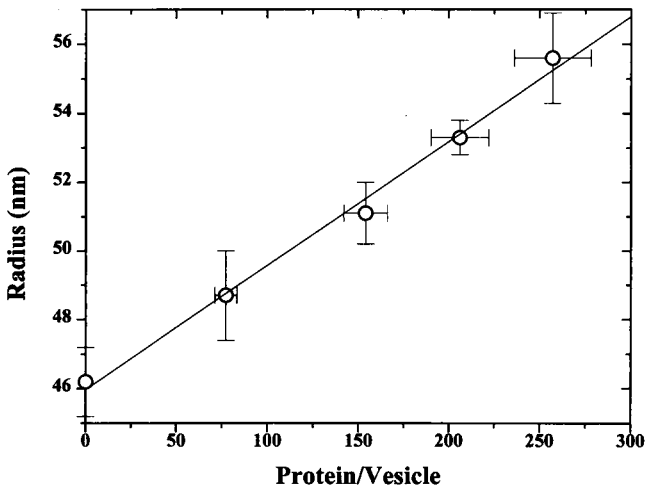

FIGURE 5 A plot showing membrane vesicle radius determined by ILS spectroscopy as a function of number of peptides added per vesicle.

is even less defined, but a value of 0.70 nm^2 was used (Marsh, 1990). An average value of 0.65 nm^2 with an uncertainty of 0.05 nm^2 was obtained by using these two areas for the POPC and POPG headgroups in a 60:40 molar ratio. Inasmuch as the concentration of lipid was known, a value was calculated for the total number of vesicles present in each sample. The concentration of protein in each sample was used to obtain a value for the number of proteins per vesicle (Table 1). This was possible because under these conditions the amount of free protein in solution was not detectable. It was previously demonstrated that the peptide binds LUV with high affinity (K_a, 2–4 × 10^9 M^{-1}, Zhang et al., 1994; A. R. Merrill, unpublished results).

Fig. 5 is a plot showing the vesicle radius as a function of the number of protein molecules per vesicle. The correlation coefficient was 0.997, which indicated good linearity. These data clearly demonstrate the dose-dependent increase in vesicle size upon titration with the channel peptide. In Fig. 6 the lipid bilayer volume was plotted as a function of number of protein molecules per vesicle. The slope of this line gives the volume per protein directly; it was found to be 177 ± 22 nm^3 (2 SD). Therefore, the protein occupies ~0.1–0.2% of the total volume of the bilayer at a protein-to-vesicle ratio of 257:1. Although other models have been proposed (Merrill and Cramer, 1990; Zhang and Cramer, 1992; Palmer and Merrill, 1994) concerning the membrane-associated structure of the closed state of the colicin E1 channel peptide, in the present study only protein-induced volume changes were determined. This is because volume changes, unlike surface area differences, are independent of the geometry of the displacing peptide moiety which, when membrane-bound, is presently not known. Furthermore, the absolute particle size depends on the accuracy of the polydisperse calibration. Uncertainties of 3–5 nm are quite plausible; this uncertainty level has a significant effect on the determination of the number of protein molecules per vesicle but does not negate the relative vesicle size increases seen upon peptide titration.

CONCLUSION

ILS spectroscopy is sufficiently sensitive to detect changes in membrane vesicle radii of a few nm. This result suggests the feasibility of measuring vesicle size changes not previously thought possible by light-scattering methods. Even though long run times were carried out to obtain the best signal possible, DLS was unable to provide the stability or the resolution to monitor these vesicles size changes. These results obtained by using ILS spectroscopy convincingly

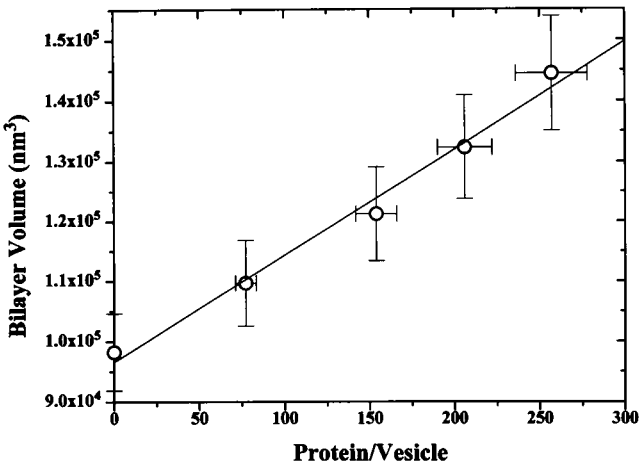

FIGURE 6 A plot showing the volume of the bilayer determined by ILS spectroscopy as a function of number of peptides added per vesicle. The slope was used to determine the volume per membrane-bound peptide of 177 nm^3.

demonstrate that the colicin E1 channel peptide penetrates into the nonpolar hydrocarbon core of the membrane upon the formation of the closed channel state. This is evidenced by the dose-dependent swelling of LUV upon titration with the channel peptide.

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