Taking deterministic control of membrane protein monomer–dimer measurements

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Forced togetherness is not ideal, whether it happens to people in the subway or to membrane proteins crowded in bilayers. The close proximity that results from the random rendezvous must not be confused with that of preferential interactions. The former arises as a trivial consequence of dense packing, whereas the latter is a manifestation of mutual attraction and is of greater interest to the natural philosopher (Kobus and Fleming, 2005; Stanley and Fleming, 2005). Distinguishing between these two types of encounters can be tricky, especially if the attraction is weak, because the situations can coexist and, in fact, do so increasingly as the density is raised. This disambiguation is the challenge faced by Chadda et al. in their quest to measure the monomer–dimer thermodynamic stability of the Cl^-/H^+ transmembrane transporter, CLC-ec1, in liposomes (Chadda and Robertson, 2016; Chadda et al., 2016, 2018). In this issue of *JGP*, they develop a new tool to simplify this herculean pursuit (Chadda et al., 2018).

Simplicity has been sought before in describing membrane protein capture by liposomes. Just 10 miles from the famous Walden Pond, where Henry David Thoreau endeavored a streamlined existence (Thoreau, 1854), another Walden followed a similar philosophy and outlined a remarkably straightforward method to extract the unitary time constant of Cl^- efflux from the same transporter captured by a population of heterogeneously sized vesicles (Walden et al., 2007). The foundation of this exposition lies in a mathematical expression for the underlying probability densities of these membrane proteins reconstituted in liposomes (Maduke et al., 1999; Walden et al., 2007). Notwithstanding the felicitous conciseness given by the Poisson equation in this treatise, the truth and beauty of the resulting microstates still ultimately rely on lipids forming vesicles with defined radial distributions. Therein lies the rub.

Lipids have sullied the picture, either by asserting their chemical identities or by varying their behavior upon cryo-electron microscopy imaging. The so-called Walden distribution was determined from *Escherichia coli* polar lipids extruded through a 400-nm filter (Walden et al., 2007). With only a small change in lipid composition (from *E. coli* polar lipid to 67% POPE and 33% POPG, no cardiolipin), Chadda et al. (2018) found that the same vesicle preparation protocol led to slight alterations in the radial distributions of the resultant vesicular population. The deviations between the Walden and the POPE/POPG radial distributions demonstrate that the latter population of vesicles has members with larger than expected radii compared with the former. Albeit small, this fraction of large liposomes deals a mighty blow to the stochastic reconstitution of CLC-ec1 because it is not the radius of a vesicle that controls membrane protein capture; rather, it is the area. Moreover, it is the fluorescent protein capture statistics per liposome that the single-molecule imaging technique detects, and the number of liposomes is, of course, a function of the area distribution for a given amount of lipid. The effect of any deviation from an expected liposome distribution is therefore a numbers game magnified by the squares of the sizes of the unanticipated outliers, weighted by their relative occurrences.

If left unchecked, the presence of these larger liposomes skews the fidelity of the photobleaching statistics when reporting membrane protein association because bigger vesicles serve as sinks for proteins. This is easily intuited by considering two homogeneous populations of vesicles prepared from the same amount of lipid. Liposomes with radii equal to 7.5 nm will be 100-fold more numerous than those 10-fold larger (75 nm). Because of the random nature of capture, which depends on the available lipid area, a higher fraction of the larger liposomes will be occupied by at least one fluorescent protein at the same protein to lipid mole ratio (Fig. 1). This is simply because there are fewer of them. This reality increases liposome occupancy by fluorescent proteins at relatively lower protein densities as well as occurrences of multistep photobleaching at higher protein to lipid mole ratios.

When mastery of the random capture statistics is in place, the single-molecule photobleaching method that measures CLC-ec1 dimerization energetics is a feat of

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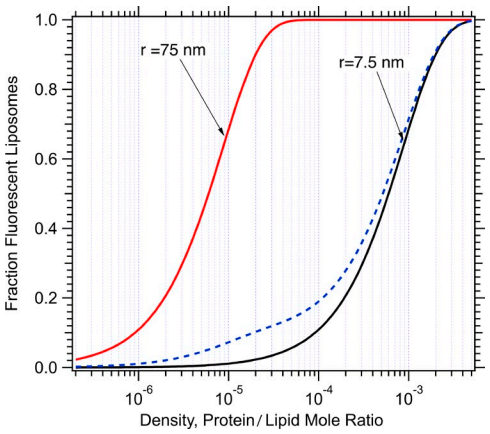

Figure 1. **Larger vesicles are sinks for randomly distributed membrane proteins.** Data were simulated for vesicles with uniform radii equal to 7.5 nm, 75 nm, or a mix of these two radii values and for a monomeric, ideal CLC-ec1 containing exactly one fluorescent label per protein. The distributions for liposomes containing at least one fluorescent protein are shown as a function of the protein/lipid mole ratio. These were calculated using the Poisson equation assuming 1.2 mg lipid with a mean surface area equal to 0.61 nm^2 $lipid^{-1}$ and mean lipid molar mass equal to 750 g mol^{-1} (Murzyn et al., 2005). The number of protein moles was incremented to generate the densities in the abscissa, and it was assumed that all vesicles were available for subunit capture. This simulation shows that a higher fraction of larger vesicles will acquire at least one fluorescent protein at lower densities as compared with smaller vesicles. At higher protein densities, there will also be an increase in the number of multistep bleaching events because a relatively higher number of the larger vesicles will stochastically contain more subunits. The mixed data in this comparison of two vesicle sizes that are 10-fold different in radius show that even a minor representation of the larger vesicles ($p(r_{75}) = 0.10$ and $p(r_{7.5}) = 0.90$) strongly affects photobleaching probabilities.

binding to behold. In particular, this technique reports on stoichiometry directly by the number of bleaching steps; it facilitates examination of an extremely wide range of protein/lipid ratios; it works in lipid bilayers; and it is amenable to manipulation by many of the thermodynamicist's favorite variables. Moreover, the issue of vesicle size distribution can be easily accounted for as long as it is known. However, under some conditions of interest for the elucidation of physical principles, it could become an onerous affair to measure the vesicle radial distribution for every single experiment. An internal control would be much better. Enter the obligate monomer and its partner, the covalent dimer.

The obligate monomer is not new, and, in fact, its presumed existence was a key clue that something might have been slightly amiss in the initial experiments. This variant of CLC-ec1, known as WW, contains two tryptophan mutations at the dimer interface (I201W/I422W). These bulky side chains introduce steric clashes at the protein–protein contact surface that abrogate dimerization. Accordingly, WW is monomeric, as assessed by gel filtration and glutaraldehyde cross-linking, and it crystallizes as a monomer (Robertson et al., 2010). Somewhat surprisingly, CLC-ec1 WW has shown some indications of weak dimerization over and above forced togetherness in POPE/POPG (2:1) at high protein densities. Given its prior status as a loner, thermodynamically meaningful dimerization of WW, with its tryptophan warts on its interface, seemed unlikely. Indeed, improved analysis in Chadda et al. (2018) using a revised liposome distribution confirms that WW meets its obligatory monomeric expectations.

At the other end of the meaningful self-association spectrum for CLC-ec1 is the dimer. Because its fluorescent microstates are distinct from those of the fluorescently labeled monomer (Chadda and Robertson, 2016), its chance liposome capture must also be known to distinguish this incidental dissemination from that of the pertinent dimer population arising from preferential monomer–dimer interactions. Further challenging this task is the fact that the CLC-ec1 dimer itself is quite large and probably cannot access the smallest of the liposomes. Chadda et al. (2018) arrived at two solutions to determine how this dimer is ideally distributed. The first makes use of a CLC-ec1 variant containing two cysteine mutations (R230C/L249C) that can be oxidized to permanently dimerize the two monomeric subunits. The second involves covalent connection of two CLC-ec1 monomers by glutaraldehyde cross-linking. Although the authors found that both obligate dimer constructs work well as stochastic standards for the dimer, a feature that is particularly pleasing about the former is that its activity is intact, thus avoiding any unforeseen uncertainties caused by malfunction.

A significant outcome of this study is that the obligate monomer and covalent dimer can now be used as models to simplify single-molecule bleaching experiments. Prepared alongside, they serve as intrinsic controls for any given liposome population, thus abrogating the need for the electron microscopy step. In cases of major, minor, or even miniscule fluctuations in liposome size distributions, the obligate monomer and covalent dimer will report this situation directly through the density dependence of their photobleaching signals. This ability greatly reduces not only the workflow of these demanding experiments, but also the underlying assumptions of the analysis. In essence, the obligate monomer and covalent dimer convert the unpredictability of liposome distributions into deterministic data, which ensures nothing will be left to chance, Poisson or otherwise.

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