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 This increase in volume is about 40% (S. G. Clerc and T. E. Thompson, unpublished results).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 $\mu$m diameter is 0.177 $\mu$m. 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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is then trapped into the cell. It is usually assumed that desesterification is complete and that Fura-2 remains within the cytosol only. This might not be the case. As pointed out by Morgan (1993), several reports identified two potential pitfalls : (i) the incomplete desesterification of Fura-2 AM, generating Ca-insensitive species with enhanced fluorescence (Blatter and Wier, 1990; Scalon et al, 1987), and (ii) the penetration of Fura-2 AM into cellular organelles before cleavage (Highsmith et al., 1986). Moreover, if the dye penetrated into Ca stores, e.g., endoplasmic reticulum or mitochondria, and if it were cleaved within them, Fura-2 would then monitor $Ca^{2+}$ concentrations considerably higher than in the cytosol. These situations might introduce uncontrolled errors, which would be of great concern in studies meant to measure $[Ca^{2+}]_i$ in *absolute values* (nM), for example, in the comparison of basal $[Ca^{2+}]_i$ in muscles fibers from normal and dystrophic mice (see Gailly et al. (1993) versus Turner et al. (1988)).

Fluorescence of Fura-2 is classically studied using two excitation wavelengths, 340 and 380 nm, chosen on both sides of the isosbestic point, and $[Ca^{2+}]_i$ is calculated from the ratio ($R$) of fluorescence intensities obtained using the formula of Grynkiewicz et al. (1985):

$$[Ca^{2+}]_i = K_d\beta(R - R_{min})/(R_{max} - R). \quad (3)$$

The calculation requires the determination of four calibration parameters ($K_d$, the Ca-Fura-2 dissociation constant; $\beta$, the fluorescence intensity ratio, excited at 380 nm, without and with Ca; $R_{min}$ and $R_{max}$, the fluorescence intensity ratio excited at 340 and 380 nm, in the absence (min) and in the presence (max) of $Ca^{2+}$, respectively). Ideally, they should be determined in situ, in the natural environment of the cytosol. This can be made by intracellular injections of Ca/EGTA buffers with the drawbacks listed by Morgan (1993); an acceptable compromise is to use cells permeabilized with the staphylococcal $\alpha$-toxin, the action of which is restricted to the plasma membrane, without effect on organelles (Nishiye et al., 1993). This requires EGTA or $Ca^{2+}$ solutions that mimic the physicochemical characteristics of the cytosol (ionic strength, pH, $[Mg^{2+}]$, [ATP], etc). However, whatever the technique, if some form of Fura-2 is trapped into organelles impermeant to EGTA, this might affect values of $\beta$ and $R_{min}$.

Uto et al. (1991) showed that by choosing 340 nm and the isosbestic point, 360 nm, as the excitation wavelength pair, constant $\beta$ is equal to 1.0, and the response curve of the indicator is shifted to the left, i.e., is sensitive to lower $Ca^{2+}$ concentrations, a substantial advantage to measure $[Ca^{2+}]_i$ in the 10–100 nM range, as in unstimulated cells. Moreover, in the case of the 340/360 nm pair, simple calculations demonstrate that the "parasitic" fluorescence contributions from Fura-2 species either insensitive to $Ca^{2+}$ and/or inaccessible to EGTA, do not affect the calculated value of $[Ca^{2+}]_i$.

Let $a$ be the Ca-sensitive fluorescence intensity of Fura-2, excited at 340 nm, and $x$, the parasitic fluorescence excited at 340 nm; $b$, the fluorescence intensity of Fura-2 at the isosbestic point (360 nm), and $y$, the parasitic fluorescence excited at 360 nm, then

$$R = (a + x)/(b + y) \quad \text{(intensity ratio in living cell)}$$

$$R_{min} = (a_{min} + x)/(b + y) \quad \text{(intensity ratio in permeabilized cell, in the presence of 1 mM EGTA)}$$

$$R_{max} = (a_{max} + x)/(b + y) \quad \text{(intensity ratio, in permeabilized cell, in the presence of } Ca^{2+}\text{, 10 mM).}$$

When these expressions of $R$, $R_{min}$, and $R_{max}$ are introduced into the Grynkiewicz et al. (1985) formula, the parasitic components $x$ and $y$ cancel out. We assume here that $x$ and $y$, though unknown, remain constant during the calibration procedure and the experiment. There is no direct proof of that. Circumstantial evidence, however, suggest that this assumption is valid, at least in the case of measurements of basal $[Ca^{2+}]_i$ in unstimulated cells. First, the fluorescence of quiescent cells, after loading with Fura-2 AM, can remain stable for long periods of time, suggesting that the generation of Ca-insensitive species of Fura-2 (incomplete desesterification) has come to an equilibrium. Second, Ca-accumulating vesicles from muscle are impermeant to EGTA (Weber et al., 1966). Thus, during calibration, the parasitic fluorescence from organelles would not be affected by EGTA (provided that cell permeabilization is achieved with $\alpha$-toxin, and not with ionomycin). Third, we observed that in CHO fibroblasts incubated in an external EGTA solution, $[Ca^{2+}]_i$ can be reduced to 2–3 nM, without depletion of the Ca stores (our unpublished results; this might not hold for all organelles in all cell types).

Thus, experimental conditions can be set where $[Ca^{2+}]_i$ can be measured free from the two possible sources of artefact, (i) and (ii), discussed above.

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