Robust hybrid thin films that incorporate lamellar phospholipid bilayer assemblies and transmembrane proteins

Gautam Gupta, Plamen Atanassov, and Gabriel P. López[a)]
Department of Chemical and Nuclear Engineering, The University of New Mexico, Albuquerque, New Mexico 87131

(Received 20 January 2006; accepted 14 February 2006; published 4 April 2006)

This study describes facile methods based on sol-gel processing for the formation of robust thin films that incorporate phospholipid bilayer membranes and transmembrane proteins as multilamellar assemblies in cross-linked silica matrices. Transmission electron microscopy and x-ray diffraction were used to examine the lamellar structure of the hybrid thin films containing 1, 2-dioleyl-*sn*-glycero-3-phospoethanolamine (DOPE), an unsaturated lipid, and 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), a saturated lipid. While the *d* spacing measured for DOPE containing films varied (from 35 to 48 Å) depending on the amount of DOPE added to the coating solution (10–1 wt %), similar changes were not observed for the films containing saturated lipid, DMPC (*d* spacing ~43 Å). Addition of purple membrane containing bacteriorhodopsin to the DOPE/silica coating solution led to the formation of multilamellar vesicle-like structures within the thin films. Mild sonication of these solutions containing the purple membrane prior to coating led to the formation thin films with planar multilamellar structures that exhibit uniform *d* spacing. The study further investigates the effects of incorporation of gramicidin and sonication on the structure of hybrid films and speculates on the eventual application of thin films prepared in this manner. © *2006 American Vacuum Society.* [DOI: 10.1116/1.2185654]

I. INTRODUCTION

Phospholipid bilayer membranes (PBMs), transmembrane proteins and associated molecules represent the quintessential biointerface. They are essential components of all cellular systems and enable a variety of functions including compartmentalization, passive and active transport, signal transduction, specific recognition, and energy utilization.[1] Because of their versatility in function, scientists have long sought to incorporate PBMs into artificial materials and devices that have a broad range of potential applications including controlled drug delivery,[2] biosensing for drug discovery, medical diagnosis and environmental monitoring,[3] chemical and biological warfare agent sequestration,[4] actuator development,[5] and bio-fuel cell development.[6] The fragility of PBMs has precluded the realization of the goal of incorporation of these versatile assemblies into many types of functional devices.

In this study we describe an approach toward the synthesis of a family of robust hybrid bio-inorganic materials that incorporate lamellar phospholipid bilayer assemblies containing transmembrane proteins into thin films and membranes. In recent years, a number of methods have been developed for facile fabrication of thin membranes and microstructures containing highly ordered lyotropic liquid crystalline surfactant phases encapsulated in rigid silica matrices.[7–10] The surfactant phases obtained (e.g., lamellar, hexagonal, or cubic) can be controlled by the type of surfactant used and the processing conditions employed. Phospholipids are the most attractive candidate and obvious choice of surfactants for incorporation of membrane proteins because they mimic the natural environment of the membrane proteins, and unlike other surfactants, they do not irreversibly denature membrane proteins.[11] Phospholipids have been used in drug delivery and biosensors because they form self-assembled structures, liposomes, which exhibit low toxicity and good biocompatibility.[12] Several examples of the synthesis of templated silicas, where lipids or liposomes have been used as structure directing agents, have been published.[13–15] In those studies that have included proteoliposomes, however it has not been demonstrated that macroscopic membrane or thin film materials can be formed which contain uniform nanostructures (e.g., a planar multilamellar architecture) that are amenable to incorporation of transmembrane proteins in nonrandom orientations.[15]

In this article we demonstrate the synthesis of hybrid thin films containing phospholipids and transmembrane proteins in a cross-linked silica matrix. The phospholipids form highly ordered lamellar assemblies in which the lamellae are parallel to the film surface. We also examine the effects of phospholipid concentration and sonication of the solution used to form the thin films. This study examines the lipid 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), a saturated lipid and 1, 2-dioleyl-*sn*-glycero-3-phospoethanolamine (DOPE), an unsaturated lipid, which exhibits a gel to liquid crystalline phase transition at a temperature of −16 °C and is shown to be very effective in introducing membrane proteins into phospholipid bilayer membranes.[16] We examined hybrid thin films that contain a model transmembrane protein, bacteriorhodopsin, and a model transmembrane peptide, gramicidin.

a)Electronic mail: gplopez@unm.edu

Bacteriorhodopsin (bR) is a 26 kDa transmembrane protein that uses a photochemical process to transport protons across the cell membrane and by doing so can create an ionic proton flux in a direction opposite to the proton gradient.[17] Bacteriorhodopsin is considered to be a prototype for a class of membrane transporters and also serves as a structural model for an important group of transmembrane receptors, the G-protein-coupled receptors. Several active transport systems have been intensively studied in recent years, including those involved in proton and ionic pumping,[18] water pumping,[19] neuro transmission,[20] and metal ion uptake up in bacteria.[21] The importance of active transport systems has led to the isolation and detailed study of several transporter proteins such as bR and their reconstitution in liposomes, vesicles, and supported lipid bilayers.[22,23] Recently, Luo *et al.* demonstrated the immobilization and functionality of proteoliposomes (bR containing liposomes) in silica matrices.[15] Gramicidin, a model ion channel, is an unusual transmembrane peptide, with alternating D and L amino acids. Gramicidin dimerizes to from a conductive state where two 15-amino acid peptides forming β-helices meet head to head at their N-termini in the interior of a lipid bilayer. The outer surface of the gramicidin that interacts with the lipids is hydrophobic in nature, and only monovalent cations pass through the more polar core of the helix.[24]

We prepared hybrid phospholipid–silica thin film assemblies by spin coating a mixture of phospholipid and a diluted silica sol which was made by a modified two-step acid catalyzed process similar to that described by Lu *et al.*[7] Figure 1(A) shows a transmission electron microscope (TEM) micrograph of the surface region of a DMPC–silica thin film (uncalcined) prepared from a diluted silica sol containing 2.5 wt % DMPC. The inset of Fig. 1(A) shows the x-ray diffraction (XRD) pattern of this hybrid lipid–silica film, which shows a lamellar mesostructure with a (001) peak at 43 Å. The presence of higher-order diffraction peaks (002), (003), (004), and the corresponding TEM image indicates the formation of a highly ordered mesophase with alternating silica and lipid bilayers. Figure 1(B) shows a TEM micrograph of the surface region of a phospholipid–silica thin film (uncalcined) prepared from a diluted silica sol containing 2.5 wt % DOPE. The inset of Fig. 1(B) shows the XRD pattern of a hybrid DOPE–silica film. The *d* spacing observed for this film is ~47 Å, with a first order diffraction peak at 47 Å and a second order peak at 24 Å in the XRD pattern. The TEM images and XRD patterns shown in Fig. 1 are representative of data obtained from many samples prepared by this method. The XRD pattern is consistent with a lamellar phase structure with alternating phospholipid and silica layers that are parallel to the film surface, although it might also be obtained from other structures [e.g., one-dimensional (1D) hexagonal]. The fact that similar TEM images were obtained from a large number of samples at a variety of orientations strongly suggests lamellar structures.

Perhaps the most definitive indication of a lamellar structure is that we observed no peaks in the XRD pattern after the phospholipid–silica thin films were subjected to calcination for 4 h at 400 °C, indicating the collapse of the lamellar structure upon calcination of these thin films. It has been observed that hexagonal ordered surfactant-templated silica thin films prepared under similar conditions do not collapse upon calcination.[9] Similar uniaxially aligned, multilamellar phospholipid bilayer assemblies (without silica) prepared by solvent evaporation have been used in investigations of peptide–phospholipid interactions by solid-state nuclear magnetic resonance (NMR).[25] We have obtained similar lamellar structures by mixing other phospholipids with silica sols.

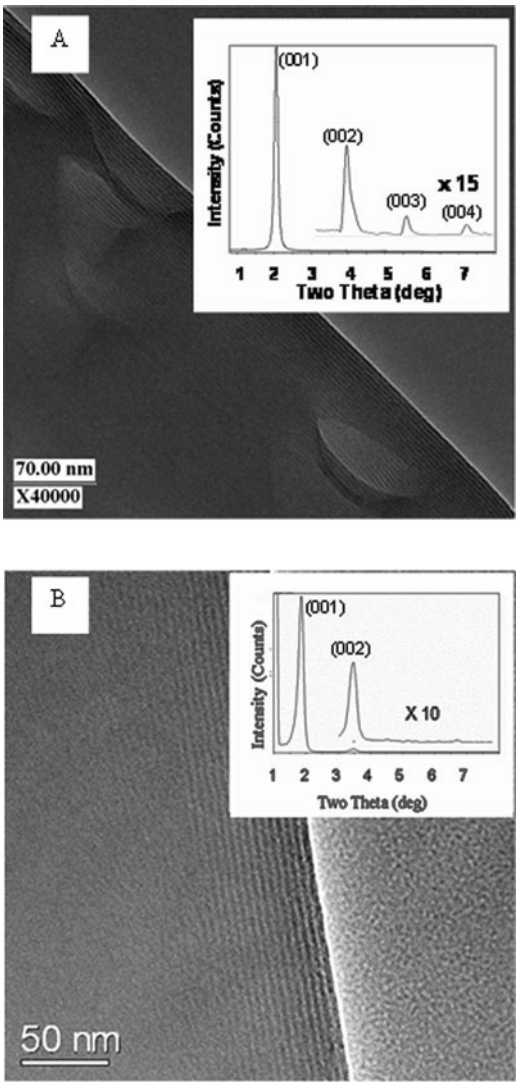

FIG. 1. TEM micrograph of the surface of lipid–silica thin film (2.5 wt % lipid in diluted silica sol): (A) DMPC–silica and (B) DOPE–silica. (Insets) XRD patterns of the same films.

Even though the structure of lipid–silica assemblies may depend on a number of parameters such as concentration of the surfactant, pH, salt concentration, type of solvent, and temperature at which the assemblies are synthesized, the packing fraction parameter for surfactants has been extensively used in the past to understand the self-assembly process.[26,27] The packing parameter or shape factor, g, of an amphiphile is given by the relation $(v_{hc}/a_o l_c)$, where a_o is the

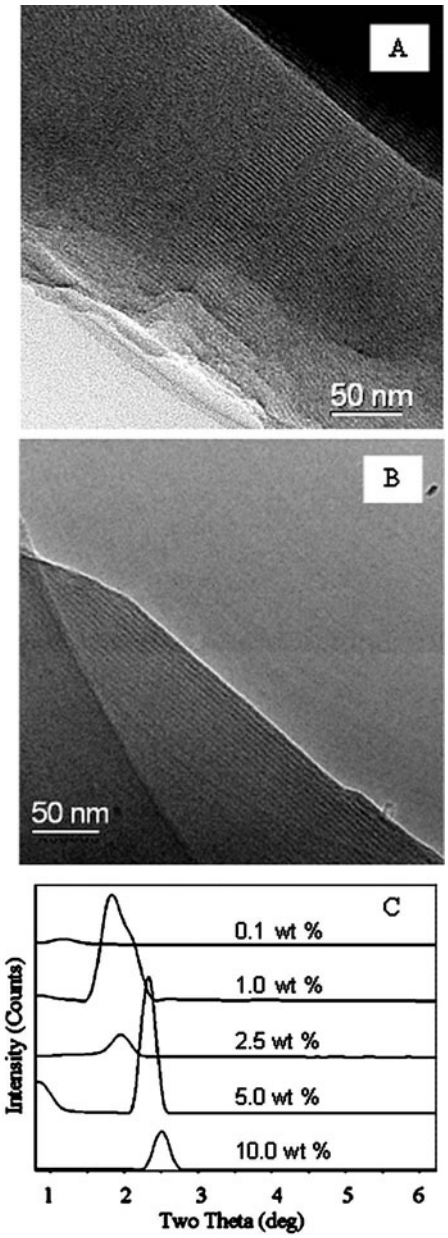

FIG. 2. TEM micrographs of the surface of DOPE–silica thin films: (A) film prepared with 10 wt % DOPE in diluted silica sol: (B) film prepared with 1 wt % DOPE, and (C) XRD patterns of DOPE–silica films prepared from 10, 5.0, 2.5, 1, and 0.1 wt % of DOPE.

head group area, l_c is the critical length of the hydrocarbon chain, and v_{hc} is the volume of hydrocarbon chains).[26] In general, phospholipids tend to have a packing parameter in the range of 0.5–1.0, which is a range in which the formation of lamellar bilayer architecture are typically expected.[26] Our results are consistent with these expectations. In this study, we restrict our further discussion to results obtained with DOPE because of our interest in incorporation of transmembrane proteins and peptides.[16]

Figures 2(A) and 2(B) show TEM micrographs of the surface region of DOPE–silica thin films (uncalcined samples) prepared from phospholipid concentrations of 10 and 1 wt % DOPE in the diluted stock sol, respectively. These micrographs clearly show the lamellae of the thin films, which are parallel to the film surface. The lamellar structure was confirmed by XRD. Figure 2(C) shows that the *d* spacing of the DOPE–silica assembly decreases as the concentration of the phospholipid in the diluted coating sol is increased. The lamellar phase obtained with 10 wt % DOPE has a *d* spacing of 35 Å and the lamellar phase obtained with 1 wt % DOPE has a *d* spacing of 48 Å, suggesting that, with increased phospholipid concentration, either the thickness of the silica wall,[28] or that of the hydrophobic bilayer,[29] or both, is reduced. It should be noted that when saturated phospholipids (characterized by a higher gel-to-fluid transition temperature) are used in the coating process, changes in *d* spacing with increased phospholipid concentration have not been observed.

We mixed bR-containing purple membranes (lyophilized) with dried phospholipid (DOPE) and then a specified amount of diluted stock sol was added to the resultant mixture. Figure 3(A) shows a TEM micrograph of a thin film prepared by spin coating 2.5 wt % DOPE and bacteriorhodopsin in 100:1 mole ratio. The TEM micrograph shows the presence of multilamellar, vesicle-like structures. TEM analysis revealed that other regions of the film contained planar lamellar structures (data not shown). The XRD pattern of the same film [Fig. 3(C)] shows two distinct peaks of different periodicities (*d* spacing); a peak corresponding to a *d* spacing of ~46 Å and another peak corresponding to 53 Å. Figure 3(B) shows a TEM micrograph of a thin film prepared from the same ratio of silica, DOPE, and purple membrane components, but with spin coating after the coating mixture had been subjected to mild sonication. The resultant thin film contained planar lamellar structures, and XRD revealed a single periodicity of ~46 Å. It is important to note that TEM did not reveal ordered multilamellar structures (neither planar nor vesiclar) when only lyophilized purple membrane (nominally 10 lipid molecules to 1 bR)[17] was added to the silica precursor sol. Similarly XRD showed only weak diffraction peaks for such thin films.

These data suggest two conclusions: that the inclusion of purple membrane components with the DOPE formulation results in the formation of multilamellar vesicle-like structures, and that sonication results in the disruption of these structures and the subsequent formation of planar lamellar structures. It is likely that the XRD peak observed at higher *d* spacing in Fig. 3(C) corresponds to the multilamellar vesicle-like structures.

We further investigated the effect of sonication on DOPE–silica thin films and also incorporated gramicidin into the films. Figure 4 shows the XRD pattern of DOPE–silica thin films with and without the addition of gramicidin. TEM analysis revealed the planar lamellar structure of these thin films, and also indicated that the structure of all these films was similar.

The *d* spacing observed for DOPE-silica assemblies before sonication is ~47 Å and after sonication was observed to be 45 Å. Further experimentation (e.g., with NMR spectroscopy) might elucidate the structural changes in these thin

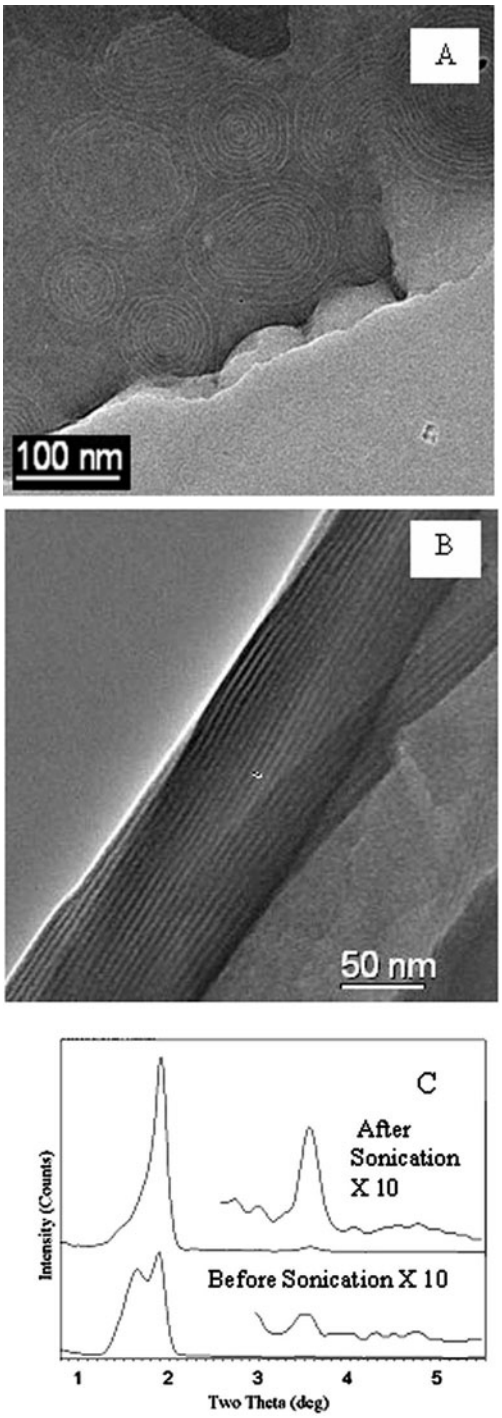

FIG. 3. TEM micrographs of DOPE–silica thin films (prepared from 2.5 wt % DOPE) containing bacteriorhodopsin (100:1, DOPE:bR): (A) before sonication, (B) after sonication, and (C) XRD pattern of the same films.

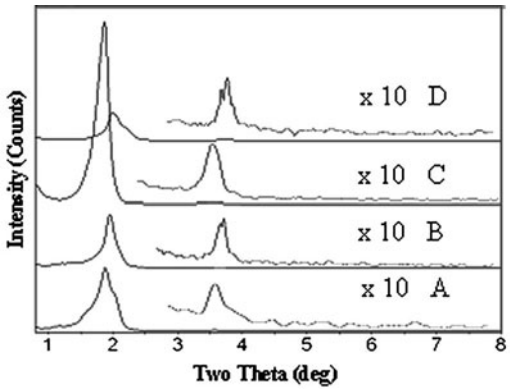

FIG. 4. XRD pattern of DOPE–silica thin films: (A) without sonication, (B) with sonication, (C) with gramicidin, but without sonication, and (D) with gramicidin and sonication.

films that accompanies sonication. Addition of gramicidin (e.g., up to 10:1, DOPE: gramicidin) resulted in planar lamellar structures, but did not result in appreciable change in the *d* spacing measured for the resulting thin film samples. Sonication of the 10:1 DOPE: gramicidin precursor resulted in a decrease in *d* spacing (to ~44 Å) similar to that observed for DOPE–silica samples. Further experimentation is underway to determine the degree of ordering of the gramicidin incorporated into the DOPE–silica thin films.

We have used a number of spectroscopic methods to confirm the presence of bacteriorhodopsin or gramicidin in our hybrid lamellar thin films. Elemental analyses via energy dispersive x-ray analysis and x-ray photoelectron spectroscopy confirm the increase in concentrations of elements associated with the protein (N,S) and the peptide (N). Furthermore, signatures characteristic of the peptide backbone can also be detected by XPS, Fourier transform infrared spectroscopy, and secondary ion mass spectrometry. Further detailed spectroscopic investigations of the structures of the hybrid lamellar thin films are ongoing to elucidate the distribution, orientation, and functionality of incorporated peptides and proteins.

In conclusion, we have demonstrated a simple method for the formation of hybrid thin films that incorporate phospholipid bilayer assemblies containing proteins and peptides in a planar lamellar structure into a robust silica matrix. The *d* spacing of the lamellar structures can be controlled to a certain extent by varying the phospholipid (DOPE) concentration. The incorporation of bR into the planar multilamellar structure was only obtained when the purple membrane was diluted with DOPE and the resultant mixture was sonicated prior to spin coating. In studies on the effect of sonication on hybrid thin films (without bR and with and without gramicidin) it was found that sonication results in a decrease of approximately 2 Å in *d* spacing for the spin coated films. No change in *d* spacing was observed upon the addition of gramicidin to lipid–silica coating solutions.

Hybrid thin films containing lamellar phospholipid membrane assemblies are currently being explored in our laboratories for utilization as selective and perhaps active transport membrane systems. Specific goals of further study include the control of the extent of crosslinking in silica (or other matrices) to optimize analyte transport, and the optimization of pH conditions for formation of the films.

II. EXPERIMENT

Silica–phospholipid thin films were synthesized using a two-step acid catalyzed sol-gel process. Tetraethyl orthosilicate (TEOS), ethanol, de-ionized water, and 0.07 N HCl were mixed in the molar ratio 1.0 TEOS: 3.8 C_2H_5OH: 1.0H_2O1: 5×10^{-5} HCl and refluxed at 60 °C for 90 min. The resulting solution is referred to as stock sol. The stock sol was diluted with ethanol (1:2) followed by addition of water and dilute HCl. Gramicidin and TEOS were purchased from Sigma Aldrich, MO. Bacteriorhodopsin was purchased from Munich Innovative Biomaterials GmbH, Germany. DOPE and DMPC were purchased from Avanti Polar Lipids, AL. The chloroform was removed from the phospholipids by evaporation followed by desiccation under vacuum for at least 12 h. The specified amount of dry phospholipids was added to the diluted stock sol and the phospholipids were hydrated for 1 h with intermittent vortexing. Calcination of samples was performed at 400 °C (heating rate of 1 °C/min) for 4 h. A similar procedure was adopted for the protein–phospholipid–silica thin films. An appropriate amount of purple membrane was added to dried phospholipids before their addition to the diluted stock sol (mole ratio = 100:1, lipid: protein). For peptide gramicidin–lipid silica assemblies, an appropriate amount of gramicidin was dissolved in methanol. This solution was mixed with 10 mg/ml lipid solution in chloroform. The final solution was dried under a stream of nitrogen and diluted stock sol was subsequently added to the dried lipid–gramicidin (mole ratio = 10:1, lipid: peptide). These solutions were hydrated for 1 h and coated onto clean silicon wafers. Sonication was performed for 5 min at room temperature for some of the samples. Resulting solutions were spin coated on "piranha" cleaned silicon wafers at a speed of 3000 rpm for 1 min. As a note of caution: Piranha solution reacts violently with organics and the handling of Piranha solutions requires special protection equipment including: a full-face shield, heavy-duty rubber gloves, and an acid apron.

In order for complete condensation of silicate species to take place, all samples were aged for 1 week before subjecting to further characterization. The films were characterized using x-ray diffraction on a Siemens D5000 diffractometer using Ni filtered Cu Kα radiation with $\lambda=1.5406$ Å in $\theta-2\theta$ ($2\theta=0.8°-8.0°$) step-scan mode using a step size 0.02° for 3 sec. Transmission electron microscopy was performed on a JEOL 2010 with 200 kV accelerating voltage, equipped with a Gatan slow scan charge coupled device camera. The samples were prepared either by scratching the films from silicon substrates using a cutting blade or using standard cross-section techniques.

ACKNOWLEDGMENTS

The work was made possible through a grant from the Army Research Office (Grant No. DAAD19-03-1-0173). XPS and SIMS data are collected at NESAC/BIO (NIH NIBIB Grant No. EB-002027).

[1]D. L. Nelson and M. M. Cox, *Lehninger Principles of Biochemistry*, 4th ed. (W. H. Freeman and Company, San Francisco, 2004), Chap. 11.
[2]T. Lian and R. J. Y. Ho, J. Pharm. Sci. **90**, 667 (2001).
[3]D. Ottenbacher, R. Kindervater, P. Gimmel, B. Klee, F. Jahnig, and W. Gopel, Sens. Actuators B **6**, 192 (1992).
[4]H. Bayley, O. Braha, and LQ. Gu, Adv. Mater. (Weinheim, Ger.) **12**, 139 (2000).
[5]C. Nicolini, Biosens. Bioelectron. **10**, 105 (1995).
[6]L. Y. Su, F. M. Hawkridge, and M. C. Rohten, Chem. Biodiversity. **1**, 1281 (2004).
[7]Y. F. Lu, Y. Yang, A. Sellinger, J. M. Lu, J. M. Huang, H. Y. Fan, R. Haddad, G. P. López, A. R. Burns, D. Y. Sasaki, J. Shelnutt, and C. J. Brinker, Nature (London) **411**, 913 (2001).
[8]G. V. Rama Rao, G. P. López, J. Bravo, H. Pham, A. K. Datye, H. Xu, and T. L. Ward, Adv. Mater. (Weinheim, Ger.) **14**, 1301 (2002).
[9]Y. F. Lu, R. Ganguli, C. A. Drewien, M. T. Anderson, C. J. Brinker, W. Gong, Y. Guo, H. Soyez, B. Dunn, M. H. Huang, and J. I. Zink, Nature (London) **389**, 364 (1997).
[10]N. R. B. Coleman and G. S. Attard, Microporous Mesoporous Mater. **44**, 73 (2001).
[11]R. M. Garavito and S. Ferguson-Miller, J. Biol. Chem. **276**, 32403 (2001).
[12]R. J. Philippot and F. Schuber, *Liposomes as Tools in Basic Research and Industry* (CRC, Boca Raton, FL, 1995), Chap. 5.
[13]S. A. Yamanaka, D. H. Charych, D. A. Loy, and D. Y. Sasaki, Langmuir **13**, 5049 (1997).
[14]H. K. Baca, PhD thesis, University of New Mexico, Albuquerque, New Mexico, 2005.
[15]T. J. M. Luo, R. Soong, E. Lan, B. Dunn, and C. Montemagno, Nat. Mater. **5**, 220 (2005).
[16]I. D. Alves, G. F. J. Salgado, Z. Salamon, M. F. Brown, G. Tollin, and V. J. Hruby, Biophys. J. **88**, 198 (2005).
[17]J.-P. Cartailler and H. Luecke, Annu. Rev. Biophys. Biomol. Struct. **326**, 1317 (2003).
[18]J. K. Lanyi and A. Pohorille, Trends Biotechnol. **19**, 140 (2001).
[19]K. Ishibashi, M. Kuwahara, and S. Sasaki, Rev. Physiol. Biochem. Pharmacol. **14**, 1 (2000).
[20]S. M. Parsons, FASEB J. **14**, 2423 (2000).
[21]G. S. Moeck and J. W. Coulton, J. Mol. Microbiol. **28**, 675 (1998).
[22]J. A. He, L. Samuelson, L. Li, J. Kumar, and S. K. Tripathy, Adv. Mater. (Weinheim, Ger.) **11**, 435 (1999).
[23]C. Nicolini, V. Erokhin, S. Paddeu, and M. Sartore, Nanotechnology **9**, 223 (1998).
[24]A. Finkelstein and O. S. Andersen, J. Membr. Biol. **59**, 155 (1981).
[25]S. Yamaguchi, T. Hong, A. Waring, R. I. Lehrer, and M. Hong, Biochemistry **4**, 9852 (2002).
[26]J. N. Israelachivili, *Intermolecular & Surface Forces* 2nd ed. (1992), Table 17.2.
[27]A. J. Karkamkar, S. S. Kim, S. D. Mahanti, and T. J. Pinnavaia, Adv. Funct. Mater. **14**, 507 (2004).
[28]M. T. Bore, S. B. Rathod, T. L. Ward, and A. K. Datye, Langmuir **19**, 256 (2003).
[29]C. G. Knight, *Liposomes from Physical Structure to Therapeutic Applications* (Elseiver/North-Holland Biomedical Press, Amsterdam, The Netherlands, 1981), Chap. 8.