Letters to the Editor

A Possible Mechanism for Vesicle Formation by Extrusion

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Despite the common use of the extrusion method to prepare large unilamellar vesicles (Hope et al., 1985; Mui et al., 1993), the mechanism of their formation has not been characterized. However, careful examination of the experimental conditions and a comparison with the theories of emulsion formation lead to the following speculative considerations about the mechanism.

The filter membranes used for extrusion have cylindrical, straight-through pores that are long and narrow tubes. The most commonly used filters have pores 0.1 μm in diameter and 6 μm in length, as stated by the manufacturer (Nucleopore, Pleasanton, CA). When a multilamellar vesicle is forced through such a pore, a cylindrical structure must be formed. The average linear flow velocity (cm/s) of the aqueous solution through one pore can be estimated as

$$\bar{u} = \frac{V}{\rho\pi^2 R^2 r^2 t}, \quad (1)$$

where V is the total volume of the suspension, ρ is the number of pores per unit area of the membrane, R is the radius of the filter membrane, r is the radius of the pore, and t is the time needed to extrude the vesicle suspension. Under the conditions usually used to make vesicles by extrusion through filters with 0.1 μm diameter pores, the average linear flow velocity is about 3 cm/s. At such a low speed, the solvent flow in the pore is laminar. The linear flow velocity of a viscous fluid through a uniform pore has a parabolic profile (Tanford, 1961)

$$u(x) = \frac{P}{4l\eta}(r^2 - x^2), \quad (2)$$

where P is the applied pressure, η is the viscosity of the fluid, l is the length of the pore, r is its radius, and x is the distance from its center. The velocity is equal to zero at the wall of the pore ($x = r$), and reaches a maximum, u_{max}, in the center ($x = 0$); under the conditions typically used to extrude vesicles, u_{max} is equal to 20 cm/s for pure water. Thus, a velocity gradient exists across the pore that has a maximum value at the wall and is equal to zero at the pore center with an average value of about 10^7 s^{-1}. When multilamellar vesicles are forced to move into the pores, cylindrical structures consisting of four or less concentric lamellae are formed (if it is assumed that one lipid lamella plus water layer is 100 Å in thickness, cylinders with five lamellae could be accommodated within the pore; however, the radius of curvature of the inner-most lamella then is 50 Å, which is too small to be tolerated by a bilayer). It seems very likely that a shear field can exist also when a suspension of multilayer vesicles is extruded (in that case, the pores are filled with water plus lipid bilayers). Then the velocity gradient produces shear between phospholipid lamellae, yielding cylindrical lipid bilayers.

When these phospholipid bilayer cylinders reach the end of the pores, they break into smaller structures. It seems possible that the mechanism of the break-up of the cylindrical structures is similar in principle to the break-up of cylindrical soap films and micelles. Simple experiments show that a cylindrical soap bubble is stable until its length reaches a critical value equal to the perimeter of the cylinder (Boys, 1911). This phenomenon has been extensively examined in the theory of emulsion formation; an emulsion shaped as a cylindrical thread of radius $\bar{r}$ and subjected to small random deformations breaks into small droplets when the wavelength of the deformation is $\lambda = 2\pi\bar{r}$ (Walstra, 1983). According to this theory, the phospholipid bilayer cylinders formed in the filter pores are broken in smaller cylindrical structures of length λ, which are unstable and seal themselves to form vesicles. For this analysis to be correct, the surface tension of the cylindrical bilayer must be non-zero (Walstra, 1983). We believe this to be the case. The surface tension of soap-film analog bilayers has been determined experimentally to be about 1 dyne cm^{-1} (for a summary of data, see Jain, 1972). The surface tension of solvent-free liposome bilayers of the type used for vesicle extrusion can be increased by mechanical tension (Cevc and Marsh, 1987). It is quite possible that the extrusion process puts the bilayers under tension. In this regard, it should also be mentioned that spontaneous shape changes in bilayer systems have been known for years. They are described in terms of the curvature elastic-energy function of the bilayer (Lipowsky, 1991).

It is interesting to compare the surface and internal aqueous volume relations in the cylindrical fragments into which the extruded cylinders break-up with the putative spherical forms that these fragments might ultimately assume. If we imagine that either the bilayer surface or the aqueous internal volume of the cylindrical fragment is conserved, it is easy to show that the ratio of the radius of the final sphere to the radius of the initial cylindrical fragment is $[\pi]^{1/2}$ (=1.77) for area conservation, and is $[3\pi/2]^{1/3}$ (=1.68) for volume conservation. This result is obtained by equating the length of

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the cylindrical fragment with the wave length ($\lambda = 2\pi\bar{r}$) of the deformation that leads to the break-up of the extruded cylinder. It is clear that, if both bilayer area and the internal volume of the cylindrical fragment are conserved, the final form of the fragment will be nonspherical. The form that is found experimentally, in fact, is an indented sphere (Clerc et al., 1992). As noted above, the radius of the putative sphere ultimately assumed by the fragment at constant volume is 5% smaller than that obtained under the constant bilayer area assumption. Thus, the indented spherical vesicle has 15% less volume than it would have if it were a true sphere. The indented spherical vesicles produced experimentally by extrusion can be osmotically expanded to spheres. The increase in volume that occurs can be determined quite accurately by measuring the increase in fluorescence of a partially self-quenched fluorophore trapped in the internal aqueous volume of the vesicles. This increase in volume is about 40% (S. G. Clerc and T. E. Thompson, unpublished results).

The maximum diameter of the constant-area spherical vesicle that can be produced in pores of 0.1 μm diameter is 0.177 μm. This size is in good agreement with that found experimentally (Clerc et al., 1992). However, because $\bar{r}$ can vary between the smallest radius of curvature the bilayer can tolerate and the radius of the pore, there must be a distribution in the sizes of the resulting vesicles. The usual extrusion procedure involves 10–15 repeated passages of the vesicle suspension through the filter membrane. Once vesicles with dimensions smaller than the pore diameter are formed, further extrusions do not affect their size. Additional extrusions, however, might have an effect on vesicle shape. Because the velocity profile in the pore is parabolic and the vesicle size approaches the pore diameter, the vesicle can be deformed as it moves along the pore adopt the lowest resistance shape. The theoretical description of an emulsion droplet in a two-dimensional laminar flow in a very narrow slit shows that the droplet adopts an arrowhead shape (Walstra, 1983). This result can be used with membrane vesicles; however, it is necessary to realize that the emulsion droplet has a constant volume with a variable area, whereas the membrane vesicle has a constant membrane area but a volume that can be altered by transient breaks in the bilayer caused by sheer stress. Therefore, a vesicle placed in a laminar flow quite possibly adopts an arrowhead morphology to accommodate the flow constrains, and its internal volume is reduced, thereby exaggerating the indented spherical morphology of the vesicles. This idea is consistent with the experimental evidence that suggests that the exaggerated nonspherical vesicle shape is the result of the passage through the pores (Mui et al., 1993).

In summary, the vesicle size in this model is determined not only by the pore geometry, but also by the velocity of the suspension through the pore, the number of extrusion cycles, and the mechanical properties of the bilayer membrane. The deformation and loss of internal volume at constant area of the unilamellar vesicles upon formation or during the subsequent extrusions cause them to be indented spheres.

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Measurements of $[Ca^{2+}]_i$ with the Diffusible Fura-2 AM: Can Some Potential Pitfalls be Evaluated?

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In a recent New and Notable, Morgan (1993) rightly questioned the accuracy of the current calibration methods when using Ca-indicators to measure the intracellular concentration of Ca^{2+}. In the literature, potential sources of artifacts are often mentioned as warnings, but it is hard to find a convincing argument to decide whether or not they do cause serious problems (except when calculations yield negative values of $[Ca^{2+}]_i$, of course!).

Fura-2 is one of the most popular Ca-indicators used in measurement of intracellular Ca^{2+} ion concentration ($[Ca^{2+}]_i$). Cells can be loaded simply by immersion in a solution of the permeant acetoxymethyl ester form (Fura-2 AM), which is fluorescent but Ca-insensitve. Subsequent cleavage by intracellular esterases liberates Fura-2, which

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