Lipid redistribution in phosphatidylserine-containing vesicles adsorbing on titania

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Lipid vesicles (liposomes) exhibit a wide range of behavior at inorganic (oxide) surfaces. A complete understanding of the vesicle-surface interactions, and of the ensuing transformations surface adsorbed liposomes undergo, has proven elusive. This is at least in part due to the large number of degrees of freedom of the system comprising vesicles with their molecular constituents, substrate surface, and electrolyte solution. The least investigated among these degrees of freedom are those intrinsic to the vesicles themselves, involving rearrangements of lipid molecules. In this study, the adsorption of two-component vesicles (phosphatidylcholine:phosphatidylserine) on titanium dioxide was investigated by dual polarization interferometry. Mixtures of these two lipids containing more than 20% of phosphatidylserine form supported bilayers on titania, with phosphatidylserine predominantly facing the surface of the oxide. The purpose of this investigation is to ascertain whether redistribution of phosphatidylserine occurs already in the adsorbing vesicles. Indeed, this was found to be the case. A possible mechanism of this process is discussed.
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I. INTRODUCTION

Solid-supported lipid systems[1,2] of various morphologies—e.g., supported planar bilayers and vesicles (liposomes)—continue to attract the attention of researchers in various communities.[1–12] This is due to both their potential applications and fundamental questions surrounding interactions between inorganic surfaces and biological systems,[13] which can be studied using lipid vesicles as a model. In this regard, it is interesting to investigate the influence of an inorganic solid support on the organization and dynamics of lipids in supported bilayers[14–22] and vesicles. Such studies furthermore advance our understanding of the supported bilayer formation process from surface-adsorbed vesicles [Fig. 1(a)]—a process that continues to intrigue investigators despite intense efforts to unravel its mysteries.[18,20,23–31,34–40]

It has been found that two-component supported lipid bilayers, where one component interacts strongly and attractively with the support and the other one interacts weakly, are asymmetric with respect to the distribution of these components between the two bilayer leaflets, with the strongly interacting component accumulating in the leaflet proximal to the substrate. Examples of such systems include those containing positively charged lipids on negatively charged surfaces (silica)[14,15] and phosphatidylserine (PS)-containing vesicles on mica and titania in the presence of Ca^{2+}.[17–19,41] In the latter case, the binding of this cation to the substrates[42,43] was found to play the crucial role in establishing the asymmetric distribution of PS in the supported bilayers [Fig. 1(b)].[17–19] Furthermore, the diffusion of PS in the proximal leaflet of the supported bilayers was severely restricted.[19,41] These effects were clearly shown to be surface-induced and Ca^{2+}-mediated and are not related to the phase behavior of the DOPC:DOPS lipid mixtures.[19]

These observations raise the question: at which point during the bilayer formation does the asymmetry appear?[17–19] Destruction of liposomes by oppositely charged poly(electrolytes) has been shown to be mediated by transfer of the charged lipids from one leaflet to the other.[44–46] This process is sometimes termed "induced flip-flop" to distinguish it from the slow, spontaneous version.[47–49] Fluorescence studies of bilayer formation point to the possibility that solid surfaces induce a similar trans-leaflet redistribution process in the adsorbing vesicles.[30,35] What, if any, role this might play in the supported bilayer formation process has not yet been investigated. In this study, the possibility that lipid asymmetry evolves at the stage of vesicle adsorption to the surface [Fig. 1(c)] is investigated.

In the free vesicles (not adsorbed to the surface), lipid molecules move rapidly in the plane of the bilayer as long as the temperature is kept above the main (gel-to-liquid) transition temperature of the lipid mixtures (<0 °C for the lipids

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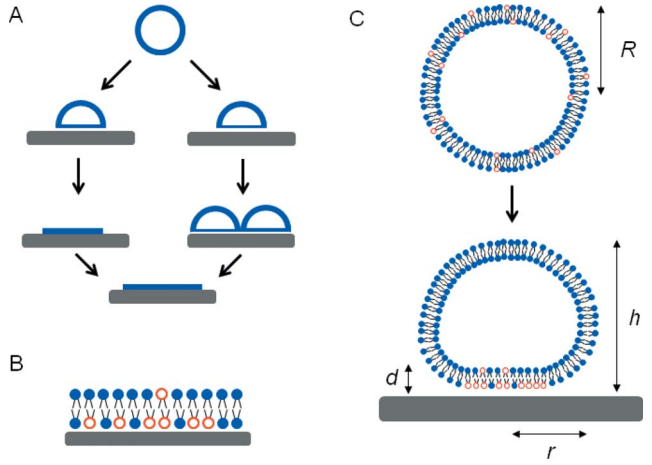

FIG. 1. Supported bilayer formation process. (a) Currently, two pathways of supported bilayer formation from vesicles are known. One (left) involves rupture of individual vesicles upon adsorption to the surface,[23–25] followed by the coalescence of resulting single bilayer disks into one continuous bilayer. The other pathway (right) proceeds via an intermediate layer of adsorbed vesicles,[23,24,26,27] which transform to the bilayer only after reaching a critical concentration.[27–29] It is not yet clear which factors determine the choice of the pathway. Both pathways begin with adsorption of vesicles to the surface. Upon adsorption, vesicles deform.[30–33] (b) A two-component supported bilayer with asymmetric lipid distribution. Such bilayers form on mica[17] and titania[18,19] from vesicles containing phosphatidylserine (PS, red open circles) and phosphatidylcholine (PC, blue filled circles). (c) In the case of free (nonadsorbed) vesicles with sizes much greater than the bilayer thickness, the asymmetry in the bilayer is negligible. In this study, the possibility that contact with the surface induces lipid asymmetry in the adsorbing vesicles is investigated. Vesicles in solution are characterized by radius R, while surface-adsorbed vesicles are characterized by their height above the surface (thickness) h and area of contact πr^2. The extent of deformation h/R is then related to the fractional area of contact $\zeta = \pi r^2/4\pi R^2$ via Eq. (1).

used in this study). When a two-component vesicle adsorbs to a surface, the strongly interacting component will tend to accumulate at the area of contact between the surface and the vesicle, simply because the diffusion of this component is restricted once it comes into contact with the surface [Fig. 1(c)].[20,50] Restriction of diffusion of PS in supported bilayers was indeed previously demonstrated on TiO_2 in the presence of Ca^{2+},[19] and a similar effect is observed on mica.[41] A relation between the extent of vesicle deformation, defined as the height of the vesicle above the surface divided by its radius in solution, h/R, and the fractional area of contact between the vesicle and the surface, ζ [area of contact divided by the total area of the vesicle, Fig. 1(c)], can be approximated as follows:

$$\frac{h}{R} = 2\sqrt{1-2\zeta}. \tag{1}$$

In deriving this equation (see the Appendix for the derivation), it was assumed that the vesicle-surface interaction represents the dominant contribution to the free energy of the system (thus neglecting the bending energy) and that the adsorbed vesicle has the shape of a spherical cap. Recent cryo transmission electron microscopy studies of lipid vesicles adsorbing on silica particles show the adsorbing liposomes exhibiting spherical caplike morphologies.[51]

According to Eq. (1), the extent of vesicle deformation varies between $h/R=2$ ($h=2R$ for an undeformed vesicle) and 0 (the contact area is such that it is no longer possible to create a spherical caplike structure above it). Adhesion energies strong enough to drive further increase in the contact area are expected to cause a change in the shape of the adsorbed liposomes, e.g., to that resembling pancakes.[31] Independently of the shape of the adsorbed liposomes (spherical caplike or not), h can never be smaller than $\sim 2d$, where d is the bilayer thickness of ~ 5 nm. Therefore, a transition to a supported bilayer is expected to occur at $h/R \sim 2d/R$. (Actually, $2d$ represents the lower limit on the smallest possible height of the adsorbed liposomes, because lipid bilayer cannot bend to arbitrarily high curvatures.[52])

In the case of vesicles used in this study, the strong-interacting component is PS, and ζ is related to the molar fraction of PS present in the outer leaflet of the bilayer, α, as follows:

$$\zeta = \alpha + \zeta_0. \tag{2}$$

ζ_0 accounts for the deformation of single-component (in this case, DOPC) vesicles upon adsorption.[32,33,52] The molar fraction of PS in the outer leaflet of the bilayer can be calculated for two extreme cases: assuming PS is distributed symmetrically across the two leaflets of the adsorbing vesicles [i.e., only 50% of all PS is allowed to interact with the surface, Eq. (3)], and assuming that *all* of the PS is accumulated in the surface-proximal (outer) leaflet of the bilayer [Eq. (4)]:

$$\alpha_{symm} = \frac{A_{PS}}{A_{PS} + A_{PC}}, \tag{3}$$

$$\alpha_{asymm} = 2\alpha_{symm}. \tag{4}$$

In Eqs. (3) and (4), A is the area per molecule of the lipid times the molar fraction of the lipid in the mixture. Molecular areas of the two lipids (0.72 and 0.63 nm^2 for dioleoyl phosphatidylcholine and dioleoyl phosphatidylserine, respectively) were taken from Refs. 53–55. Equation (4) assumes that the area of each of the two bilayer leaflets remains constant, so the transfer of PS to the outer leaflet is compensated for by the transfer of PC to the inner leaflet.

II. MATERIALS AND METHODS

A. Materials

Phospholipids—dioleoyl phosphatidylcholine (DOPC), dioleoyl phosphatidylserine (DOPS), palmitoyl oleoyl phosphatidylcholine (POPC), palmitoyl oleoyl phosphatidylserine (POPS)—were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). The buffer used throughout the study consisted of 10 mM HEPES, 100 mM NaCl, and 2 mM $CaCl_2$, *p*H 7.4,[56] unless specified otherwise. Chemicals for buffer preparation and sodium dodecyl sulfate used for cleaning surfaces of the waveguides were purchased from Sigma-Aldrich (Buchs, Switzerland).

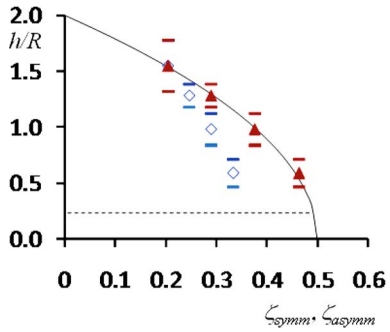

FIG. 2. The extent of deformation of DOPC:DOPS vesicles upon adsorption to TiO_2 surface, h/R, is plotted as a function of the area of contact, ζ [calculated according to Eq. (2)]. R is the number-averaged mean radius of vesicles in solution measured by dynamic light scattering. Vesicles of five different compositions were examined, containing 0%, 5%, 10%, and 15%, and 20% PS (by weight). Values for four of these are plotted in this figure, while the h/R value observed in the case of 20% PS vesicles (bilayer formation) is not shown (see text for discussion). Each datum point corresponds to one composition, with ζ calculated in two different ways: solid (brown) triangles—the distribution of PS in the adsorbing vesicles is assumed to be asymmetric [see Eq. (4)]; open (blue) rhombi—the distribution of PS is in the adsorbing vesicles assumed to be symmetric [see Eq. (3)]. The deformation in this case is larger than expected based on the amount of PS present in the vesicles. Error bars are calculated based on standard deviations of the thickness values h. Black solid line is the plot of h/R vs ζ according to Eq. (1). The dashed black horizontal line (guide to eye) corresponds approximately to $2d/R$, where d is bilayer thickness or ~5 nm. It indicates the extent of deformation at which the vesicles are expected to rupture.

B. Vesicle preparation and characterization

Multilamellar vesicles (MLVs) containing various DOPC:DOPS ratios were prepared by mixing chloroform solutions of lipids in the desired ratio, evaporating the chloroform first with a stream of argon, and then under vacuum (provided by an oil-free diaphragm vacuum pump), and finally dispersing the lipid film in the buffer by vortexing. Unilamellar vesicles were obtained by extruding the MLV suspensions through polycarbonate membranes with nominal pore size of 50 nm with a Lipofast extruder (Avestin Inc., Ottawa, Canada) . The resulting vesicles were characterized by dynamic light scattering (DLS) using a Malvern Zetasizer 3000 HAS (Malvern, U.K.) instrument. The mean of the number-averaged size distribution obtained from the second-order cumulant analysis was used in the calculations of h/R ratio [Eq. (1), Fig. 2], since it reflects the number density of the adsorbing vesicles. Sizes of vesicles of various compositions are shown in Table I. Vesicle solutions were stored under argon until used.

C. Dual polarization interferometry measurements

Dual polarization interferometry measurements were performed with an Analight® BIO200 (Farfield Sensors Ltd., Crewe, U.K.) instrument. A detailed description of the technique and its application to studying biomolecular adsorption can be found in Refs. 57 and 58. The instrument is based on an integrated Young's interferometer implemented in a stack of silicon oxinitride slabs illuminated with an alternating polarized laser beam (632.8 nm). The sensing arm of the interferometer is exposed to the environment and the reference arm is buried beneath. The spatial characteristics of the resulting interference pattern depend on the properties (refractive index, thickness of the adsorbed film) of the environment of the sensing arm. Using two orthogonal polarizations allows the refractive index (density) and thickness of optically isotropic adsorbed films to be resolved.

TABLE I. DOPC : DOPS vesicles in solution and adsorbed on titania: size, layer thickness, refractive index, and extent of deformation.

% PS (by weight)	Vesicle radius R (nm)[a]	Layer thickness h (nm)[b]	Layer refractive index[b]	Extent of deformation, h/R
0	33	51 ± 8	1.36 ± 0.005	1.5 ± 0.1
5	25	32 ± 3	1.38 ± 0.004	1.3 ± 0.1
10	28	27 ± 4	1.39 ± 0.006	1 ± 0.2
15	27	16 ± 3	1.40 ± 0.008	0.6 ± 0.4
20	28	27 ± 10	1.38 ± 0.02	N/A

[a]The number-averaged mean vesicle radius determined by dynamic light scattering is quoted.
[b]Calculated from the DPI measurements assuming an isotropic layer.

Silicon oxinitride waveguide sensor chips (24×6 mm^2, Farfield Sensors Ltd., Crewe, U.K.) used in the Analight instrument were sputter-coated with 6–8 nm of TiO_2 in a Leybold DC-magnetron Z600 sputtering unit at the Paul Scherrer Institut (Villigen, Switzerland) as described previously.[59] These titania-coated waveguides were cleaned with 2% SDS solution for 30 min, rinsed with ultrapure water, dried with a stream of nitrogen gas, and then placed in a preheated UV ozone cleaner (model 135500, Boekel Industries, Feasterville, PA, USA) for 30 min.[56] The clean waveguides were installed in the Analight instrument and allowed to equilibrate in water overnight to minimize baseline drift.

An experiment began with the calibration of the equilibrated waveguides by measuring the interference pattern in 80% ethanol: water solution and in ultrapure water of known refractive indexes. Calibration was followed by equilibration in buffer, an injection of 0.5 mg/ml vesicle solution, and a final rinse with the buffer. During each stage, the phase shift angles for the two polarizations were measured in real time and converted into optical path length changes. The isotropic refractive index n and thickness h of the adsorbed layer were calculated by comparing these experimentally determined optical path length changes with predictions of a model based on the optical transfer matrix approach.[60] Waveguide structure and sensitivity parameters, as well as the refractive index of the buffer, were obtained from the calibration. The values are presented in Table I.

An alternative *in situ* cleaning procedure was explored in an attempt to save time: after each vesicle adsorption experiment, the measurement chamber was rinsed with 2% SDS solution. The nominally clean surface was then used in the next experiment, while the overnight equilibration in water was required only before the first experiment, i.e., immediately after the UV-ozone treatment. However, it was observed that the values for the thickness of the vesicular layers

TABLE II. Thicknesses of supported lipid bilayers from DPI measurements. The thicknesses for different supported lipid bilayers calculated from four independent DPI measurements per system using an isotropic layer model are presented. The true thickness of all these supported bilayers is ~5 nm. In all cases, DPI thickness is much larger due to the optical anisotropy of the ordered film.[63–66] The differences between the different systems are not well-understood at present, but are probably related to the variations in the magnitude of molecular alignment. All results except DOPC:DOPS TiO_2 (Ca^{2+}) are obtained on silicon oxinitride waveguides. The buffer used in the experiments on silicon oxinitride was 150 mM NaCl, 10 mM HEPES with or without 2 mM $CaCl_2$.

Lipid system	POPC	POPC Ca^{2+}	POPC:POPS 20% Ca^{2+}	DOPC	DOPC Ca^{2+}	DOPC:DOPS 20% Ca^{2+}	DOPC:DOPS 20% TiO_2 Ca^{2+}
DPI thickness (nm)	30 ± 4	24 ± 0.8	53 ± 12	46 ± 14	16 ± 2	34 ± 5	27 ± 10
Refractive index	1.363 ± 0.003	1.367 ± 0.001	1.354 ± 0.003	1.349 ± 0.002	1.378 ± 0.005	1.360 ± 0.003	1.38 ± 0.02

prepared on such nominally clean surfaces increased with the number of the experiment (up to five sequential experiments were attempted), indicating that the properties of the SDS-cleaned surfaces are somehow different from those subsequently cleaned with UV-ozone. This was indeed confirmed by elipsometric measurements of titania surfaces before and after an SDS cleaning step (not shown). [A similar observation has previously been made using quartz crystal microbalance (Reviakine *et al.*, unpublished).] It therefore appeared that the SDS/UV-ozone combination was required before each experiment and all experiments reported in this study were performed in this way.

III. RESULTS AND DISCUSSION

In this work, the thickness of vesicular layers prepared from vesicles containing various amounts of phosphatidylserine adsorbed on TiO_2, h, is measured as a function of the PS contents, by dual polarization interferometry (DPI; Table I). DPI is based on an integrated Young's interferometer illuminated with two orthogonally polarized light beams. The changes in the interference pattern due to changes in optical path length caused by, e.g., vesicle adsorption, observed for each polarization, are converted into the refractive index and thickness of the adsorbed layer based on the assumption that the film is optically isotropic. The validity of this assumption for the case of an adsorbed liposome layer is discussed below.

The thickness obtained from DPI measurements is then scaled by the average vesicle radius in solution, R, determined by dynamic light scattering, to obtain the extent of vesicle deformation, h/R (Table I). In the case of PC vesicles, $h/R \sim 1.54$ is obtained. This compares favorably with estimates of $\sim 1-2$ calculated from our previous QCM-D results for vesicles of similar size,[32,33] but note that the properties of the titania surfaces used are likely to differ between the studies. The value of 1.54 for h/R at 0% PS is used to obtain $\zeta_0 = 0.205$ from Eq. (1). The extent of vesicle deformation is then plotted against the fractional area of contact between the vesicle and the surface, ζ_{symm} and ζ_{asymm}, calculated according to Eq. (2) from the molar fractions of PS present in the outer leaflet of the bilayer, α_{symm} and α_{asymm}, respectively [Eqs. (3) and (4)], and ζ_0. The resulting plots are presented in Fig. 2 for vesicles containing 0%, 5%, 10%, and 15% PS (by weight). It is apparent that the extent of vesicle deformation increases (h/R decreases) with increasing PS content of the vesicles, indicating increasing interaction area between the vesicles and the surface as a function of PS content of the vesicles. This observation is consistent with our previous findings.[56,61] Second, the agreement between the measurements and Eq. (1) is much better if the distribution of PS is assumed to be asymmetric. That is to say, redistribution of PS between the two leaflets occurs already in adsorbed vesicles, before the vesicle-to-bilayer transformation takes place.

Finally, the extent of deformation of vesicles containing 15% PS is approaching the limit of $2d/R$ set on the extent of vesicle deformation by the finite bilayer thickness (see above). In this system, the transition from adsorbed vesicles to supported bilayers occurs in the case of vesicles containing 20% PS or more.[56] The h/R value observed for the 20% PS vesicles is not shown in Fig. 2 to avoid confusion: it corresponds to a thickness of $h = 27 \pm 10$ nm, instead of the ~5 nm expected for the bilayer. This can at first look surprising, but the overestimated thickness is a consequence of ignoring the anisotropy of the lipid bilayer when interpreting the DPI results. Significantly larger than expected values for supported bilayer thicknesses have previously been reported, derived from measurements by ellipsometry[62] and waveguide spectroscopy.[63,64] This effect is further discussed in a recent theoretical treatment of optically anisotropic layers probed by waveguide spectroscopy by Horváth and Ramsden.[65] In support of this interpretation of the unrealistically large value for the thickness of PC:PS bilayers, a range of values for the thickness of bilayers obtained on the bare DPI waveguide silicon oxinitride surface for several different systems is presented in Table II. It is rather clear from the results presented in Table II that the bilayer thickness derived from the DPI measurements based on an isotropic layer model is systematically and significantly larger than that known for these systems from many other measurements.[23,24,55,67] A more thorough analysis of the optical anisotropy of lipid systems and its implications for studying formation kinetics of supported membranes with DPI is given elsewhere.[66] In the case of layers comprised of spherical shell structures, such as a liposomes, it is the polarizability of the entire shell that determines the optical properties of the film.[68] Thus, the induced average anisotropy of the lipid alignment in the vesicle shell upon deformation

is not expected to affect the calculated thickness of vesicular layers to any significant degree. Intuitively, this can be further understood by considering that the effect of the anisotropic component on the overall optical properties of a layer is related to its volume fraction in the layer. In the case of a lipid bilayer, this volume fraction is close to 1, while in the case of a layer of vesicles, it never exceeds 0.2.

Interestingly, the data for the extent of liposome deformation in the entire range of PS concentrations studied (0% – 15%) agree with predictions of an equation that assumes a spherical caplike morphology. No deviation, indicative of a transition to a pancakelike shape[31] that could occur at high PS contents, is observed. Although it might be worth searching for evidence of such shape changes in the range of 15% – 20% PS, this observation may also be taken to suggest that the collective liposome-to-bilayer transition [Fig. 1(a), right] occurs at sufficiently small adhesion energies for the adsorbed vesicles to maintain the spherical caplike shape. Supporting this possibility are the recent atomic force microscopy studies of liposome ensembles on the verge of the bilayer formation, which do not show pancakelike morphologies,[24,29,69] as well as recent cryo transmission electron microscopy studies of liposomes adsorbing on silica particles, which show the adsorbed liposomes maintaining spherical caplike shapes over a wide range of deformations.[51]

The main conclusion of our study is that lipids in the bilayers of adsorbing vesicles redistribute between the two leaflets.[70] This leads to a larger (negative) adhesion energy[52] and a higher rate constant for rupture[38] than for the case of symmetric distribution of lipids. The time scales normally associated with the flip-flop process—tens of hours[47–49]—are clearly not compatible with those of vesicle adsorption or supported bilayer formation processes (seconds to minutes). The slow rate is a consequence of the large energy barrier associated with the transfer of the hydrophilic headgroups through the hydropohobic interior of the bilayer. There are a number of ways in which this barrier may be lowered. Dedicated proteins increase the rate of flip-flop by orders of magnitude in membranes of native cells—though the mechanism of this action is not yet known.[49,71] In the absence of such proteins, pores could provide the means for lowering the energy barrier associated with the redistribution of lipids between the two leaflets of the bilayer. Indeed, theoretical and experimental studies show that lipid redistribution between leaflets occurs many orders of magnitude faster in strained membranes due to pore formation.[72,73] Recent studies of the effect of the average spontaneous curvature on the supported bilayer formation process also support the idea that formation of pores in the lipid membranes is instrumental in the process of the adsorbed vesicle-SLB transformation.[25,40] In this regard, our findings lend further credence to the idea that increased pore formation occurs in adsorbed lipid vesicles, leading to rapid redistribution of lipids.

IV. CONCLUSIONS

Recent findings of asymmetric lipid distribution in two-component (phosphatidyl choline/phosphatidyl serine) lipid bilayers on the surfaces of mica and titania raise a question that concerns the stage of supported lipid bilayer formation process at which lipid redistribution occurs. To address this question, the extent of deformation of two-component liposomes adsorbing on titania was measured by dual polarization interferometry as a function of the contents of a strongly interacting component, phosphatidyl serine. It was found that adsorbing liposomes deformed to a greater extent than would be expected based on the phosphatidyl serine contents in the outer leaflet of the liposomes. It is therefore concluded that lipid redistribution occurs in the adsorbing liposomes before the liposome-to-bilayer transition step. This process is likely to rely on the formation of transmembrane pores, recently implicated in the supported lipid bilayer formation.

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APPENDIX

For the sake of completeness, Eq. (1) is derived here. It is assumed that the surface-lipid interactions are much stronger than the bending energy and, hence, upon adsorption, a vesicle will form a spherical cap. Furthermore, it is assumed that the total area of the vesicle does not change in the process. Then, if $A=4\pi R^2$ is the area of the free vesicle and πr^2 is the area in contact with the surface (see Fig. 1 for definitions), the ratio of the two quantities gives us the fractional area of contact:

$$\zeta = \frac{\pi r^2}{4\pi R^2}. \tag{A1}$$

If a vesicle has two components, and only one of the two interacts attractively with the surface, this is the areal fraction of the strongly interacting component.

The total area of the adsorbed vesicle is given by

$$4\pi R^2 = \pi(h^2 + r^2) + \pi r^2. \tag{A2}$$

The first term on the right hand side is the area of the spherical cap, and the second is the area of the supporting circular base. Rearranging and substituting Eq. (A1) gives Eq. (1).

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