Sizing Membrane Pores in Lipid Vesicles by Leakage of Co-Encapsulated Markers: Pore Formation by Melittin

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ABSTRACT Many toxins and antimicrobial peptides permeabilize membrane vesicles by forming multimeric pores. Determination of the size of such pores is an important first step for understanding their structure and the mechanism of their self-assembly. We report a simple method for sizing pores in vesicles based on the differential release of co-encapsulated fluorescently labeled dextran markers of two different sizes. The method was tested using the bee venom peptide melittin, which was found to form pores of 25–30 Å diameter in palmitoyloleoylphosphatidylcholine (POPC) vesicles at a lipid-to-peptide ratio of 50. This result is consistent with observations on melittin pore formation in erythrocytes (Katsu, T., C. Ninomiya, M. Kuroko, H. Kobayashi, T. Hirota, and Y. Fujita. 1988. Action mechanism of amphiphatic peptides gramicidin S and melittin on erythrocyte membrane. Biochim. Biophys. Acta. 939:57–63).

INTRODUCTION

Induced permeabilization of cell membranes is one of the most common killing mechanisms of peptides that have cytotoxic or host-defense functions. For several such peptides, formation of multimeric pores has been suggested to be responsible for membrane permeabilization (Rapaport et al., 1996; Rex, 1996; Schwarz and Arbuzova, 1995; Matsuzaki et al., 1994; Wimley et al., 1994; Schwarz et al., 1992; Parente et al., 1990). The first step toward understanding the structure and function of such pores is the determination of their size as gauged by the dimensions of the molecules able to pass through them. This is usually done by measuring the leakage of markers of different sizes through the pore in consecutive experiments (Rex, 1996; Wimley et al., 1994; Ostolaza et al., 1993; Sabirov et al., 1990; Parente et al., 1990; Katsu et al., 1988; Tosteson et al., 1985). In addition to the time required to carry out consecutive experiments, unavoidable variations in experimental conditions, such as the concentrations of lipid and peptide, can make the approach cumbersome to implement and interpret. The basic approach would be greatly simplified, and strengthened, if the sizing of the pores could be accomplished in a single experiment.

We describe here a simple method for sizing pores in lipid vesicles based on the differential release of co-encapsulated markers of different sizes. Vesicles are preloaded with fluorescently labeled dextrans FD-4 (4.4 kDa mol wt) and FD-70 (50.7 kDa mol wt) and then incubated with the leakage-inducing agent. The relative release of the two markers is determined quantitatively by means of a flow-through cuvette placed in a fluorescent spectrometer that monitors the elution profile from a gel filtration column. The method was tested using the bee venom peptide melittin, which is known to cause hemolysis (Katsu et al., 1988; Tosteson et al., 1985; DeGrado et al., 1982) and to induce leakage of fluorescent dyes from lipid vesicles (Rex, 1996; Benachir and Laffleur, 1995; Ladokhin et al., 1995; Schwarz et al., 1992). In our study, melittin was found to release 88% of FD-4 but only 22% of FD-70 from large unilamellar POPC vesicles at a lipid-to-peptide ratio of 50. Based on these data, we estimate that the average pore diameter is 25–30 Å, consistent with observations on melittin hemolysis of erythrocytes (Katsu et al., 1988).

MATERIALS AND METHODS

Materials

POPC was obtained from Avanti Polar-Lipids (Birmingham, AL). Melittin (sequencing grade), Triton X-100, FD-4 dextran, and FD-70 dextran were obtained from Sigma (St. Louis, MO) and Sepahcryl S-400 HR from Pharmacia Biotech (Uppsala, Sweden). The buffer solution (pH = 7.0) used for most experiments contained 10 mM HEPES, 50 mM KCl, 1 mM EDTA, and 3 mM NaN3. Buffer for melittin samples contained 4 mM EDTA. The buffer was carefully degassed before each run to avoid formation of air bubbles on the column.

Choice of column media for gel filtration

The implementation of the method depends crucially on the quality of the separation of dextran-loaded vesicles from the two sizes of released dexo-
trans. The gel filtration system used must satisfy three criteria. First, a good general resolution of the components must be achieved. Second, because the three components in the injected mixture (vesicles, larger marker, and smaller marker) cover a wide range of sizes, the range must be matched by the resolving ability of the column. Third, both vesicles and dextrans have a distribution of sizes that causes broadening of the elution profiles. This has implications which are discussed in Results and Discussion. We found that Sephacryl S-400 HR satisfied these criteria and permitted separation of LUV from FD-70 and FD-4.

Preparation of dextran-loaded vesicles and leakage experiments

LUV of ~0.1 μm diameter were formed by extrusion under N₂ pressure through Nucleopore polycarbonate membranes using the method of Mayer et al. (1986). To prepare LUV with entrapped solutes, the lipid was suspended in buffer containing 2 mg/ml FD-4 and 4 mg/ml FD-70, and was then frozen and thawed 20 times before extrusion, and several additional times during the extrusion process. Unentrapped dextran was removed by gel filtration using Sephacryl S-400 HR packed into a 38 × 1-cm column run at 12 ml/h controlled by a P-1 peristaltic pump (Pharmacia Biotech, Uppsala, Sweden). At the concentration of dextran used for encapsulation, no self-quenching was observed. Fluorescein dextran-containing LUV were treated either with Triton, to determine the ratio of entrapped dextran, or with melittin, to examine differential dextran release. The treated LUV were applied at 4.6 ml/h to a column connected to a 176.752-QS flow-through fluorescence cuvette (Hellma, Germany) placed in an SLM-8100 fluorimeter (SLM/Aminco, Urbana, IL). Excitation and emission wavelengths were 490 nm (4-nm slits) and 530 nm (16-nm slits), respectively. During the initial separation, the slits were 1 nm and 2 nm, respectively. A polarizer oriented horizontally was introduced into the emission path to minimize scattering artifacts. All experiments were carried out at room temperature (23°C).

Data analysis

Elution profiles were determined by measuring relative fluorescence intensity I (sampled at 10-s intervals) as a function of time t. We found that the elution profiles for a single dextran species could be represented accurately as log-normal distributions, as has been observed in diverse spectroscopic analyses of complex spectra (Donoso et al., 1993; Johnson and Metzler, 1970). We therefore estimated the fractions of FD-4 and FD-70 released by determining the areas of elution profiles obtained from best-fit log-normal distributions:

\[ I(t) = \sum_i I_i \cdot \exp \left( -\frac{\ln 2}{(\ln \rho_i)^2} \left[ \ln \left( 1 + \frac{(t - t_i) \cdot (\rho_i^2 - 1)}{\rho_i \cdot \Gamma_i} \right) \right]^2 \right) \]

\[ I_i \neq 0 \text{ for } t > t_i - \frac{\rho_i \Gamma_i}{\rho_i^2 - 1} \]

\[ I_i = 0 \text{ for } t < t_i - \frac{\rho_i \Gamma_i}{\rho_i^2 - 1} \]

where i = LUV, FD-70, or FD-4; and \( t_i, \Gamma_i, \) and \( \rho_i \) are four parameters of the distribution, corresponding to maximal intensity, position of maximum, width, and asymmetry, respectively.

The typical release experiment results in an overlapping three-peak elution profile so that 12 parameters are required to describe it. This large number of parameters was found to cause instabilities if fitted simultaneously in a single pass. The following procedure was therefore used. The data in the early phase of release (1 × 10⁵ to 1.5 × 10⁵ s) corresponding to the LUV-encapsulated dextrans were fitted with a single log-normal distribution using the Marquardt-Levenberg fitting algorithm (Press et al., 1989). The resulting computed log-normal distribution was then subtracted from the complete profile, leaving a two-peak distribution corresponding to the two classes of dextrans released from the vesicles. This residual distribution was then fitted to a sum of two log-normal distributions corresponding to released FD-70 and FD-4. During the latter fit, a single parameter (of the remaining eight) corresponding to the asymmetry of the least-resolved FD-70 peak was fixed at \( \rho_{FD-70} = 1.3 \) value determined independently from the elution profile of the free dextran (data not shown). The areas under the peaks were calculated numerically using software package Origin 3.5 (MicroCal, Inc., Northampton, MA). The relative fractions of individual markers were calculated from the ratios of the areas of the individual peaks for samples with partially and completely released contents. Complete release was achieved by treating vesicles with 0.5% Triton X-100. The uncertainties in fractions were estimated to be 0.05.

RESULTS AND DISCUSSION

The first step in the development of the method was the characterization of the encapsulation process. Fig. 1 shows the elution profile associated with the separation of LUV-encapsulated FD-4 and FD-70 from unencapsulated dextrans. A 0.3-mL sample containing 70 mM POPC vesicles was applied to a 38 × 1 cm Sephacryl S-400 HR column connected to a flow-through cuvette placed in the SLM-8100 fluorescence spectrometer. Conditions of excitation and emission were selected to follow the fluorescence of labeled dextrans and to minimize the contribution from scattering (see Methods). Dextran-loaded LUV appear close to the void volume at 15 ml and are well separated from the free FD-70 and FD-4 peaks, which appear at 23 and 28 ml, respectively.

![FIGURE 1 Elution profile demonstrating the separation of LUV with co-encapsulated fluorescently labeled dextrans FD-4 and FD-70 from unencapsulated markers. A 0.3-mL sample containing 70 mM POPC vesicles formed in the presence of FD-4 and FD-70 was applied to a 38 × 1 cm Sephacryl S-400 HR gel filtration column connected to a flow-through cuvette placed in a fluorescence spectrometer. The flow rate was 12 ml/h. The shaded area corresponds to the fraction of LUV used in further studies.](image-url)
The fraction of LUV eluted between 14.0 and 16.5 ml was used for all further studies (shaded area in Fig. 1). This fraction, when run on the same column, elutes as a single peak as shown by the dashed curve in Fig. 2. To verify that both markers were indeed encapsulated, the vesicles were disrupted with Triton X-100 before application on the column. Fig. 2 shows that the single LUV peak is replaced by two well-resolved peaks corresponding to the now-free FD-70 and FD-4 (solid curve). The change in the relative areas of the FD-4 and FD-70 peaks seen in Fig. 2, compared with Fig. 1, indicates less efficient encapsulation of the larger dextran. To check for the possibility of interactions of the dextrans with vesicle surfaces, we added dextrans externally to “empty” LUV, and then passed the mixture through the column. No fluorescence was observed at elution volumes corresponding to the LUV (data not shown). We therefore concluded that the fluorescence corresponding to the LUV peak arose solely from entrapped dextrans.

The Triton disruption experiment serves two purposes: it allows one to estimate the ratio of the entrapped dyes and to control the quality of the separation of unentrapped dextrans from dextran-loaded LUV. This is necessary because commercially available dextrans have a distribution of sizes so that a dextran of specified molecular weight contains significant percentages of dextrans that are both larger and smaller than the specified characteristic size. If the initial separation of the large unentrapped dextrans from the vesicles is inadequate, there will be a population of large dextrans that can co-migrate with the loaded vesicles. When the sample is subsequently re-run through the column after incubation with a permeabilizing peptide, this fraction can cause the amount of release to be underestimated. The addition of Triton allows this fraction of dextrans to be easily detected as an extra peak early in the elution. Experiments of this kind showed that other gels, such as Sephadex G-100, for example, do not give a complete separation of LUV and dextrans larger than FD-4 (data not shown). The use of Sephacryl S-400 HR, however, resulted in complete separation, as evidenced by the data of Fig. 2.

A peptide that forms pores, defined as passageways of any kind that have limited apertures, will lead to leakage of vesicle contents in a manner that depends on the size of the solute. If, however, the peptide acts in a detergent-like manner and “dissolves” (micellizes) the membrane, there should be no preference in the release of markers of different sizes. If pores or other leakage pathways are very small, then no release of dextrans will be detected. The application of the method was tested using the well-studied membrane-active peptide, melittin.

Melittin was found to cause the release of both markers, but to significantly different extents as shown in Fig. 3 by the fact that the FD-4 peak is much larger than the FD-70 peak in the elution profile (solid line). This indicates the formation of a pore rather than disruption through a deter-

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**FIGURE 2** Elution profile for the LUV with co-encapsulated FD-4 and FD-70 in the absence and presence of Triton X-100. Dashed curve on left is the profile for untreated (intact) vesicles. The twin-peaks profile, solid curve, results when vesicles are disrupted with 0.5% reduced Triton X-100, and demonstrates that both FD-4 and FD-70 are co-encapsulated in vesicles. For both elution profiles, 0.2-ml samples containing 2 mM POPC were run through the same column as in Fig. 1, but at 4.6 ml/h.

**FIGURE 3** Release of fluorescently labeled dextrans from POPC vesicles induced by melittin. A 0.3-ml sample containing 28 µM melittin and 1.4 mM POPC LUV preloaded with FD-4 and FD-70 was incubated for 3 h and then applied to the gel filtration column (same conditions as in Fig. 2). The solid curve is the observed time trace of the elution profile. The dashed lines are the log-normal approximations of individual components corresponding to (from left to right) LUV encapsulated dextrans, released FD-70, and released FD-4. The dotted curve is the sum of the log-normal distributions of the individual components. It demonstrates that the relative amounts of FD-4 and FD-70 released can be accurately determined by means of the log-normal fitting procedure. The release of FD-4 by melittin is much more effective as compared with the release of FD-70, indicating the formation of pores ~25–30 Å in diameter under these conditions (see text).
gent-like action or some other "collective membrane perturbation" (Benchar and Lafleur, 1995). To quantitate the preference of the release, the elution profile was decomposed into three components: unreleased dextran, released FD-70, and released FD-4, each of which can be approximated as log-normal distributions as described in Methods (dashed lines, Fig. 3). The sum of these computed log-normal distributions, shown as the dotted line in Fig. 3, coincides well with the actual data (solid line). The fractions of FD-4 and FD-70 released from the vesicles by melittin are estimated to be 88% and 22%, respectively. This result was reproduced within the uncertainty of the method (see Methods) in a second run under identical conditions (not shown).

The effective hydrodynamic radii for FD-4 and FD-70 are estimated to be 18 and 50 Å, respectively, from the data of Bohrer et al. (1979). However, as the study of Bohrer et al. indicates, the dextrans are best modeled as prolate ellipsoids. By extrapolation from Bohrer et al.'s data, we estimate that the full length of the short axis is ∼20 Å for both dextrans, while the long axes are ∼40 and 440 Å for FD-4 and FD-70, respectively. A pore aperture of 25–30 Å should therefore allow a relatively free passage of FD-4 while reducing significantly the leakage of FD-70, which might pass through the pore by reptation. A pore of this aperture should allow complete leakage of smaller molecules such as calcein, carboxyfluorescein, and ANTS/DPX. Indeed, all of those markers have been reported to be completely released from vesicles at lipid-to-protein ratios below the value of 50 used here (Rex, 1996; Benchar and Lafleur, 1995; Ladokhin et al., 1995; Schwarz et al., 1992).

Consistent with our results, studies on osmotic protection of erythrocytes led to an estimated pore size of ∼20–30 Å at high melittin concentrations (Katsu et al., 1988). These protection experiments also showed that the size of pores formed by melittin or another peptide, gramicidin S, increased with peptide concentration. For gramicidin S, the degree of hemolysis dropped abruptly with an increase of solute size (Katsu et al., 1988), whereas for melittin, a rather broad transition occurs (Sabirov et al., 1990). This behavior can be rationalized by assuming that melittin forms pores that have a rather wide distribution of sizes. This suggests that our estimate for the pore diameter should be attributed to a mean pore size characteristic of the distribution. A significant population of larger pores could result in enhanced release of FD-70 while having little effect on the release of FD-4. In such a case, the ratio of the fractional release of two dextrans is expected to decrease as the width of distribution of pore sizes increases.

Melittin's secondary structure in membranes is well established to be highly α-helical, but its degree of aggregation and its membrane orientation are subjects of continuing debate [see Dempsey (1990) for review]. A reasonable view at the present time is that multiple aggregated forms of melittin can exist in membranes that are affected by temperature, lipid composition, and lipid-to-peptide ratio. One such form may consist of aggregates containing transbilayer helices (Vogel and Jähnig, 1986). In that case, our estimate for the pore size of 25–30 Å in inner diameter suggests that 10–15 monomers would be required. This is quite reasonable, because under conditions used in our measurement each vesicle, on average, should contain 2000 melittin molecules, assuming a mole-fraction partition coefficient of 4 · 10^5 (W. C. Wimley, A. S. Ladokhin, and S. H. White, unpublished observations). Thus, the number of monomers is more than sufficient to form at least one pore per vesicle, assuming a modest equilibrium constant between monomers and multimers.

Leakage induction by melittin has been studied extensively and the formation of small pores (10 Å in diameter) has been suggested to be responsible for leakage of the small dye carboxyfluorescein (Rex, 1996; Schwarz et al., 1992). These studies were, however, confined to relatively low peptide concentrations (R_i = 200–2000). On the other hand, at extremely high concentrations (R_i < 20) a complete loss of vesicular structure has been demonstrated (Dufourcq et al., 1986; Monette and Lafleur, 1996). One can rationalize two possibilities for the intermediate case of moderate concentrations: 1) the number of pores increases, but their size remains fixed; or 2) the average multiplicity of aggregates increases, leading to larger pores, which allow the leakage of larger molecules. Our result on melittin pore formation in POPC vesicles obtained for R_i = 50 suggest the second possibility, consistent with studies on erythrocytes (Katsu et al., 1988).

We have demonstrated that measurement of the leakage of co-encapsulated markers of different sizes provides a fast and convenient way of sizing membrane pores. The method eliminates the unavoidable variations in experimental conditions that occur when pores are sized through measurements of leakage in consecutive experiments. The complete characterization of pore formation by uncharacterized peptides will require studies with several sets of co-encapsulated markers to explore a wide range of concentrations and sizes. For an extremely well-studied peptide, a single experiment can yield new information as we have demonstrated in the case of melittin.

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