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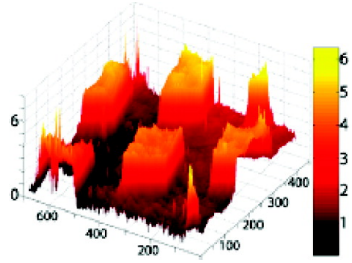

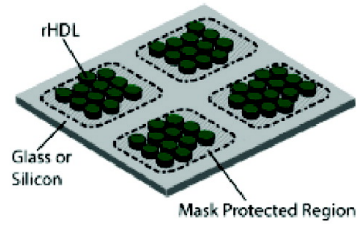

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Bridging Across Length Scales: Multi-Scale Ordering of Supported Lipid Bilayers via Lipoprotein Self-assembly and Surface Patterning

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Abstract: We show that a two-step process, involving spontaneous self-assembly of lipids and apolipoproteins and surface patterning, produces single, supported lipid bilayers over two discrete and independently adjustable length scales. Specifically, an aqueous phase incubation of DMPC vesicles with purified apolipoprotein A-I results in the reconstitution of high density lipoprotein (rHDL), wherein nanoscale clusters of single lipid bilayers are corralled by the protein. Adsorption of these discoidal particles to clean hydrophilic glass (or silicon) followed by direct exposure to a spatial pattern of short-wavelength UV radiation directly produces microscopic patterns of nanostructured bilayers. Alternatively, simple incubation of aqueous phase rHDL with a chemically patterned hydrophilic/hydrophobic surface produces a novel compositional pattern, caused by an increased affinity for adsorption onto hydrophilic regions relative to the surrounding hydrophobic regions. Further, by simple chemical denaturation of the boundary protein, nanoscale compartmentalization can be selectively erased, thus producing patterns of laterally fluid, lipid bilayers structured solely at the mesoscopic length scale. Since these aqueous phase microarrays of nanostructured lipid bilayers allow for membrane proteins to be embedded within single nanoscale bilayer compartments, they present a viable means of generating high-density membrane protein arrays. Such a system would permit in-depth elucidation of membrane protein structure−function relationships and the consequences of membrane compartmentalization on lipid dynamics.

Introduction

The ability to pattern or compartmentalize materials at multiple, independent length scales offers interesting opportunities to integrate length-scale dependent material properties. In this regard, approaches that merge bottom-up self-assembly with top-down microfabrication have proven highly valuable.[1−4] Bottom-up approaches typically yield ordering at the level of the building blocks, namely molecular to nanometer length scales, whereas top-down lithographic approaches routinely allow for the creation of patterns with microscopic feature dimensions. Material scientists have fruitfully employed these notions to build diverse classes of hierarchically structured solids and thin-films using many different building blocks including crystallizing molecules,[5] colloids,[6] phase-separating polymers,[7] and sol−gel precursors.[8] In all of these cases, the hierarchy is obtained in static structural organization. Here, we extend this notion to develop a fluid structure, which incorporates a hierarchy of spatial confinements. Specifically, we show that spontaneous self-assembly of lipids and apolipoproteins[9−11]—which mimics the biological construction of high-density lipoproteins (HDL)—can be combined with spatially templated surface self-assembly (or photochemical patterning) to organize single, fluid supported lipid bilayers[12] over two discrete and independently adjustable length scales.

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High-density lipoprotein (HDL) are naturally occurring core−shell nanoparticles (∼10−20 nm dia.) which circulate in the blood collecting cholesterol and other lipids from peripheral tissues and transporting them to the liver for efflux.[13] Structurally, they represent a co-operative self-assembly of lipids and apolipoproteins, where the apolar core, bounded by the hydrophobic residues of apolipoprotein A-I (apoA-I), solvates the hydrophobic lipid tails (as well as hydrophobic triglycerides and cholesteryl esters), while phospholipid head-groups and polar side chains of apoA-I comprise the polar outer shell.[14,15] It is now well documented that recombination of the total apolipoproteins with the total original HDL lipids (including cholesterol and triglycerides) reforms the native structure.[9] However, reconstitution of purified apolipoprotein A-I alone with small unilamellar vesicles (SUVs) consisting only of pure phospholipid (sans cholesterol) results in the formation of nascent or pre-β HDL particles. The phospholipids in these reconstituted lipoproteins (rHDL also referred to as nanodiscs or nanolipoproteins (NLPs)) reorganize to adopt a disc-shaped planar bilayer configuration with two or more amphipathic protein molecules fencing the single bilayer disc in a looped-belt conformation.[16] Simply by changing the lipid−protein ratio in solution, lipid composition, phase state, and vesicle curvatures, shapes and precise dimensions of rHDL can be systematically tuned.[17,18]

In the work reported here, we show that the bulk phase reconstitution of nanodiscs, followed by their surface adsorption onto prepatterned hydrophilic/hydrophobic surfaces produces lipid bilayers over two discrete and independently adjustable length scales. Alternatively, uniformly adsorbed nanostructured rHDL at hydrophilic surfaces can also be patterned using short-wavelength UV photolithography at microscopic length scales. The lipid−protein coassembly of rHDL structures the bilayer at the nanometer length scale and template recognition (or photopatterning) allows for higher order organization. The ability to independently engineer both nanoscopic and microscopic length scales is particularly important for systematic studies of how lateral confinement influences fluid cooperativity and modulates function in both lipids and proteins. Two generic examples include the importance of sizes of fluid compartments attributed to cytoskeleton barriers in cellular membranes[19] and of cholesterol-enriched lipid rafts implicated in many signaling functions.[20] Further, we show that by simple chemical denaturation of the boundary protein, nanoscale compartmentalization can be selectively erased, thus producing patterns of laterally fluid lipid bilayers structured solely at the mesoscopic length scale. Since these aqueous phase microarrays of nanostructured lipid bilayers allow for membrane proteins to be embedded within single bilayer discs,[21,22] they may prove useful for elucidating membrane protein structure−function relationships. Moreover, multiscale patternability of lipid bilayers should prove useful in understanding consequences of membrane compartmentalization on lipid dynamics.[23]

Detailed experimental methods are described in the Supporting Information (SI).

Results and Discussion

A simple chemical incubation of small unilamellar vesicles (SUVs, ∼100 nm) consisting of DMPC (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine) phospholipids with purified apoA-I in 100:1 stoichiometry produces discoidal rHDL particles as illustrated in Figure 1a. A negative-stain transmission electron microscopy measurement confirms the discoidal shape of the particle (Figure 1b) and also provides a rough estimate of ∼20 nm for particle diameter. Using an independent dynamic light scattering measurement (SI), we confirm that the nanodiscs so formed are predominantly 22 nm (∼35% of the population), although a distribution of sizes between 19 and 26 nm was found. A detailed characterization of similarly reconstituted HDL reported recently, finds that such size variations are strongly dependent on the precise conformation and the stoichiometry of apoA-I at the lipoprotein surface.[24] The rHDL disk height of ∼4 nm, in good agreement with the expected height of a single DMPC bilayer,[25] is established using atomic force microscopy (AFM) measurements of isolated rHDL particles on mica[26] (Figure 1c). The formation of these discoidal rHDL particles from parent vesicles must proceed via vesicle to micelle structural change.[27]To confirm this structural transformation, we performed experiments, which utilized DMPC SUVs loaded with carboxy-fluorescein (CF) at sufficiently high concentrations to induce self-quenching (SI). Following incubation with apoA-I, an increase in CF fluorescence emission is clearly evident. This increase in CF fluorescence can be straightforwardly attributed to the diminution of self-quenching caused by the release of CF from initial vesicle confinement into the large extra-vesicular solution—consistent with vesicle−micelle transition (SI).

rHDL Particles Can Be Further Self-Assembled at Solid Surfaces via Simple Physisorption. This is achieved by simply incubating freshly oxidized solid substrates (e.g., glass, silicon, or mica) in dilute aqueous solutions (<1 mM) of preformed rHDL. Previous studies establish that this simple solution-phase self-assembly is marked by a self-limiting surface adsorption, culminating in the formation of a uniform monolayer of densely packed, uniformly oriented rHDL particles.[26] Our preliminary characterization using lipid- and protein-labeled samples reveals a uniform fluorescence signature (SI) consistent with these claims. Note that subsequent patterning affords further characterization of thickness and film uniformities via ellipsometry, by allowing a direct comparison of pattern features in single

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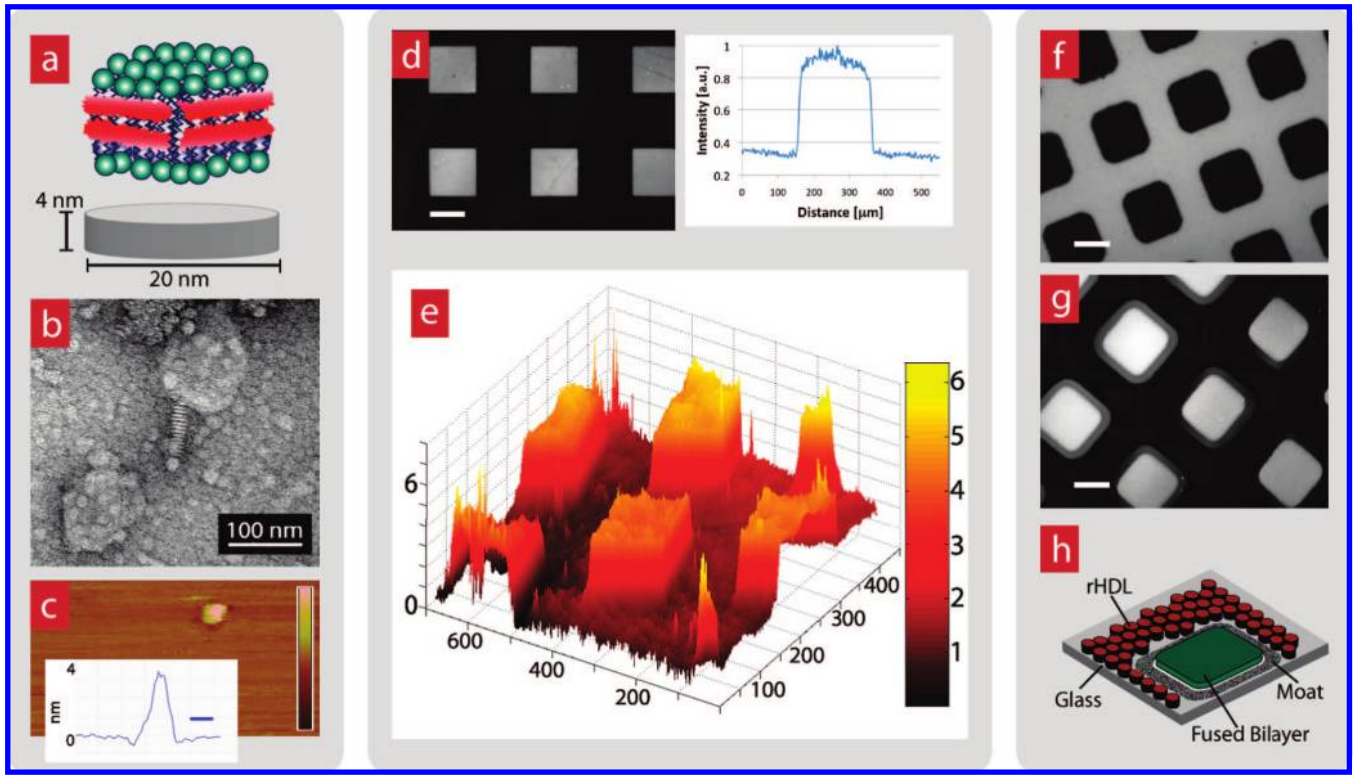

Figure 1. Ordering lipid bilayers at two independent length scales by lipoprotein coassembly and photolithography. (a) Schematic depiction of an rHDL particle; (b) TEM image of lipid-labeled rHDL obtained using a negative staining technique; (c) Contact-mode Atomic force microscopy (AFM) image of isolated lipid-labeled rHDL on mica (scale bar is 20 nm and color scale maximum is 5.0 nm); (d) Epifluorescence image of a UV patterned, lipid-labeled rHDL monolayer on a silicon substrate and a spatial profile of intensity spanning the UV illuminated and unilluminated regions; (e) A spatial map of ellipsometrically derived thicknesses of a UV patterned, lipid-labeled rHDL monolayer on silicon substrate (lateral dimensions are μm and vertical dimensions are nm); (f) Epifluorescence image of a UV patterned, lipid-labeled rHDL monolayer on a glass coverslip; (g) Epifluorescence image of the UV patterned, lipid-labeled rHDL monolayer after secondary backfilling using POPC vesicles doped with NBD-PE; (h) Cartoon illustration of the rHDL/nanodisc patterns obtained after lipid-backfilling of photolithographically generated rHDL patterns. For all epifluorescence images, the scale bar represents 100 μm.

samples. These results (see below) lend additional support to the formation of dense, uniform, and oriented rHDL monolayers at hydrophilic surfaces. These observations are consistet with those described by Malmsten et al.[28] for authentic HDL particles.

The persistence of nanoscale confinement following adsorption of rHDL onto freshly oxidized glass is verified by a modified fluorescence recovery after photobleaching (FRAP) experiment. In lipid-labeled samples, it is clear that at temperatures (32 °C) significantly above the DMPC phase transition point (T_m, 24 °C), photobleached areas retain their original shape even after several hours (SI). This absence of long-range translational mobility confirms that the apoA-I, which corrals the individual bilayer discs, serves as an efficient diffusional barrier. Recently, it has been shown that the effective main phase transition temperature for DMPC[29] in nanodiscs is shifted to higher temperatures as compared to pure lamellar lipid phase, lending additional support to the findings above.

Additional Structural Features at Hierarchically Larger and Arbitrarily Chosen Length Scales Can Be Engineered in Nanostructured Lipid Bilayers via a Variety of Top-Down Lithographic or Template-Directed Processes. Below, we illustrate two such approaches to build micrometer scale patterns of these nanostructured supported lipid bilayers.

First, the monolayer of discoidal rHDL (or nanopatterned lipid bilayer assemblies) can be directly modified at microscopic length scales using spatially patterned photolysis by short-wavelength ultraviolet (UV) radiation (187−254 nm) and a physical photomask. It is well-known that short-wavelength UV light degrades exposed organics in aqueous phases via a combined effect of their energy and the potent cocktail of oxidants (e.g., singlet molecular oxygen and ozone) produced during exposure.[30] The optically defined transfer of the mask pattern onto the rHDL monolayer is manifest in epifluorescence images as illustrated in Figure 1d. A high-contrast fluorescent pattern is visible, with bright squares corresponding to the protected areas (where the rHDL assembly remains unperturbed) separated by a dark background devoid of fluorescence emission (where UV illumination occurs and rHDL is removed). Imaging ellipsometry data shown in Figure 1e reveals the topography of the corresponding fluorescence pattern. Note that the ellipsometric evidence for 4 nm height and the uniformity of film thicknesses (±0.5 nm) in unexposed areas further confirms the foregoing inference that rHDL particles assemble in a dense uniform orientation and at monolayer coverage onto hydrophilic surfaces.

Complete removal of rHDL from illuminated areas is established by two parallel lines of evidence: (a) restoration of original, bare substrate ellipsometric values after photolysis (data not shown) and (b) subsequent fusion of differently labeled secondary vesicles (Figure 1, parts f and g).[31] It appears instructive to note that the latter could also serve as a means to construct complex lipid and fluidity patterns,[32] wherein the

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apolipoprotein-corralled lipid bilayer discs are spatially arrayed within independently chosen, fluid lipid surroundings as depicted in Figure 1h. The back-filling of secondary vesicles in photopatterned rHDL arrays often reveals a boundary region or moat, wherein vesicle fusion is at least partially inhibited. The formation of such a lipophobic moat region, which also serves as a diffusional barrier, has been previously[33] attributed to topochemical roughening at the mask edges due to penumbral blurring in the photolithography approach used here.

Our second approach relies on the surface recognition properties of rHDL particles. Previous studies indicate that while HDL adsorbs both to hydrophilic and hydrophobic surfaces, they adsorb at substantially higher coverage to polar hydrophilic surfaces.[28] Thus, by using predetermined spatial patterns of substrate wettability (and charge) surface density of rHDL can be spatially modulated. The result of this templating strategy is a supported lipid bilayer prestructured by the apolipoproteins at the nanoscale and organized into arbitrary microscopic patterns.

Self-assembled monolayers or SAMs are excellent candidates for preparing two-dimensional substrate templates displaying microscopic patterns of surface wettability. We used photopatterned *n*-octadecyltrichlorosilane (OTS) monolayers on glass and oxidized silicon substrates. OTS SAMs are prepared by following a standard solution-phase self-assembly procedure and subsequently patterned as described above using ozone-generating, short-wavelength ultraviolet radiation in conjunction with a photomask. This treatment results in binary surface-energy patterns of intact OTS in UV-protected regions (hydrophobic) and oxidized silica in the UV-exposed regions (hydrophilic) separated by the aforementioned moat region. These photopatterned OTS substrates are then exposed to aqueous solutions of preformed discoidal rHDL (see SI for detailed description).

The formation of surface density patterns that reflect the underlying template of surface wettability is established using a complementary suite of fluorescence, imaging ellipsometry, and AFM. Figure 2a shows a typical epifluorescence image observed for lipid-labeled rHDL particles adsorbed onto a patterned OTS surface. The pattern of fluorescence emission mirrors that of the initial surface wettability. Further, the fluorescence intensity due to lipid-labeled rHDL adsorption is *1.5 to 2.0*× greater in hydrophilic regions compared to the surrounding hydrophobic regions, likely caused by a greater affinity of rHDL for adsorption onto hydrophilic surfaces. Additional support was obtained in supplementary experiments performed at low rHDL bulk concentrations (1:100 dilution). In these experiments, surface characterization using AFM (Figure 2c) and large-area analysis of ellipsometric images (SI) shows an approximate *5-fold* increase in adsorption of rHDL particles to the hydrophilic regions of exposed silicon oxide over OTS-derivatized, hydrophobic regions, as determined by analyzing the appearance of elevated features in each region.

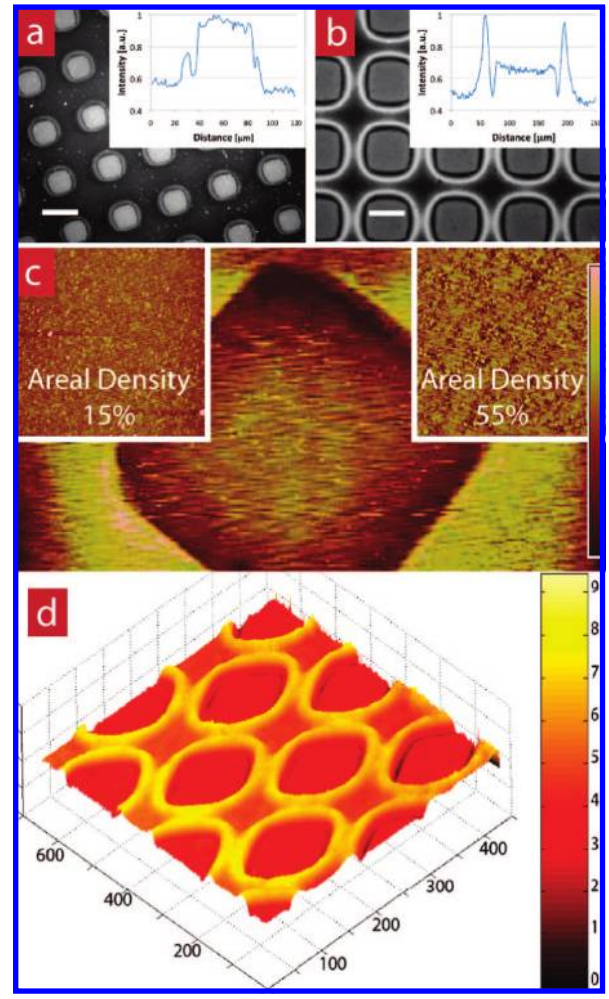

Figure 2. Templating rHDL organization using surface recognition. (a,b) Epifluorescence images of lipid-labeled rHDL (a) and protein-labeled rHDL (b) adsorbed on a prepatterned hydrophilic/hydrophobic (silicon, square region; OTS, surrounding region) surface. Insets show a spatial profile of intensity; (c) tapping-mode atomic force microscopy (AFM) image of the surface phase derived from the recognition of patterned OTS monolayer by lipid-labeled rHDL (fluorescence image appears in (a)). Five × five micron square insets reveal the differences in rHDL packing for hydrophobic (areal density 15%) and hydrophilic (areal density 55%) regions of the patterned OTS monolayer. Color scale maximum is 5.0 nm; and (d) thickness map derived from an ellipsometric contrast image (lateral dimensions in μm and vertical dimensions in nm) of rHDL adsorbed to silicon (inside squares) and OTS (surrounding area).

Protein-labeled rHDL particles adsorbed at saturating concentrations also transcribe the underlying pattern of surface wettability but reveal intriguing features due to protein-surface interactions as detailed below. The epifluorescence image in Figure 2b shows that for protein-labeled samples, the fluorescence intensity in the hydrophilic and the hydrophobic areas are almost indistinguishable. This appears contradictory to the observed adsorption preference of rHDL for hydrophilic substrates of lipid-labeled rHDL (see above). Maps of surface topography obtained from ellipsometric contrast images (Figure 2d) further reiterate the similarity in coverage between hydrophilic and hydrophobic regions (4.55 nm for hydrophilic v 4.48 nm for hydrophobic after correcting for OTS thickness). The apparent discrepancy in the results obtained by using the two labeling schemes is resolved by investigating the interaction

(31) The small difference in fluorescence contrast and pattern fidelity between data reported in Figure 1f and similar data in Fig. 1d can be straightforwardly attributed to the differences between glass (Figure 1f) and oxidized silicon (Figure 1d) substrates used in the two experiments. oxidized silicon wafers are smoother allowing for a better fidelity in pattern transfer and their reflective properties induces well-known interference contrast in the measured fluorescence emission Lambacher, A.; Fromherz, P. *J. Opt. Soc. Am. B−Opt. Phys.* **2002**, *19*, 1435–1453.

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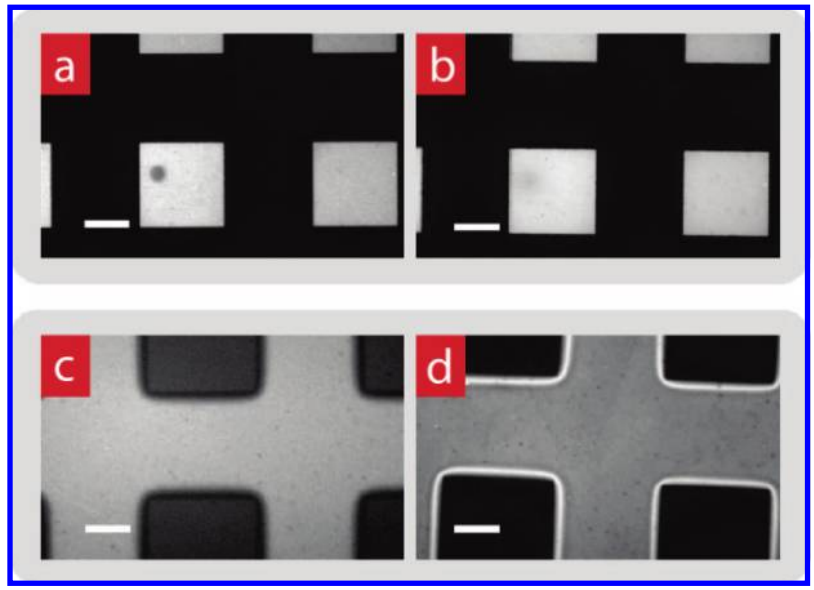

Figure 3. Preferential erasure of nanoscale compartmentalization in patterned rHDL monolayers | (a,b) Epifluorescence images of a photolithographically generated lipid-labeled rHDL monolayer pattern on a glass coverslip after a 5 h incubation with denaturant, GdmHCl (6M) at 22 and 30 °C. The sharp circular spot in (a) and a corresponding blurred spot in (b) show the establishment of a long-range lateral fluidity following protein denaturation. (c,d) Epifluorescence images for protein-labeled rHDL patterns derived by recognition of a (hydrophilic/hydrophobic) patterned OTS monolayer (c) before and (d) after incubation with 6 M GdmHCl for 24 h. For all images, scale bar represents 100 μm.

between lipid-free protein and the surface. In the presence of a patterned OTS surface, unlipidated protein displays a strong preference for the hydrophobic OTS regions (SI), resulting in a compositional rather than topographical pattern. The wettability-dependent behavior stems from a difference in surface interaction: both lipid-bound (rHDL) and lipid-free protein adsorb in hydrophobic regions while lipid-bound protein alone adsorbs in hydrophilic areas.

An interesting feature of the patterns so formed is a striking decoration of the edge (i.e., the boundary between hydrophilic and hydrophobic) in both fluorescence and imaging ellipsometry data. For protein-labeled samples, a markedly higher fluorescence intensity and ellipsometric thickness (7−10 nm) is observed in the outer moat, the portion nearest the hydrophobic region, suggesting adsorption of free protein there. Additional experiments reveal that lipid-free apoA-I does indeed exhibit a strong affinity for the moat and maintains an aversion to fused lipid bilayers (SI). This is consistent with prior observations,[33] where the hydrophobic edge of this boundary region is shown to be accessible for protein adsorption after vesicle fusion, while the hydrophilic edge remains resistant. In sum, these results suggest that the differential lipoprotein and free protein assembly at hydrophilic and hydrophobic regions of patterned wettability surfaces produces unique topographical and compositional surface density patterns.

Can Chemical Denaturation of apoA-I Be Used to Irreversibly "Erase" the Nanoscale Compartmentalization? Such an ability to selectively lift the nanoscale confinement should prove useful in many fundamental biophysical studies. For instance, the initial compartmentalization ensures molecular-level mixing of lipid components as determined by the nanoscale system dimensions. Subsequent protein denaturation should then provide a window into how the molecular distributions are influenced by changes in system size, surface interactions, and emergence of lateral mobility. To begin the development of these capabilities, we explore the erasure of apoA-I-induced nanoscale confinement of the lipid bilayer using a chemical denaturant. It is well-known that interaction between rHDL and concentrated guanidinium hydrochloride (GdmHCl) results in complete denaturation of lipid-associated apoA-I in solution.[34] Incubation of labeled rHDL with 6 M GdmHCl induces protein denaturation, in solution, of DMPC-apoA-I rHDL particles as indicated by the change in tryptophanyl fluorescence (SI). The efficacy of this treatment for surface-supported rHDL monolayers is verified by a simple FRAP measurement. Epifluorescence images of the UV-patterned lipid-labeled rHDL, shown in Figure 3, parts a and b, reveal that after treatment with GdmHCl, photobleached areas regain their fluorescence intensity via lipid diffusion. The establishment of long-range translational mobility in probe lipids is consistent with the dissociation of apoA-I from the lipoprotein complex and the release of lipid confinement. Note that this approach of releasing nanoscale confinement may also denature any membrane proteins embedded within rHDL particles, but low concentrations of highly water-soluble denaturants (e.g., urea) can allow selective denaturation of surface apoproteins, without perturbing lipid-soluble membrane proteins.[35]

Treatment with protein denaturant also provides some further insight into the relationship between adsorption of unlipidated apolipoprotein and surface chemistry. A typical epifluorescence emission image of protein-labeled rHDL adsorbed on to a hybrid OTS-glass surface is shown in Figure 3c. Subsequent treatment of the sample with 6 M GdmHCL reduces fluorescence intensity in the hydrophilic region as the labeled protein dissociates from the lipoprotein assembly. The fluorescence in the hydrophobic region decrease only slightly further suggesting the tenacious adhesion of unlipidated protein there (see above). The final pattern of fluorescence (Figure 3d) further reveals a preference for freed apolipoprotein to resettle in the topochemically roughened moat over the hydrophobic silane region.

The facile methods for multiscale patterning of supported lipid bilayers introduced here, via apolipoprotein coassembly and

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either surface-directed self-assembly or photochemical patterning, suggest several interesting possibilities. First, this approach provides a simple means to induce membrane compartmentalization at two independently selected length scales. By appropriately selecting the lipid−protein ratio and sizes of starting lipid vesicles, shapes and sizes of nanoscale compartments can be chosen. Higher level ordering at microscopic length scale imposed in independent surface processes affords a separate control. Second, the ability to selectively remove the nanoscale confinement following surface adsorption should allow fundamental biophysical studies on how system sizes influence lateral membrane dynamics. Third, several recent studies have successfully shown the incorporation of functional membrane proteins (e.g., GPCRs) within single nanodiscs.[22,36,37] The methods developed here, in conjunction with membrane protein-incorporating nanodiscs, should allow organization of the functionalities of these proteins into predetermined and possibly high-density surface microarrays such as for screening of potential drug candidates. In this same vein, the use of functional substrates (e.g., optical or electrical transducers) should further enable integrating functionality of membrane proteins in membrane-based biomimetic devices.

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Supporting Information Available: Detailed experimental methods and results from supporting experiments are provided. This information is available free of charge via the Internet at http://pubs.acs.org/.

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