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A DETERGENT DEPLETION TECHNIQUE FOR THE PREPARATION OF SMALL VESICLES

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ABSTRACT: A simple method for the preparation of small phospholipid vesicles is presented. This method utilizes the rapid dilution of octyl glucoside-egg lecithin mixed micelles to a final detergent concentration below the critical micelle concentration, at which the bilayer state is favored. Electron microscopic and gel filtration chromatographic analysis of the resulting vesicle preparations show that the rapid dilution technique results in a uniform population of vesicles with a mean diameter of 20-28 nm. This result supports the hypothesis that vesicle formation occurs through the fusion of discoidal mixed micelles, the geometry of which is determined by the phospholipid and detergent content.

INTRODUCTION

A number of methods are reported for the preparation of phospholipid vesicles. These methods utilize sonication,[1,2] solvent injection,[3,4,5] reverse phase evaporation,[6] filter extrusion,[7] and detergent depletion from mixed micelles with dialysis[3,8,9,10] or gel filtration chromatography[3,11,12] to produce vesicles. Each method has advantages and disadvantages, and produces a characteristic vesicle size distribution. Ether removal and dialysis generally produce larger vesicles (100 to 500 nm diameters) which are useful for, among other things, encapsulation and transport studies. Solvent injection, sonication, and gel filtration methods can yield smaller vesicles (10 to 50 nm diameters), which are desired for many types of experiments, including NMR studies where peak resolution is needed. This communication describes a method for producing homogeneously sized small vesicles by rapidly diluting octyl glucoside-egg lecithin mixed micelles to final detergent concentrations (below the critical micelle concentration) at which micelles are not stable. This method yields a population of vesicles with diameters of about 25 nm that is approximately homogeneous with respect to size distribution and stable with respect to further size changes.

EXPERIMENTAL

Egg phophatidylcholine (egg PC) was obtained from Lipid Products, U.K., and used without further purification.

Octyl glucoside (1-octyl-β-glycopyranoside) was obtained from Calbiochem, while sodium dodecyl sulfate was purchased from New England Nuclear. Polystyrene beads and Sepharose CL-2B were respectively obtained from Polyscience, Inc. and Pharmacia. All buffers and salts were reagent grade. Hemoglobin and Hemocyanin were obtained from Sigma.

The preparation of small vesicles proceeded as follows: First, octyl glucoside-egg PC mixed micelles were prepared with 100 mM octyl glucoside and 10 mM egg PC, by adding the desired amount of solid octyl glucoside to a multilamellar dispersion of 10 mM egg PC in buffer A (10 mM Tris/Tris HCl, 1.0 mM EDTA·Na_2, pH = 7.4). The dilution was performed by adding, from a syringe, 1.0 ml of the mixed micelles at a rate of 1.0 ml/min to 9.0 ml of buffer A. During the addition, the vial containing the buffer was vortexed at top speed. The actual dilution event took place on a millisecond timescale, as determined by preforming a similar dilution with solutions containing dye. After dilution, the suspension was dialyzed for 48 hrs against two 2.0-liter changes of buffer A at 22°C. The resulting vesicles were characterized with respect to phospholipid and octyl glucoside content with the phosphate determination of Bartlett[13] and the glucoside analysis of Kowald and McCormak.[14] The essential feature of this method for preparing small vesicles was that the dilution event occured as fast as possible. The initial and final detergent and phospholipid concentrations were not critical if the requirements that the phospholipid was initially fully solubilized and that the final octyl glucoside concentration was well below the critical micelle concentration (25 mM)[15] was met. Also, factors such as temperature and dialysis time

had little effect on the final result, as long as excess detergent was adequately removed.

After dialysis, the resulting vesicles were analyzed with negative staining EM techniques and with gel filtration chromatography. In preparation for electron microscopic examination, carbon coated grids were blotted first with vesicle suspensions, followed by 2% (w/v) uranyl acetate or 2% (w/v) sodium phosphotungstinic acid, pH = 6.5. Gel filtration chromatography of vesicle suspensions utilized a Sepharose CL-2B column. The 1.3 x 48.0 cm column was pre-equilibrated and eluted with buffer A. Three ml of dialyzed vesicle suspension was applied to the column and eluted at a flow rate of approximately 7.5 ml/hr. Chromatography was performed at 22°C. Chromatography fractions were then analyzed for phosphate content using Bartlett's procedure.[13] The void volume (V_o) and total column volume (V_t) of the column were respectively determined with blue dyed polystyrene beads (diameter = 500 nm) and bromophenol blue as follows: The CL-2B column was preincubated and eluted with 20 mM sodium dodecyl sulfate in buffer A. One ml of a solution containing 12.5 mg beads, 0.1 mM bromophenol blue and 20 mM sodium dodecyl sulfate in buffer A was applied to the column. The absorbance at 595 nm of the eluted fraction was noted. Sodium dodecyl sulfate was required to prevent aggregation of the beads.

The dependence of elution position on particle size of the CL-2B column was investigated by independently chromatographing 60 nm polystyrene beads, hemocyanin and hemoglobin. The 60 nm beads were chromatographed under the same conditions as the 500 nm beads. The turbidity at 340 nm was measured to determine the elution position of the beads.

Hemocyanin and hemoglobin were chromatographed under the same conditions as the small vesicles. Hemocyanin and hemoglobin solutions in buffer A, with protein concentrations of 1 mg/ml, were applied to the column. The elution profiles of hemoglobin and hemocyanin were respectively measured by noting the absorbence at 410 and 280 nm.

RESULTS

The method used to produce vesicles was based upon the rapid dilution of mixed micelles to a final concentration at which the bilayer state was favored. Upon diluting optically transparent octyl glucoside-egg PC mixed micelles to 10 mM octyl glucoside, 1 mM egg PC, a slight amount of turbidity (A_{340} = 0.04, 1 cm path length) was noted. No further change in turbidity was noted as a function of time after dilution, indicating that no additional particle growth occurred. After two days of dialysis of the diluted suspensions, a further increase in turbidity was noted (A_{340} = 0.08, 1 cm path length). Further dialysis or storage for up to three weeks (at 3°C, with EDTA) of vesicle suspensions did not result in the development of additional turbidity. This indicated that the vesicles were stable with respect to further growth. Based on phosphate and glucoside analysis, the phopholipid/octyl glucoside ratio was 12/1.

Electron microscopic examination of the vesicles revealed a population of particles with 15 to 35 nm diameters, with a mean diameter of 20 nm, as demonstrated in Fig. 1. This result was obtained with both uranyl acetate

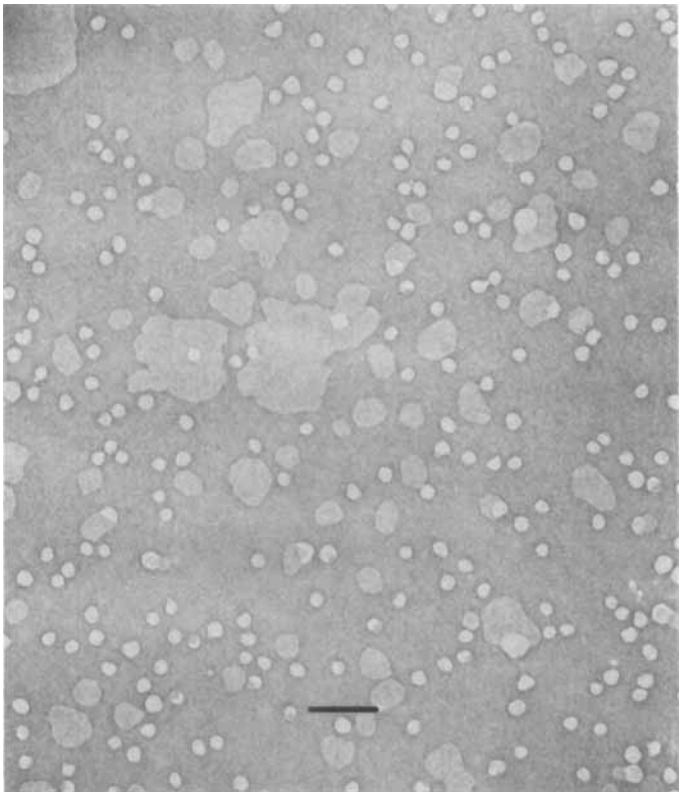

FIGURE 1. Negative stain electron micrograph of vesicle preparation with phosphotungstinic acid stain. Bar at the bottom represents 100 nm.

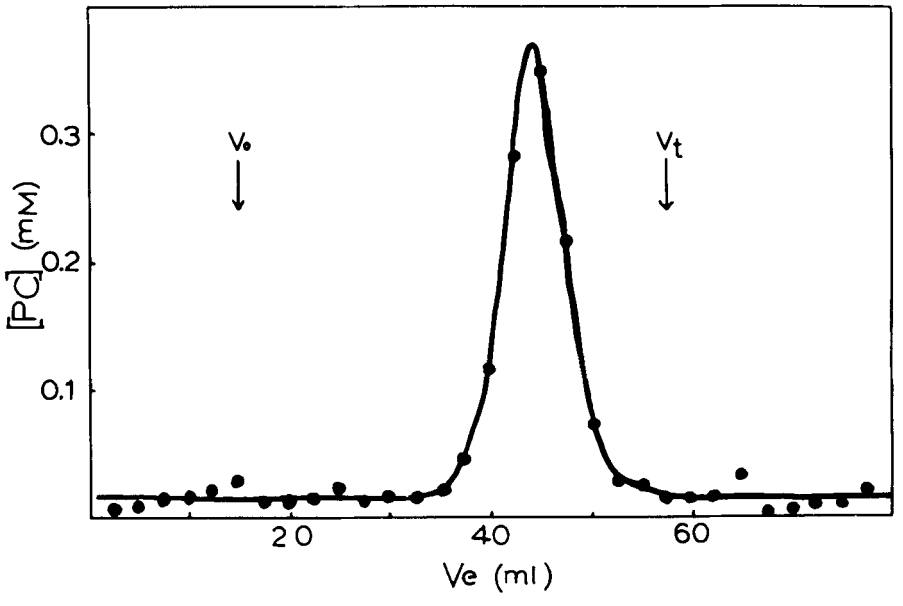

FIGURE 2. Elution profile of small vesicle preparation on Sepharose CL-4B. Arrows refer to the void volume (V_o) and total column volume (V_t) positions.

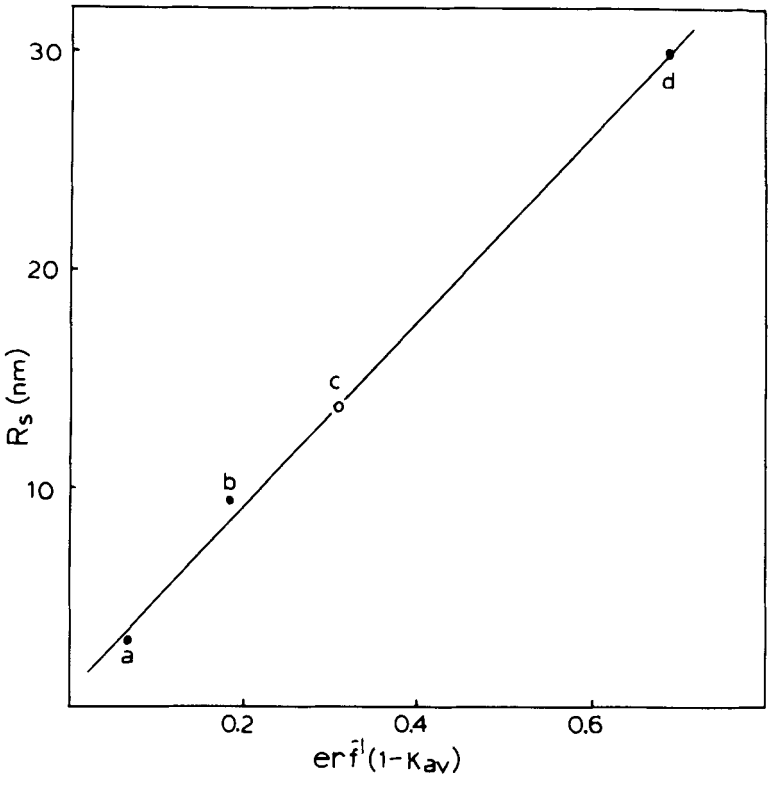

FIGURE 3. Stokes Radius (R_s) vs $erf^{-1}(1-K_{av})$ for hemoglobin (a), hemocyanin (b), small vesicles (c) and polystyrene (d).

and phosphotungstinic acid stains. The size distribution of particles was also analyzed with gel-filtration chromatography. As demonstrated in Fig. 2, chromatography of the vesicle suspensions on Sepharose CL-2B yielded a single, approximately symmetrical peak with a K_{av} value of 0.66. Yield of phospholipid from the column was essentially quantitative. These results indicated that the mixed micelle dilution procedure described in this communication yielded a uniform population of small vesicles which was largely free of contamination by larger vesicles and/or multilammelar structure.

The size extimate based on EM measurements was further supported by the data depicted in Fig. 3. A comparison of the elution positions of the small vesicles with hemoglobin (Stokes radius (R_s) = 3.0 nm), hemocyanin (R_s = 9.5 nm) and polystyrene beads (R_s = 30.0 nm) gave a vesicle diameter of 28 nm.

To obtain a uniform population of small vesicles, it was absolutely necessary to maximize the dilution rate of the mixed micelle suspension. We attempted to develop a procedure for the production of larger vesicles that was based on a controlled rate of dilution (-0.01 to -10 mM octyl glucoside/sec) of the mixed micelle suspension. This procedure resulted in the production of vesicles with a heterogeneous (20 to 200 nm) size distribution. Furthermore, the characteristics of the size distribution seemed to be a stronger function of rheological factors related to the stir rate and vessel shape than to the dilution rate. A heterogeneously sized vesicle population was probably obtained because microscale dilution rate was not uniform.

DISCUSSION

The results presented in this study demonstrate that small vesicles are formed when the detergent depletion rate is maximized. This result, as well as the tendency for methods that utilize slower detergent depletion rates to produce larger vesicles,[3,8,9,10] can be understood by considering recently developed theoretical treatments of the vesicle formation process.[16,17]

The vesicle formation theory of Danieli[16,17] predicts that a homogeneous population of small vesicles results when the dilution rate is maximized as follows: When the mixed micelle suspension is diluted below the critical micelle concentration (25 mM), the octyl glucoside rapidly partitions into the aqueous phase, depleting the micelles of detergent. To protect the diacyl hydrocarbon chains from a polar environment, the micelles must fuse into disc-like structures with the detergent largely distributed at the disc edges. As detergent depletion continues, the disc-like micelles undergo additional fusion events to minimize the total disc circumference of the system. The disc structures bend, reducing the circumference even more. Ultimately, when the disc-like structures obtain a certain size and/or phospholipid-to- detergent ratio, they vesiculate. The final vesiculation step eliminates the disc edges.

This mechanism for vesicle formation can be applied only above a certain critical time (probably on a millisecond timescale) required for micelles to fuse into particles large enough to vesiculate. The minimum particle size required for vesiculation is expected to be a function

of factors that effect the elastic properties of the membrane, such as acyl chain structure and residual detergent content. The critical time probably depends on factors that influence the fusion frequency, such as concentration, diffusion coefficients, and efficency of fusion. In the hypothetical experiment where the detergent is removed infinitely fast, the phospholipid would probably flocculate. Utilizing the rapid dilution technique outlined in this paper, vesicle formation occurs near, but not below, the critical time. The disc-like micelles evidently close upon themselves as quick as possible, when a geometry suitable for vesiculation is obtained, resulting in a homogeneously sized preparation of small vesicles. In contrast to this result, when detergent is depleted at a slower rate (by slowing the dilution rate or with dialysis), larger vesicles are obtained. In this case, the disc-like micelles have additional time to undergo collision-induced fusion before vesiculation occurs.

In summary, a technique for preparing a homogeneous preparation of small vesicles is presented. This result can be understood in terms of a) a reduced mixed micelle fusion time and b) a substantial residual detergent content, which permits a high radius of curvature.

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