Asymmetric Distribution of Phosphatidyl Serine in Supported Phospholipid Bilayers on Titanium Dioxide

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Supported phospholipid bilayers (SPBs) are useful for studying cell adhesion, cell−cell interactions, protein−lipid interactions, protein crystallization, and applications in biosensor and biomaterial areas. We have recently reported that SPBs could be formed on titanium dioxide, an important biomaterial, from vesicles containing anionic phospholipid phosphatidyl serine (PS) in the presence of calcium. Here, we show that the mobility of the fluorescently labeled PS present in these bilayers is severely restricted, whereas that of the zwitterionic phosphatidyl choline is not affected. Removal of calcium alleviated the restriction on the mobility of PS. Both components were found to be mobile in SPBs of identical compositions prepared in the presence of calcium on silica. To explain these results, we propose that, on TiO_2, PS is trapped in the proximal leaflet of the bilayers. This proposal is supported by the results of protein adsorption experiments carried out on bilayers containing various amounts of PS prepared on silica and titania.

Introduction

Solid-supported phospholipid bilayers (SPBs)[1−3] are useful tools for studying cell adhesion and cell−cell interactions,[2,4−8] protein−lipid interactions, and protein crystallization[9−15] as well as membrane properties in general.[16−18] SPB-based applications in biosensor and biomaterial areas are also under intense investigation,[8,19−24] driven by the unique properties of SPBs that include a degree of biological inertness,[25] electrical insulating properties,[26] and the ability to serve as matrixes for the surface immobilization of transmembrane proteins.[27−32]

Recently, the properties of supported bilayers, such as lipid mobility, lateral distribution, and transbilayer asymmetry, have come into focus.[13,33−44] Of specific interest is the effect of the underlying substrate surface on these properties,[13,33,35,36,41,45,46] because it might affect the ability of SPBs to mimic cell membranes successfully or perform other functions. Therefore,

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(1) Brian, A. A.; McConnell, H. M. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 6159−6163.
(2) McConnell, H. M.; Watts, T. H.; Weis, R. M.; Brian, A. A. *Biochim. Biophys. Acta* **1986**, *864*, 95−106.
(3) Sackmann, E. *Science* **1996**, *271*, 43−48.
(4) Simson, R.; Sackmann, E. In *Physical Chemistry of Biological Interfaces*; Baszkin, A., Norde, W., Eds.; Marcel Dekker: New York, 2000.
(5) Orth, R. N.; Wu, M.; Holowka, D. A.; Craighead, H. G.; Baird, B. A. *Langmuir* **2003**, *19*, 1599−1605.
(6) Groves, J. T.; Dustin, M. L. *J. Immunol. Methods* **2003**, *278*, 19−32.
(7) Wu, M.; Holowka, D.; Craighead, H. G.; Baird, B. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 13798−13803.
(8) Kasemo, B. *Surf. Sci.* **2002**, *500*, 656−677.
(9) Giesen, P. L. A.; Willems, G. M.; Hermens, W. T. *J. Biol. Chem.* **1991**, *266*, 1379−1382.
(10) Pearce, K. H.; Hiskey, R. G.; Thompson, N. L. *Biochemistry* **1992**, *31*, 5983−5995.
(11) Terrettaz, S.; Stora, T.; Duschl, C.; Vogel, H. *Langmuir* **1993**, *9*, 1361−1369.
(12) Lopez-Marin, L. M.; Quesada, D.; Lakhdar-Ghazal, F.; Tocanne, J.-F.; Lanéelle, G. *Biochemistry* **1994**, *33*, 7056−7061.
(13) Cézanne, L.; Lopez, A.; Loste, F.; Parnaud, G.; Saurel, O.; Demange, P.; Tocanne, J.-F. *Biochemistry* **1999**, *38*, 2779−2786.
(14) Reviakine, I.; Bergsma-Schutter, W.; Morozov, A. N.; Brisson, A. *Langmuir* **2001**, *17*, 1680−1686.
(15) Richter, R. P.; Him, J. L. K.; Tessier, B.; Tessier, C.; Brisson, A. R. *Biophys. J.* **2005**, *89*, 3372−3385.
(16) Mou, J.; Yang, J.; Shao, Z. *Biochemistry* **1994**, *33*, 4439−4443.
(17) Dufrêne, Y. F.; Lee, G. U. *Biochim. Biophys. Acta* **2000**, *1509*, 14−41.
(18) Milhiet, P. E.; Vie, V.; Giocondi, M. C.; Le Grimellec, C. *Single Mol.* **2001**, *2*, 109−112.
(19) Erb, E. M.; Chen, X. Y.; Allen, S.; Roberts, C. J.; Tendler, S. J. B.; Davies, M. C.; Forsen, S. *Anal. Biochem.* **2000**, *280*, 29−35.
(20) Cheng, Y. L.; Bushby, R. J.; Evans, S. D.; Knowles, P. F.; Miles, R. E.; Ogier, S. D. *Langmuir* **2001**, *17*, 1240−1242.
(21) Olofsson, L.; Rindzevicius, T.; Pfeiffer, I.; Käll, M.; Höök, F. *Langmuir* **2003**, *19*, 10414−10419.
(22) Svedhem, S.; Pfeiffer, I.; Larsson, C.; Wingren, C.; Borrebaeck, C.; Höök, F. *ChemBioChem* **2003**, *4*, 339−343.
(23) Yoshina-Ishii, C.; Boxer, S. G. *J. Am. Chem. Soc.* **2003**, *125*, 3696−3697.
(24) Suzuki, H.; Tabata, K.; Kato-Yamada, Y.; Noji, H.; Takeuchi, S. *Lab Chip* **2004**, *4*, 502−505.
(25) Glasmästar, K.; Larsson, C.; Höök, F.; Kasemo, B. *J. Colloid Interface Sci.* **2002**, *246*, 40−47.
(26) Wiegand, G.; Arribas-Layton, N.; Hillebrandt, H.; Sackmann, E.; Wagner, P. *J. Phys. Chem. B* **2002**, *106*, 4245−4254.
(27) Contino, P. B.; Hasselbacher, C. A.; Ross, J. B.; Nemerson, Y. *Biophys. J.* **1994**, *67*, 1113−1116.
(28) Salafsky, J.; Groves, J. T.; Boxer, S. G. *Biochemistry* **1996**, *35*, 14773−14781.
(29) Heyse, S.; Stora, T.; Schmid, E.; Lakey, J. H.; Vogel, H. *Biochim. Biophys. Acta* **1998**, *85507*, 319−338.
(30) Goryll, M.; Wilk, S.; Laws, G. M.; Thornton, T.; Goodnick, S.; Saraniti, M.; Tang, J.; Eisenberg, R. S. *Superlattices Microstruct.* **2003**, *34*, 451−457.
(31) Graneli, A.; Rydstrom, J.; Kasemo, B.; Höök, F. *Langmuir* **2003**, *19*, 842−850.
(32) Hussain, M. A.; Agnihotri, A.; Siedlecki, C. A. *Langmuir* **2005**, *21*.
(33) Käsbauer, M.; Junglas, M.; Bayerl, T. M. *Biophys. J.* **1999**, *76*, 2600−2605.
(34) Dietrich, C.; Bagatolli, L. A.; Volovyk, Z. N.; Thompson, N. L.; Levi, M.; Jacobson, K.; Gratton, E. *Biophys. J.* **2001**, *80*, 1417−1428.
(35) Stottrup, B. L.; Veatch, S. L.; Keller, S. L. *Biophys. J.* **2004**, *86*, 2942−2950.
(36) Richter, R. P.; Maury, N.; Brisson, A. R. *Langmuir* **2005**, *21*, 299−304.
(37) Crane, J. M.; Kiessling, V.; Tamm, L. K. *Langmuir* **2005**, *21*, 1377−1388.
(38) Dolainsky, C.; Karakatsanis, P.; Bayerl, T. M. *Phys. Rev. E* **1997**, *55*, 4512−4521.
(39) Linseisen, F. M.; Hetzer, M.; Brumm, T.; Bayerl, T. M. *Biophys. J.* **1997**, *72*, 1659−1667.
(40) Marxer, C. G.; Kraft, M. L.; Weber, P. K.; Hutcheon, I. D.; Boxer, S. G. *Biophys. J.* **2005**, *88*, 2965−2975.
(41) Deverall, M. A.; Gindl, E.; Sinner, E. K.; Besir, H.; Ruehe, J.; Saxton, M. J.; Naumann, C. A. *Biophys. J.* **2005**, *88*, 1875−1886.
(42) Ratto, T. V.; Longo, M. L. *Biophys. J.* **2002**, *83*, 3380−3392.
(43) Almeida, P. F. F.; Vaz, W. L. C.; Thompson, T. E. *Biochemistry* **1992**, *31*, 7198−7210.
(44) Saurel, O.; Cézanne, L.; Milon, A.; Tocanne, J.-F.; Demange, P. *Biochemistry* **1998**, *37*, 1403−1410.
(45) Hetzer, M.; Heinz, S.; Grage, S.; Bayerl, T. M. *Langmuir* **1998**, *14*, 982−984.
(46) Junglas, M.; Danner, B.; Bayerl, T. M. *Langmuir* **2003**, *19*, 1914−1917.

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in this study, we examine the lateral organization and mobility of phospholipids in supported bilayers prepared on TiO_2[47] and specifically focus on the influence of surface−lipid interactions on these properties. TiO_2 is responsible for the biocompatibility of titanium,[48,49] an important implant material,[50] and is also used in sensing applications based on optical detection methods.[51] Despite their potential relevance to elucidating the basis for the oxide's biocompatibility[8,48,50,52] and the importance of TiO_2 as a biomaterial, its interactions with liposomes have only recently begun to be investigated.[47,53−57] Thus, there is virtually no information concerning the dynamics of phospholipids on this surface in the literature. Our results represent an important step toward filling this knowledge gap.

Materials and Methods

Materials. Dioleoyl phosphatidyl choline (DOPC), dioleoyl phosphatidyl serine (DOPS), and their fluorescently labeled derivatives—1-oleoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl-sn-glycero-3-phosphocholine and 1-oleoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl-sn-glycero-3-phospho-L-serine (ammonium salt), abbreviated NBD-PC and NBD-PS, respectively—were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). The fluorescent label is located on one of the acyl chains and is not expected to interfere with the headgroup−surface interactions. Human serum (control serum Precinorm U) was purchased from Roche (Basel, Switzerland).

Two different buffers were used throughout this study: Ca^{2+} buffer, containing 10 mM HEPES, 100 mM NaCl, and 2 mM $CaCl_2$ and EDTA buffer, containing 10 mM HEPES, 100 mM NaCl, and 2 mM EDTA. In both cases, the pH was adjusted to 7.4 with NaOH. Chemicals were purchased from Sigma or Fluka (Buchs, Switzerland).

Vesicle Preparation. Unilamellar vesicles containing 0−30% DOPS in DOPC (by weight) were prepared by extrusion with a Lipofast extruder (Avestin Inc., Ottawa, Canada) following established protocols (e.g., see refs 58−60). The pore size of the extrusion filters was 100 nm.

Substrate Preparation and Cleaning. Round glass coverslips (Menzel Gläser, Braunschweig, Germany) to be used in fluorescence microscopy experiments and gold-coated quartz crystals (Q-Sense AB, Gothenburg, Sweden) to be used in the QCM-D experiments were coated with either 12 nm of TiO_2 or (12 nm TiO_2 + 12 nm SiO_2) by reactive sputtering in a Leybold dc-magnetron Z600 sputtering unit at the Paul Scherrer Institut (Villigen, Switzerland), as described previously.[51] The substrates were cleaned in a 2% SDS solution for at least 30 min, rinsed with ultrapure water, and treated in a preheated UV/ozone cleaner (model 135500, Boekel Industries Inc., Feasterville, PN) for 30 min.

Fluorescence Microscopy (FM). Fluorescence microscopy experiments were performed with a Zeiss LSM 510 confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany) equipped with a 25 mW argon laser (the 488 nm line was used for excitation), a 100× oil-immersion objective with a 1.4 numerical aperture, and a photomultiplier tube for detecting the emitted fluorescence. The resolution of the images was set at 512 pixels × 512 pixels to allow for fast scanning (maximum resolution: 2048 pixels × 2048 pixels). NBD-PC, NBD-PS, or mixtures of both lipids were used as the fluorescent species.

SPBs were prepared by exposing the freshly cleaned substrates (mounted at the bottom of a homemade sample holder shaped as a well) with a solution of vesicles in Ca^{2+} buffer, at a lipid concentration of 0.5 mg/mL, for 10 min. Excess vesicles were removed by rinsing with pure buffer prior to observation.

Fluorescence Recovery after Photobleaching (FRAP). We followed the protocol for data acquisition and evaluation published by Berquand et al.[61] based on the theory published by Axelrod et al.[62] and Soumpasis.[63] Equation 3 that appears on page 1703 of Berquand et al.[61] contains an error; it is the sum, and not the difference, of the Bessel functions that enters this equation. See Lopez et al.[64] for the correct equation. A round spot of 33.8 μm diameter was bleached in the supported bilayer. The bleaching time was kept at less than 5% of the characteristic recovery time of the bleached spot, as recommended in the literature.[62] In the first minute after bleaching, images were acquired every 5 s, in the second minute, every 10 s, and from the third through the seventh minutes, every 20 s. Additional images were recorded after 5 and 10 additional minutes. For selected samples, additional FRAP experiments were carried out after rinsing the samples with EDTA buffer.

Quartz Crystal Microbalance with Dissipation (QCM-D).[65] QCM-D measurements were performed with a QE301 (electronics unit)/QAFC301 (axial flow chamber) instrument from Q-Sense AB (Gothenburg, Sweden) as described elsewhere.[60,65] Briefly, a clean crystal was mounted in the flow chamber, which was then filled with Ca^{2+} buffer. After the acquisition of a baseline, 0.5 mL of a temperature-equilibrated suspension of unilamellar vesicles at 0.1 mg/mL lipid concentration was injected into the measurement chamber. After the formation of a supported bilayer, excess vesicles were removed by rinsing the measurement chamber with 0.5 mL of a temperature-equilibrated buffer. Full human serum (containing ∼52 mg/mL of serum proteins and 140 mM salt) was then introduced into the chamber for 15 min. Subsequently, the chamber was repeatedly rinsed with Ca^{2+} buffer until the frequency and dissipation signals stabilized. Measurements were performed on the third, fifth, and seventh overtones. For clarity, only the results obtained on the third overtone are reported, scaled by the overtone order.

Results

The formation of supported bilayers on TiO_2 from DOPC:DOPS mixtures requires both the presence of Ca^{2+} in solution and a DOPS content of at least 20%.[47] These conditions were used in this study for FRAP experiments with SPBs formed on titania. For comparison, SPBs containing 20% DOPS prepared on silica in the presence of Ca^{2+} were investigated as well.

To study the mobility of both the zwitterionic (DOPC) and anionic (DOPS) components of the SPBs, two different fluorescent lipids—NBD-PC and NBD-PS—were used. Because lipid−surface interactions, dominated by the headgroup region, were the focus of these experiments, acyl chain-labeled lipids were chosen. Both labeled lipids were found to be fully mobile in supported bilayers formed on silica in the presence of Ca^{2+} (Figure

(47) Rossetti, F. F.; Bally, M.; Michel, R.; Textor, M.; Reviakine, I. *Langmuir* **2005**, *21*, 6443−6450.
(48) Kasemo, B. *J. Prosthet. Dent.* **1983**, *49*, 832−837.
(49) Huang, N.; Yang, P.; Leng, Y. X.; Chen, J. Y.; Sun, H.; Wang, J.; Wang, G. J.; Ding, P. D.; Xi, T. F.; Leng, Y. *Biomaterials* **2003**, *24*, 2177−2187.
(50) Brunette, D. M., Tengvall, P., Textor, M., Thomsen, P., Eds. *Titanium in Medicine*; Springer-Verlag: Berlin, 2001.
(51) Kurrat, R.; Textor, M.; Ramsden, J. J.; Böni, P.; Spencer, N. D. *Rev. Sci. Instrum.* **1997**, *68*, 2172−2176.
(52) Parsegian, V. A. *J. Prosthet. Dent.* **1983**, *49*, 838−842.
(53) Bennett, T. C.; Creeth, J. E.; Jones, M. N. *J. Liposome Res.* **2000**, *10*, 303−320.
(54) Reimhult, E.; Höök, F.; Kasemo, B. *J. Chem. Phys.* **2002**, *117*, 7401−7404.
(55) Reimhult, E.; Höök, F.; Kasemo, B. *Langmuir* **2003**, *19*, 1681−1691.
(56) Jiang, C. H.; Gamarnik, A.; Tripp, C. P. *J. Phys. Chem. B* **2005**, *109*, 4539−4544.
(57) Reviakine, I.; Rossetti, F. F.; Morozov, A. N.; Textor, M. *J. Chem. Phys.* **2005**, *122*, 204711.
(58) MacDonald, R. C.; MacDonald, R. I.; Menco, B. P. M.; Takeshita, K.; Subbarao, N. K.; Hu, L.-R. *Biochim. Biophys. Acta* **1991**, *1061*, 297−303.
(59) Reviakine, I.; Brisson, A. *Langmuir* **2000**, *16*, 1806−1815.
(60) Rossetti, F. F.; Reviakine, I.; Csúcs, G.; Assi, F.; Vörös, J.; Textor, M. *Biophys. J.* **2004**, *87*, 1711−1721.
(61) Berquand, A.; Mazeran, P.-E.; Pantigny, J.; Proux-Delrouyre, V.; Laval, J.-M.; Bourdillon, C. *Langmuir* **2003**, *19*, 1700−1707.
(62) Axelrod, D.; Koppel, D. E.; Schlessinger, J.; Elson, E.; Webb, W. W. *Biophys. J.* **1976**, *16*, 1055−1069.
(63) Soumpasis, D. M. *Biophys. J.* **1983**, *41*, 95−97.
(64) Lopez, A.; Dupou, L.; Altibelli, A.; Trotard, J.; Tocanne, J.-F. *Biophys. J.* **1988**, *53*, 963−970.
(65) Rodahl, M.; Höök, F.; Krozer, A.; Brzezinski, P.; Kasemo, B. *Rev. Sci. Instrum.* **1995**, *66*, 3924−3930.

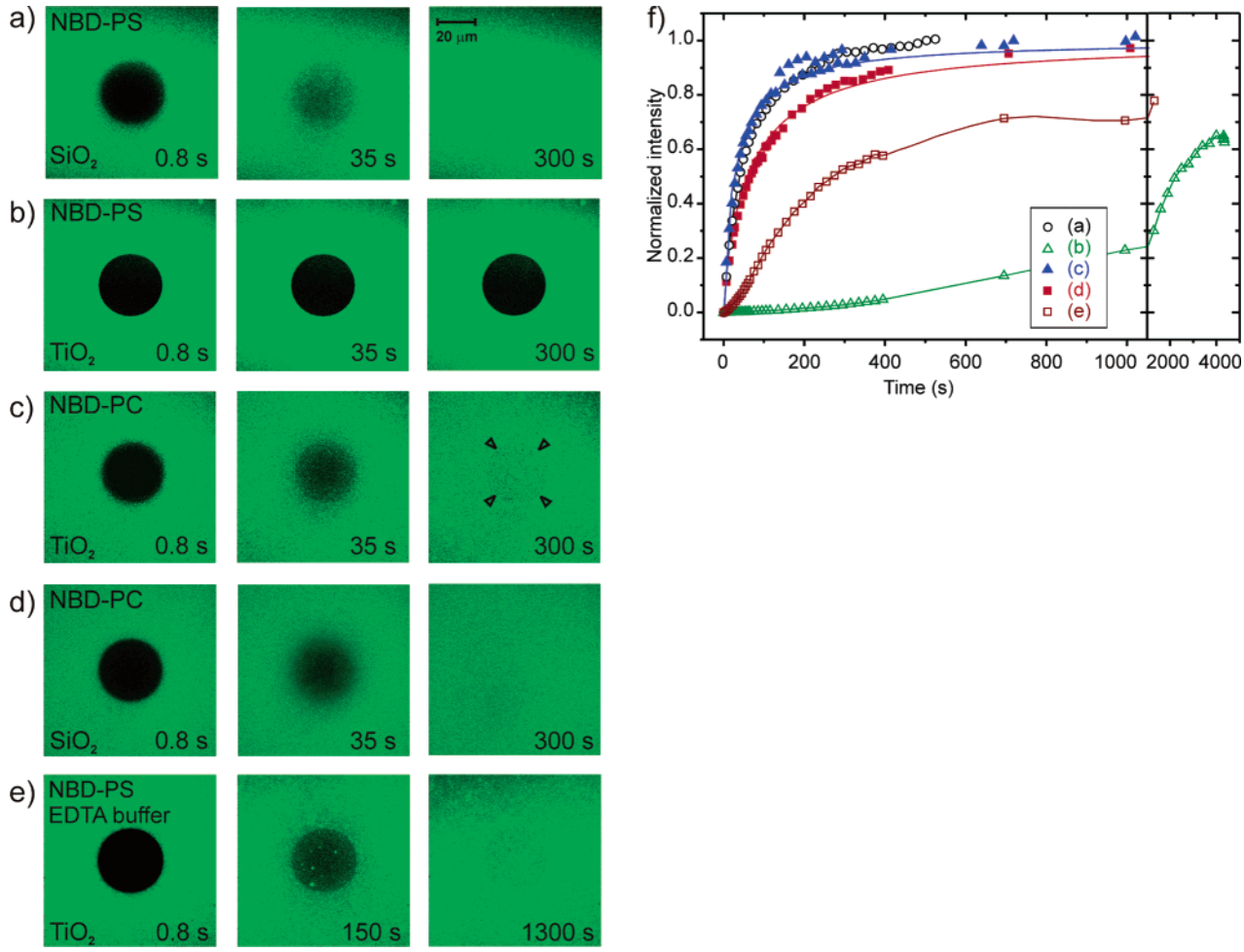

Figure 1. Mobility of the NBD-PC and NPD-PS lipids in 80:20 DOPC:DOPS bilayers formed on SiO_2 and TiO_2. Images a−d were recorded in Ca^{2+} buffer, and image e was recorded in EDTA buffer. (a) NBD-PS is fully mobile on SiO_2: when a spot is bleached in the bilayer, the lipid molecules diffuse laterally, and the fluorescence intensity inside the spot recovers to the value it had before bleaching. (b) On the same time scale as in panel a, NBD-PS appears to be immobile on TiO_2: the fluorescence intensity inside the bleached spot does not recover to more than 4% of the original intensity after up to 300 s. (c) The actual presence of a supported bilayer on TiO_2 (versus, for instance, a vesicular layer) is proven by the fact that when NBD-PS is substituted with NBD-PC full recovery of the bleached area is observed. However, a well-defined edge along the perimeter of the bleached spot (arrowheads) is present up to 300 s after bleaching before eventually disappearing, indicating that a slow-diffusing population of PC is present in these bilayers. (d) For comparison with panel c, the mobility of NBD-PC in an SPB formed on SiO_2 is presented. The fluorescence recovery is complete after 300 s, and no edge along the perimeter of the bleached spot is visible. (e) Removing Ca^{2+} from solution by introducing the Ca^{2+} chelator EDTA restores the lateral mobility of PS in SPBs prepared on TiO_2 (cf. panel b). For reasons that are not entirely clear, the diffusion coefficient of PS in these bilayers is an order of magnitude smaller than that in SPBs prepared on silica (Table 1). This is reflected in longer times needed to reach recovery. The presence of a well-defined edge (similar to the one shown in panel c) suggests that not all of the Ca^{2+} is removed from the TiO_2 surface by the EDTA rinse, and a slow-diffusing population of PS persists. (f) Fluorescence intensity, measured within the bleached area, is plotted as a function of time for the cases shown in panels a−e. The time scale is split to accommodate the very slow diffusion of NBD-PS on TiO_2 in the presence of Ca^{2+} (curve b). Please note that the intensities in the 0−1000 s and 2000−4000 s regions are plotted on different time scales. In the cases where complete recovery of the fluorescence intensity occurs, the lines drawn are fits to the model,[61−64] whereas in the remaining cases they are mere guidelines for the eye.

1a, d, and f, Table 1), as expected.[13,44,66] Similarly, NBD-PC was found to be fully mobile in SPBs prepared on TiO_2 (Figure 1c and f, Table 1), as reported by us previously.[47]

The behavior of NBD-PS in SPBs prepared on TiO_2 was quite different. The recovery was much slower, taking more than 1 h to reach 60% (Figure 1f; note that the accuracy with which the extent of recovery can be determined after such a long time is limited by the overall bleaching of the sample and by instrumental drift). Within the initial 300 to 400 s (i.e., the time it takes for complete recovery of fluorescence when NBD-PC is used as a label), at most 4% of the original fluorescence intensity has recovered (Figure 1b and f); therefore, on the time scale of PC

Table 1. Diffusion Coefficients at Room Temperature of NBD-PC and NBD-PS in SPBs Containing 20% DOPS, Formed on SiO_2 and TiO_2 in the Presence of Ca^{2+}

		SiO_2		TiO_2	
probe	% DOPS	D (10^{-8} cm^2/s)	n	D (10^{-8} cm^2/s)	n
NBD-PC	20	1.1 ± 0.2	13	2.3 ± 0.7	12
NBD-PS	20	2.0 ± 0.1	5	[a]	16

[a] The diffusion coefficient for NBD-PS on TiO_2 was not determined because the data could not be fit to the model used to interpret the recovery data. Apparently, the diffusion of PS in these bilayers did not satisfy the assumptions of the model (e.g., a negligible loss of fluorescence during imaging[61−64]). It is roughly estimated to be about 2 orders of magnitude smaller than the diffusion coefficient of NBD-PC.

(66) Tamm, L. K.; McConnell, H. M. *Biophys. J.* **1985**, *47*, 105−113.

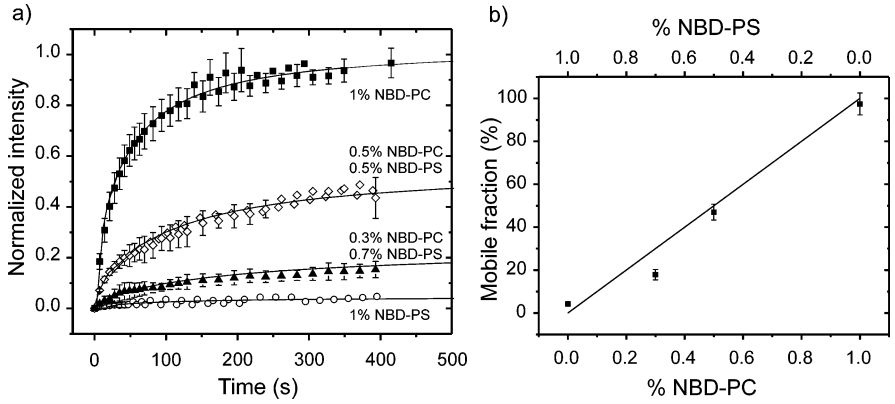

Figure 2. Fluorescence recovery after photobleaching for 80:20 DOPC:DOPS bilayers containing mixtures of NBD-PC and NBD-PS lipids in the presence of Ca^{2+}. The substrate is TiO_2. The amount of labeled lipids (PC + PS) is always 1% of the total lipids. (a) Normalized fluorescence intensities as a function of time. The extent of recovery of the fluorescence intensity after bleaching at 0 s depends on the amount of NBD-PC in the bilayer. The plots were obtained by averaging over at least three recovery curves for each composition. The lines are fits to the model.[61−64] (b) Mobile fractions as a function of NBD-PC content for the data shown in part a. The extent of recovery of the fluorescence intensity after 400 s correlates very well with the relative amount of NBD-PC in the mixture. On this time scale, the error introduced by the very slow diffusion of NBD-PS is negligible.

diffusion, PS is essentially immobile. The analysis of the long-term mobility of PS in these bilayers goes beyond the scope of this study because it requires a different experimental setup suitable for the analysis of slow-diffusing species (cf. Miehlich et al.[67] and Wang et al.[68]).

Both probes were distributed homogeneously on the micrometer scale (Figure 1b and c), suggesting that no phase separation occurs in these SPBs. This conclusion is confirmed by the observation that the diffusion of NBD-PC was, on average, not restricted (see above).

It therefore appears that on the TiO_2 surface an SPB is formed in which the mobility of NBD-PS is severely restricted, whereas PC molecules are freely mobile. To confirm this idea, experiments were performed with 80:20 DOPC:DOPS SPBs containing mixtures of the two probes in various ratios (Figure 2). The extent of recovery, determined 400 s after bleaching (cf. Figure 2a), was found to correlate linearly with the content of NBD-PC (Figure 2b).

Rinsing bilayers prepared on TiO_2 in the presence of Ca^{2+} with a buffer containing the calcium chelator EDTA alleviated the restriction on the mobility of PS, although PS was still found to diffuse at a significantly reduced rate (∼1 order of magnitude slower) as compared to that in bilayers prepared on SiO_2 (Figure 1e and f).

Glasmästar et al.[25] showed previously that egg PC bilayers prepared on silica were resistant to protein adsorption, whereas Richter et al.[36] have recently used the adsorption of the protein prothrombin on PS-containing bilayers prepared on mica and on silica to study the distribution of PS between the two leaflets of the SPBs. Building upon these findings, we investigated protein adsorption from undiluted human serum (∼52 mg/mL protein concentration) on SPBs of various compositions prepared on titania and on silica (Figure 3). On silica, far less protein adsorbed on SPBs that contained only PC (∼100 ng/cm^2) than on those that contained PS (∼1000 ng/cm^2 in the case of 20% PS, Figure 3). (The masses derived from the QCM-D tend to be higher than those determined by optical techniques[69]). Even lower levels of protein adsorption on PC bilayers reported by Glasmästar et al.[25]

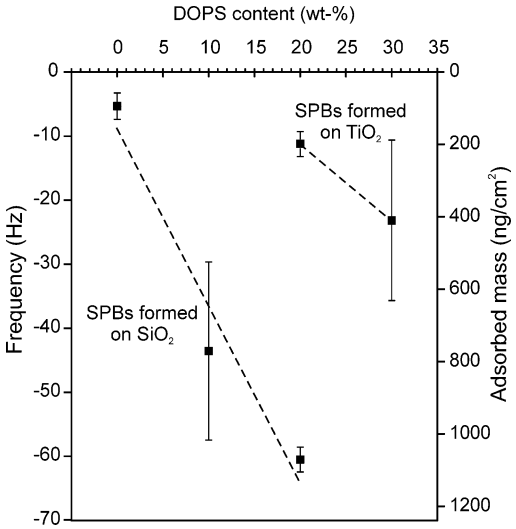

Figure 3. Adsorption of serum proteins from human serum (undiluted) on SPBs of various compositions prepared on SiO_2 and TiO_2, as measured by QCM-D. Pure PC bilayers are very effective in preventing protein adsorption (see also Glasmästar et al.[25]). Increasing the content of PS in the SPBs results in an increased amount of adsorbed proteins on both surfaces. However, the adsorbed mass is significantly less on SPBs prepared on TiO_2, indicating that only a few of the PS molecules contained in the bilayer are exposed in the solution-facing (distal) leaflet. The mass of the adsorbed proteins was calculated from the frequency shifts by using Sauerbrey's equation.[101]

are likely due to the use of diluted serum (10% fetal bovine serum) and better-quality bilayers prepared from sonicated vesicles in their study. Electrostatic interactions, or Ca^{2+}-mediated specific effects, between anionic PS and serum proteins are most likely responsible for the enhanced protein adsorption on PS-containing SPBs.

Far less protein was found to adsorb on PS-containing bilayers prepared on titania: ∼200 ng/cm^2 in the case of 20% PS and ∼400 ng/cm^2 in the case of 30% PS (Figure 3). This suggests that the distal (solution-facing) leaflet of TiO_2-supported bilayers is essentially devoid of PS, containing at most a few percent of it.

The behavior of PS described in this section was observed in samples that were analyzed immediately after preparation, as

(67) Miehlich, R.; Gaub, H. E. *Rev. Sci. Instrum.* **1993**, *64*, 2632−2638.
(68) Wang, L. Y.; Schönhoff, M.; Möhwald, H. *J. Phys. Chem. B* **2002**, *106*, 9135−9142.
(69) Höök, F.; Vörös, J.; Rodahl, M.; Kurrat, R.; Boni, P.; Ramsden, J. J.; Textor, M.; Spencer, N. D.; Tengvall, P.; Gold, J.; Kasemo, B. *Colloid Surf., B* **2002**, *24*, 155−170.

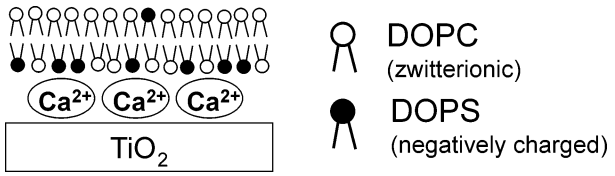

Figure 4. Lipid distribution in PS-containing supported bilayers on titania: most of the PS (black headgroups; i.e., 96−98% in the case of NBD-PS in 80:20 DOPC:DOPS SPBs) is immobilized in a Ca^{2+}-mediated fashion in the proximal leaflet of the SPB.

well as following up to 5 h of incubation in buffer after the preparation.

SPBs could be prepared on TiO_2 from vesicles containing up to 50% DOPS (see Figure 2 in Supporting Information). NBD-PC was fully mobile in all of these bilayers ($D \approx 10^{-8}$ cm^2/s), whereas the mobility of NBD-PS was significantly restricted in all of them. The addition of EDTA alleviated this restriction for bilayers of all compositions tested, just as it did in the case of SPBs containing 20% PS. The extent of recovery of fluorescence observed within the first 400 s increased somewhat with the amount of PS, reaching 12% in the case of SPBs containing 50% PS. This mirrors the increase in the amount of protein adsorbed to the bilayers that contained more PS. (See Figure 3 and the discussion above.) However, even though the SPBs containing more than 30% PS prepared on TiO_2-coated glass coverslips were usually of sufficient quality for FRAP studies, SPBs of identical composition prepared on the rougher QCM-D crystals were affected by the phenomenon of restructuring.[70] The resulting poor reproducibility prevented protein adsorption studies from being carried out on SPBs containing more than 30% PS. Therefore, the data obtained with those bilayers are not shown and not discussed further.

Discussion

We have shown above that in supported bilayers formed on TiO_2 in the presence of Ca^{2+} the mobility of NBD-PS is severely hindered, whereas the mobility of NBD-PC is, on average, not restricted. This effect is surface-specific because it was not observed on SiO_2 for identical lipid and buffer compositions (Figure 1), clearly pointing to an interaction between TiO_2 and PS as its cause. As discussed in our previous publication,[47] this interaction is most likely Ca^{2+}-mediated (Figure 4). Indeed, rinsing the bilayers with an EDTA-containing buffer alleviated the restriction on the mobility of PS (Figure 1e and f).

The somewhat surprising finding is that the mobility of most (96−98%) of the NBD-PS present in the SPBs is restricted, as opposed to the fraction of it expected to be present in the leaflet facing the surface (i.e., ∼50%, assuming a symmetric distribution of lipids between the two leaflets of the bilayer). This suggests that most of the PS that is present in SPBs formed on TiO_2 is sequestered in the proximal leaflet, forming an arrangement such as that shown in Figure 4. The limited extent of protein adsorption on PS-containing SPBs prepared on TiO_2 (Figure 3) strongly supports the idea that PS in SPBs prepared on TiO_2 is distributed asymmetrically, with most of it facing the oxide surface. A similar strategy—the adsorption of prothrombin to PS-containing bilayers prepared on mica and on silica (prothrombin is a plasma protein that specifically interacts with PS)—was recently used by Richter et al.[36] to demonstrate that PS was distributed asymmetrically in SPBs prepared on mica in the presence of Ca^{2+}.

A possible alternative explanation is that the distribution of PS across the two leaflets is symmetrical, but that PS in the distal leaflet is immobilized by trans-leaflet coupling to the immobile PS in the proximal leaflet. This explanation, however, is not only inconsistent with the results of the serum adsorption experiments, but is also at odds with what is known about trans-leaflet coupling: numerous studies have shown that trans-leaflet coupling occurs only over gel-phase domains in the proximal leaflet, and not over lipids (mobile or immobile) that have their hydrophobic chains in a disordered configuration.[34,35,45,46,71,72] The formation of DOPS gel-phase domains in PC:PS mixtures has been observed only in the case of cochleates—multilayered structures held together by trans-bilayer $Ca(PS)_2$ complexes[73−78]—but not in the case of single bilayers. We have previously shown that single bilayers are formed on TiO_2.[47] Furthermore, the formation of gel-phase domains, even with sizes below the resolution of an optical microscope, would significantly impair the diffusion of PC molecules in both leaflets of a symmetric bilayer.[13,34,35,38,41−44,79] An analysis of the diffusion coefficients of NBD-PC (Table 1) suggests that this is not the case. In fact, PC was found, on average, to diffuse *faster* on TiO_2 than on SiO_2 (Table 1). We attribute this faster diffusion to the fact that in titania-supported bilayers PC is enriched in the distal leaflet as compared to the silica-supported ones. The overall diffusion coefficient measured by FRAP contains contributions from lipids located in both leaflets. Because the mobility of PC in the proximal leaflet of silica-supported bilayers is reduced because of its interactions with the surface,[45] the enrichment of PC in the distal leaflet of titania-supported bilayers leads to the larger overall diffusion coefficient as measured by FRAP. It is worth noting that the distal leaflet of silica-supported bilayers may be further depleted of PC, thus further exacerbating the difference in the overall diffusion coefficients: Käsbauer et al.[33] have previously shown that cationic lipids favor the proximal leaflet of silica-supported bilayers; therefore, the anionic PS may favor the distal leaflet.

It should be clear from the above discussion that the mobility of both lipids in these mixed SPBs is quite intricate and certainly deserves further study. In the case of PC in SPBs formed on TiO_2, for example, a close inspection of the fluorescence images shown in Figure 1c reveals a well-defined edge along the perimeter of the bleached area that is visible for up to 300 s. (See also Figure 1a in Supporting Information for the complete recovery series.) No such edge was observed on SiO_2 (Figure 1d, Figure 1b in Supporting Information). This indicates that on TiO_2 there exists a fraction of PC molecules with impaired mobility that is most likely due to the PS molecules present in the proximal leaflet that act as obstacles.[13,41,44,79] This explanation is supported by the fact that after rinsing with EDTA buffer, which alleviates the mobility restriction of PS, as discussed above (Figure 1e, f), this slower PC fraction disappears (Figure 2 in Supporting Information). The overall fast diffusion of PC, in conjunction with the presence of this slow-diffusing fraction in Ca^{2+} buffer,

(70) Richter, R.; Mukhopadhyay, A.; Brisson, A. *Biophys. J.* **2003**, *85*, 3035−3047.

(71) Seul, M.; Subramaniam, S.; McConnell, H. M. *J. Phys. Chem.* **1995**, *89*, 3592−3595.

(72) Feng, Z. V.; Spurlin, T. A.; Gewirth, A. A. *Biophys. J.* **2005**, *88*, 2154−2164.

(73) Papahadjopoulos, D.; Vail, W. J.; Jacobson, K.; Poste, G. *Biochim. Biophys. Acta* **1975**, *394*, 483−491.

(74) Tokutomi, S.; Lew, R.; Ohnishi, S. *Biochim. Biophys. Acta* **1981**, *643*, 276−282.

(75) Feigenson, G. W. *Biochemistry* **1986**, *25*, 5819−5825.

(76) Florine, K. I.; Feigenson, G. W. *Biochemistry* **1987**, *26*, 1757−1768.

(77) Feigenson, G. W. *Biochemistry* **1989**, *28*, 1270−1278.

(78) Coorssen, J. R.; Rand, R. P. *Biophys. J.* **1995**, *68*, 1009−1018.

(79) Huang, Z. P.; Pearce, K. H.; Thompson, N. L. *Biochim. Biophys. Acta* **1992**, *1112*, 259−265.

in our opinion further supports the notion of the asymmetric distribution of PS in bilayers prepared on TiO_2.

Furthermore, PS molecules themselves are mobile at long times. Without detailed knowledge of the nature of the Ca^{2+}-mediated TiO_2−PS interaction, it is difficult to speculate on the mechanism behind this mobility. Deuterium NMR, which has previously been used to resolve diffusion coefficients of PC in each of the two leaflets of bilayers supported on silica particles,[45] might be a useful way to investigate the various aspects of the intricate diffusion processes found in these bilayers.

The asymmetric distribution of PS in SPBs prepared on TiO_2 or on mica[36] from vesicles that have their lipids distributed symmetrically implies that a redistribution of lipids across the two leaflets occurs either during bilayer formation or in the SPB itself after its formation is complete. As discussed by Richter et al.,[36] the latter of these two possibilities is not likely: lipid redistribution is favored by the presence of defects in the bilayer,[37] which are more abundant during SPB formation than in a completely formed SPB. (Defects are expected to reduce the activation energy associated with the transfer of the lipids from one leaflet to the other.) Specifically with respect to bilayers on mica, Richter et al. found their SPBs to be stable against redistribution once formed.[36] (The difference between their results and those of Käsbauer et al.,[33] who observed redistribution as long as 12 h after bilayer formation was complete, is most probably due to the difference in ionic strength employed in the two studies.)

The intriguing question of whether lipid redistribution occurs at the stage of surface-adsorbed vesicles and the role that this process may play in bilayer formation remains to be investigated. This possibility is supported by at least two observations: on one hand, lipid vesicles containing ∼6% negatively charged, fluorescent lipids were observed to leak their contents upon adsorption to a charged surface;[80,81] on the other hand, the adsorption of poly(electrolytes) on oppositely charged liposomes resulted in lipid redistribution and ultimately in the destruction of liposomes that contained sufficient amounts of charged lipids.[82]

The importance of asymmetric SPBs has been recognized for some time.[37,83] There have been a number of attempts to prepare them by Langmuir−Blodgett or Langmuir−Schaefer methods, but these systems suffered from the presence of defects and were not stable with regard to lipid distribution.[37,83,84] SPBs prepared from vesicles are superior in both regards.

In the context of biocompatibility research, the asymmetric distribution of PS across the leaflets of TiO_2-supported bilayers is interesting because it mimics that in the membranes of eukariotic cells (where phosphatidyl serine is confined to the inner leaflet).[85−90] Exposure of PS in the outer leaflet activates macrophages,[89] initiates blood coagulation at the site of injury or at an implant surface,[49,87] and activates the inflammatory response[91] − reactions that are typically associated with implant rejection.[50,92] It is therefore quite interesting that the material that performs relatively well when implanted into the blood stream[49,93] has the property of sequestering PS. In this context, it should be mentioned that platelets, which play a key role in the process of blood coagulation, are found to adsorb to implant surfaces within 5 s of exposure to blood.[94] This time scale is comparable to that of protein adsorption on implant surface, a process that is thought to play a defining role in implant biocompatibility.[8,94] Therefore, platelets as well as microvesicles[95] may very well interact with the implant surface not yet covered with the adsorbed proteins, and the effect of the implant surface on lipid distribution in their membranes may play a role in determining the response of the host.

Finally, as discussed above, the effect of titania on PS is Ca^{2+}-dependent (cf. Figure 1b, d, and f). As such, it also serves as an indirect indication that Ca^{2+} binds to TiO_2, as previously suggested.[96,97] The importance of surface ion exchange processes in the context of interactions between biological systems and artificial surfaces has been discussed by Kasemo et al.[8] On model surfaces, such as mica, Ca^{2+} binding is well known to affect the behavior of biological entities,[36,59] but little experimental information concerning the role of this process in determining implant performance is available. Of note in this context are studies on the effects of Ca^{2+} ion implantation on the osteointegration of titanium implants[98] and the findings by Satsangi et al.[99,100] of the enhancement of osteoblast growth and differentiation by titanium implants coated with Ca−PS−PO_4 complexes.

The findings reported here will provide a starting point for further investigations of these phenomena.

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(80) Johnson, J. M.; Ha, T.; Chu, S.; Boxer, S. G. *Biophys. J.* **2002**, *83*, 3371−3379.
(81) Schönherr, H.; Johnson, J. M.; Lenz, P.; Frank, C. W.; Boxer, S. G. *Langmuir* **2004**, *20*, 11600−11606.
(82) Yaroslavov, A. A.; Kiseliova, E. A.; Udalykh, O. Y.; Kabanov, V. A. *Langmuir* **1998**, *14*, 5160−5163.
(83) Kalb, E.; Frey, S.; Tamm, L. K. *Biochim. Biophys. Acta* **1992**, *1103*, 307−316.
(84) Liu, J.; Conboy, J. C. *J. Am. Chem. Soc.* **2004**, *126*, 8376−8377.
(85) Yeagle, P. L. *The Membranes of Cells*, 2nd ed.; Academic Press: San Francisco, 1987.
(86) Zwaal, R. F. A.; Schroit, A. J. *Blood* **1997**, *89*, 1121−1132.
(87) Bevers, E. M.; Comfurius, P.; Dekkers, D. W. C.; Harmsma, M.; Zwaal, R. F. A. *Biol. Chem.* **1998**, *379*, 973−986.
(88) Bevers, E. M.; Comfurius, P.; Dekkers, D. W. C.; Zwaal, R. F. A. *Biochim. Biophys. Acta* **1999**, *1439*, 317−330.
(89) van Heerde, W. L.; Robert-Offerman, S.; Dumont, E.; Hofstra, L.; Doevendans, P. A.; Smits, J. F. M.; Daemen, M.; Reutelingsperger, C. P. M. *Cardiovasc. Res.* **2000**, *45*, 549−559.
(90) Balasubramanian, K.; Schroit, A. J. *Annu. Rev. Physiol.* **2003**, *65*, 701−734.
(91) Esmon, C. T. *Br. J. Haematol.* **2005**, *131*, 417−430.
(92) Hu, W. J.; Eaton, J. W.; Tang, L. P. *Blood* **2001**, *98*, 1231−1238.
(93) Biehl, V.; Wack, T.; Winter, S.; Seyfert, U. T.; Breme, J. *Biomol. Eng.* **2002**, *19*, 97−101.
(94) Nygren, H.; Tengvall, P.; Lundstrom, I. *J. Biomed. Mater. Res.* **1997**, *34*, 487−492.
(95) Giesen, P. L. A.; Rauch, U.; Bohrmann, B.; Kling, D.; Roque, M.; Fallon, J. T.; Badimon, J. J.; Himber, J.; Riederer, M. A.; Nemerson, Y. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 2311−2315.
(96) We have measured the ζ potential of ∼200 nm TiO_2 particles as a function of Ca^{2+} concentration in the buffer. From 0 to 2 mM Ca^{2+}, we found a dramatic reduction of the ζ potential from −33 ± 1 mV to −3 ± 1 mV. At 3 mM, the ζ potential was inverted (+2.2 mV). For comparison, the concentration of Ca^{2+} in human blood is ∼ 2.5−3 mM, of which 50% is in the ionized form. Our measurements of IEP and of the ζ potential in the absence of Ca^{2+} are consistent with published values, Rezwan, K. Protein treated aqueous colloidal oxide particle suspensions: driving forces for protein adsorption and conformational changes. Dissertation Nr. 15882, Department of Materials, ETH Zürich: Zürich, 2005, 174 pages.
(97) Ellingsen, J. E. *Biomaterials* **1991**, *12*, 593−596.
(98) Nayab, S. N.; Jones, F. H.; Olsen, I. *Biomaterials* **2005**, *26*, 4717−4727.
(99) Satsangi, N.; Satsangi, A.; Glover, R.; Ong, J. L.; Satsangi, R. K. *J. Mater. Sci.: Mater. Med.* **2004**, *15*, 693−697.
(100) Satsangi, A.; Satsangi, N.; Glover, R.; Satsangi, R. K.; Ong, J. L. *Biomaterials* **2003**, *24*, 4585−4589.
(101) Sauerbrey, G. *Z. Phys.* **1959**, *155*, 206−222.

Supporting Information Available: Fluorescence recovery images of SPBs prepared on TiO_2 and SiO_2. Effect of rinsing with EDTA buffer on the mobility of NBD-PC in SPBs formed on TiO_2. This material is available free of charge via the Internet at http://pubs.acs.org.

Note Added in Proof. In their recent article, Richter et al.[102] also show that DOPS is distributed asymmetrically in TiO_2-supported bilayers, and discuss the influence of the lipid redistribution process on the behavior of the surface-adsorbed vesicles. Their determination of DOPS content in the distal leaflet of the supported bilayers is based on the analysis of prothrombin adsorption by QCM-D.[36] DOPS content of $<3\%$ and $\sim17\%$ in TiO_2-supported SPBs prepared from liposomes containing 20% and 50% DOPS, respectively, reported by these authors (Table 1 in ref. 102) agree very well with our FRAP-based estimates of $\sim2-4\%$ and $\sim12\%$ for the DOPS content in the distal leaflets of TiO_2-supported SPBs of identical nominal compositions. However, there is also a curious difference between our observations: while we observed that the process of SPB formation from liposomes containing more than 30% DOPS on QCM crystals was affected by the phenomenon of restructuring,[70] Richter et al. report that bilayers could be prepared from liposomes containing up to 80% of DOPS. There are three possible reasons for these different observations: liposomes used in the two studies were prepared by different methods (sonication in ref 102, extrusion in our case); buffers used in the two studies were of different ionic strengths; and the QCM crystals used in these two studies were from different sources.

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(102) Richter, R. P.; Bérat, R.; Brisson, A. R. *Langmuir* **2006**, in press. DOI: 10.1021/la052687c.