We present the construction of microscopic vesicular particles comprising phospholipids and polydiacetylene (PDA), a polymer with unique color and fluorescence properties. We show that the vesicle-embedded PDA domains function as chromatic reporters of membrane events, undergoing dramatic colorimetric and fluorescence transformations induced by interactions with membrane-active species. In particular, the micrometer sizes of the giant vesicles facilitate their utilization for visual inspection of membrane events by conventional microscopy techniques. The morphology, size, and chromatic properties of the vesicular aggregates depend upon the type of phospholipids and the molecular ratio between the phospholipids and diacetylene, indicating that the lipids and polymer form interdependent domains within the vesicles. The giant chromatic aggregates have been employed for detection and microscopic visualization of varied membrane processes, including lipid interactions of lipophilic drugs, binding of antimicrobial peptides, and membrane attachment by virus particles.

1. Introduction

The fundamental roles of membranes and membrane processes in the cellular world have led to the development of diverse molecular approaches for elucidating the characteristics of membrane events. The structural and functional complexities of actual cellular membranes, however, have dictated the use of varied model systems in membrane research, including unilamellar and multilamellar vesicles,[1] planar lipid bilayers,[2] lipid monolayers and micelles,[1b,3] and others. Specifically, studies of membrane processes have overwhelmingly employed small lipid bilayer vesicles (50–500 nm), due to the ease of preparation and molecular simplicity of such systems.[4] Small vesicular systems, however, have notable drawbacks as reliable tools for membrane analysis, particularly their high surface curvature (significantly different from the situation in most cells), and the fact that only averages of molecular information can be inferred from spectroscopic analysis of vesicle populations.

Recently introduced giant lipid vesicles have attracted interest as a promising platform for studying membrane properties.[5] Giant vesicles, with diameters of between few micrometers and hundreds of micrometers, exhibit sizes that are comparable to actual cells and have been shown to effectively mimic cell membranes.[5] In particular, the primary advantage of giant vesicle systems is the feasibility of their direct observation employing giant vesicles have facilitated real-time visualization of fundamental processes such as shape transformations of membranes, fusion and fission events,[6] membrane budding and endocytosis,[6,7] and others.

Here we describe the construction of new multicomponent giant vesicles comprising phospholipids and polydiacetylene (PDA), a unique chromatic polymer, for detection and visualization of membrane processes. PDA vesicular aggregates and films have been previously shown to undergo distinct blue-red colorimetric changes owing to conformational transitions in the conjugated (ene-yne) polymer backbone, induced by external structural perturbations.[6] Furthermore, PDA also exhibits interesting fluorescence properties; no fluorescence is emitted by the initially polymerized blue-phase PDA, while the red-phase PDA strongly fluoresces at 560 nm and at 630 nm.[9] The colorimetric and fluorescence properties of PDA have made the polymer an attractive reporter component in varied sensing assemblies. In particular, chromatic transformations of PDA have been induced by membrane-active compounds in phospholipid/PDA vesicle assemblies[10] and PDA-labeled live cells,[11] making PDA-containing assemblies useful biosensors.

2. Results and Discussion

2.1. Particle Morphology

Lipid/PDA aggregates were prepared through a simple procedure based on dissolution of the individual components in an organic solvent (chloroform), addition of water, and evaporation of the organic solvent (see Experimental Sec.). Figure 1 depicts the overall structure and morphology of the newly synthesized mixed phospholipid/PDA vesicles in comparison to giant vesicles comprising only phospholipids. The phase-contrast microscopy image of a typical vesicle composed of egg-
phosphatidylcholine (egg-PC) and PDA (Fig. 1A) indicates that the lipid/polymer aggregate adopts the overall spherical shape generally produced in conventional vesicular systems.[4] However, different from the previously-reported giant vesicles comprising pure phospholipids (Fig. 1B),[5] the incorporation of PDA seems to radically alter the classical “bubble” organization of a lipid-encapsulated fluid. Rather, the egg-PC/PDA vesicles appear optically dense, without a clear distinction between an inner fluid volume and a bilayer lipid surface (Fig. 1A). Cryo-scanning electron microscopy (cryo-SEM) experiments (Supporting Information) point to a condensed sheet-like structure of the lipid/PDA vesicle interior, further highlighting the pronounced difference compared to conventional giant phospholipid vesicles.

The SEM image in Figure 1C further demonstrates that the surface of the giant lipid/PDA vesicles features protruding irregular semi-rectangular sheets. These surface fragments most likely correspond to the PDA assemblies that often exhibit organized structures.[12] Importantly, even though the overall vesicular structure of the PC/PDA aggregates seem highly perturbed in Figure 1, fluorescein release experiments[13] (see Supporting Information) confirmed that the phospholipid/PDA assemblies contain an enclosed aqueous volume, thus can be referred to indeed as “vesicles”.

Comprehensive SEM analysis, depicted in Figure 2, reveals that the size and surface morphology of the phospholipid/PDA vesicles are affected by the molar ratio between the lipid and diacetylene components, and by the type of phospholipids incorporated. Figure 2A depicts representative SEM images of vesicles prepared with different ratios between egg-PC and diacetylene. The SEM analysis clearly shows that increasing the mole ratio of diacetylene in the mixed aggregates disrupted the smoothness of the vesicle surface, giving rise to formation of abundant PDA plates. Moreover, the sizes of the vesicles appear sensitive to the lipid:diacetylene ratio, reaching above 10 μm diameter in vesicles comprising ratios close to unity (Fig. 2A). Observation of additional vesicles in the SEM experiment and application of statistical image analysis (Supporting Information) pointed to some variability in vesicle sizes, however the dependence of average vesicle size and surface morphologies upon the lipid:diacetylene mole ratio were confirmed.

The composition of the vesicles also seems to influence their morphologies (Fig. 2B). The SEM images in Figure 2B indicate that the surface organization and appearance of the protruding PDA “sheets” in DMPC/PDA vesicles (1:1.5 mole ratio) were different compared to DMPE/DMPG/PDA vesicles (1:1.3 mole ratio). Specifically, the polymeric leafs adopt smaller and more rectangular structures in DMPC/PDA vesicles (Fig. 2Bi). The dependence of vesicle morphologies upon their compositions indicates that even though the polymeric units seem to form distinct domains, the phospholipids still exert a considerable influence within the mixed assemblies.

2.2. Spectroscopic Properties

The apparent interactions between the phospholipid and polymer constituents in the mixed vesicles, as inferred from the microscopy analysis in Figure 2, are also manifested in spectroscopic experiments (Figs. 3 and 4). Figure 3A depicts the visible spectra of egg-PC/PDA vesicles with different ratios between the lipid and diacetylene molecules. The peak at around 640 nm, appearing in vesicles exhibiting specific lipid:diacetylene ratios, corresponds to the blue-phase PDA. Indeed, the graphical representation depicting the relationship between the peak intensity at 640 nm and mole-percent of diacetylene in the vesicles (Fig. 3B) clearly demonstrates that the blue PDA moieties were formed only in a narrow range of the lipid:diacetylene mole ratio.
In particular, Figure 3B indicates that the highest intensity of the 640 nm peak in the visible spectrum was recorded in egg-PC/PDA assemblies exhibiting a 1:1.5 mole ratio between the lipid and diacetylene constituents. Intriguingly, increasing further the concentration of the diacetylene in the mixed giant vesicles yielded a significant attenuation of the blue color, with complete disappearance of the 640 nm peak in vesicles comprising only PDA (Fig. 3). This result stands in dramatic contrast to small unilamellar PDA-based vesicles in which aggregates containing only polydiacetylene exhibit the most intense blue appearance.[14] The dampening of the blue PDA appearance in giant vesicles containing high PDA concentration is surprising, indicating a significant disruption of the conjugated polymer network in such assemblies. Previous studies of small PDA vesicles have reported that some synthetically-modified monomers do not facilitate efficient conjugation and thus no blue color observed.[14b] However the disappearance of the chromatic signal in vesicles comprising pure PDA, which is detected in the giant lipid/PDA vesicle system reported here, has not been observed before. Overall, the relationship exposed in Figure 3 between vesicle color and lipid:diacetylene ratio again points to a significant interplay between the phospholipids and the PDA moieties in the giant mixed vesicles.

DSC analysis, depicted in Figure 4, provides additional evidence for the occurrence of significant interactions between the phospholipids and PDA in the mixed giant vesicles. Specifically, the DSC thermograms show that the peak area under the DMPC endothermic transition at around 24 °C[4] depends upon the mole ratio between the phospholipids and diacetylene in the mixed vesicles. For example, vesicles exhibiting 1:1.5 DMPC:diacetylene mole ratio (spectrum iii in Fig. 4) displayed the smallest peak area – indicating a pronounced effect of the interspersed PDA domains upon the organization and cooperative properties of the phospholipids. The broad PDA transitions at around 55 °C also appear highly dependent upon vesicle composition. Importantly, the PDA peak disappear in the pure PDA vesicle sample (spectrum i)-consistent with the absence of blue color in these vesicles (Fig. 3).

2.3. Bio-Analytical Applications

The phospholipid/PDA vesicles constitute a useful platform for studying and visualization of membrane interactions. Figure 5 presents spectroscopic and microscopic data recorded after addition of the antibiotic cyclic peptide polymyxin-B (PMB)[15] to giant DMPC/PDA vesicles (1:1.5 mole ratio). Figure 5A depicts a scanned image of the vesicle solution before (Fig. 5Ai), and after mixing with increasing concentrations of PMB. Figure 5A clearly demonstrates the induction of blue-red transformation following interactions of the peptide with the DMPC/PDA vesicles.

The chromatic transformations of the PDA matrix induced by membrane-active molecules in lipid/PDA systems have been thoroughly studied.[10c,16] The color changes have been generally ascribed to structural perturbations of the pendant side-chains of the polymer, primarily induced through surface interactions by the membrane-active species. Specifically in
case of PMB interactions with small lipid/PDA vesicles, previous studies have shown that the chromatic transformations of the PDA matrix were induced by association of the peptide with the phospholipid domains in the mixed systems[10d]. Indeed, the ESR data in Figure 5B provides evidence for a similar preference of PMB to insert into the phospholipid moieties in the giant DMPC/PDA vesicle system. The graph shown in Figure 5B depicts the hyperfine splitting calculated from electron spin resonance (ESR) spectra of 5-doxyl-stearate, a spin probe incorporated only in the phospholipids domains in the mixed PC/PDA vesicles.[10c] Figure 5B clearly shows that the hyperfine splitting progressively increased in vesicle solutions containing higher concentrations of PMB, up to a plateau at approximately 56 Gauss. The higher hyperfine splitting corresponds to lower mobility of the spin probe within the lipid domains, ascribed to peptide insertion into the bilayers.[17]

Interactions of PMB with the giant DMPC/PDA vesicles can be inspected microscopically as well (Fig. 5C). The fluorescence microscopy images in Figure 5C reveal that addition of PMB to the DMPC/PDA vesicles gave rise to distinct fluorescent regions on the vesicle surface, corresponding to the transformed red PDA. The control vesicles did not emit fluorescence because blue-phase PDA is non fluorescent (Fig. 5C, top). However, gradually raising the PMB concentration increased the abundance and intensity of the fluorescent areas. Echoing the color transitions apparent in Figure 5A, the fluorescent PDA regions captured in Figure 5B most likely correspond to perturbations within the vesicle surface induced by peptide binding.[10d] Indeed, the phase-contrast microscopy images in Figure 5C confirm that surface domains displaying significant distortions are correlated with the appearance of intense fluorescence. Detergent-like effects of PMB and other cytolytic peptides have been previously reported.[15b]

The giant lipid/PDA vesicles can be employed for screening membrane interactions of pharmaceutical compounds (Fig. 6). Figure 6 depicts the phase-contrast and fluorescence microscopy images of giant DMPC/PDA vesicles following addition of nortriptyline, a lipophilic anti-depressant.[18] Nortriptyline is believed to permeate lipid barriers through passive diffusion, although the exact penetration mechanism has not been fully elucidated.[19] The microscopy data in Figure 6 show that addition of nortriptyline to the vesicles gave rise to increased PDA fluorescence, indicating vesicle binding. However, in contrast to the highly localized surface perturbations observed in case of PMB (Fig. 5C), nortriptyline binding to the vesicle surface appears uniform, giving rise to an even distribution of fluorescence emission in the DMPC/PDA vesicles (Fig. 6B and C). This result most likely attests to the pronounced lipid affinity and passive diffusion of nortriptyline through hydrophobic barriers, rather than processes of bilayer disruption leading to pore formation and membrane destruction, apparent following surface interactions of membrane-active peptides such as PMB.[15b]

The giant lipid/PDA vesicles can be also employed for analysis of membrane activities of complex biological entities, such as viruses (Fig. 7). Gaining insight into membrane interactions and penetration by viruses is important because the exact
mechanisms of cell internalization and infection by viral particles are still not fully understood. In particular, shedding light on virus-membrane interactions could help decipher the processes responsible for cross-species viral infections, such as bird influenza. Figure 7 depicts microscopy experiments designed to probe membrane binding of *vaccinia virus* (VV), a member of the *Poxviridae* family, which infects a wide variety of mammalian cells. The precise mechanisms of VV insertion into the host cell have not been fully elucidated, although viral penetration is believed to be closely associated with its binding and fusion to the plasma cell membrane.

Figure 7 depicts fluorescence microscopy images collected at distinct time intervals after addition of VV (2 millions pfu/ml) to DMPE/DMPG/PDA vesicles (1:1:3 mole ratio). The microscopy experiment presented in Figure 7 essentially provides “snapshots” of the progression of viral binding to the vesicles. Specifically, Figure 7 confirms that viral docking onto the vesicle surface occurred, inducing the appearance of PDA fluorescence areas on the vesicle surface. Similar virally-induced fluorescence was previously observed when VV particles were added to PDA-labeled epithelial cells. Importantly, Figure 7 indicates that *vaccinia* binding to the membrane is not an instantaneous event, but rather a process that progressed over more than 30 minutes. This observation suggests that virus internalization into the host cell does not occur immediately upon virus contact, an inference that carries important implications when devising molecular therapeutics to prevent or reduce viral infection.

3. Conclusions

This work describes construction and characterization of giant phospholipid/PDA vesicles and their utilization for detection and analysis of membrane processes. Structural and spectroscopic characterization indicates that the vesicles exhibit distinct chromatic and structural properties that are different than previously reported small lipid/PDA vesicles. In particular, the presented data point to the existence of intimate molecular interactions between the lipid and diacetylene constituents which affect both the phospholipids, as well as the organization and conjugation network of the polymer.

One of the primary advantages of the giant chromatic lipid/PDA vesicle platform is the large, micrometer size of the particles. This feature is significant for several reasons. Scientifically – the vesicular aggregates mimic actual cells in term of size and possibly also surface curvature within the lipid regions, making them a model system for studying cell-surface processes. From practical, bio-analytical point of view, the micrometer dimensionality of the lipid/PDA vesicles makes possible their observation and analysis using conventional optical and microscopy instruments. For example, the microscopy experiments in Figures 5–7 underscore the difference between the highly localized membrane interactions of PMB and *vaccinia virus* on the one hand, and the uniform binding of nortriptyline on the other hand.

The giant lipid/PDA platform exhibits other noteworthy features as an effective membrane probe. In particular, the color and fluorescence signals of the PDA domains appear only in conjunction with the occurrence of membrane events, a prop-
tery that is fundamentally different from conventionally-employed fluorescence or luminescent dyes for which emission does not generally depend upon an external stimulus. Furthermore, PDA does not undergo photo-bleaching or fluorescence quenching, thereby significantly enhancing its practical applicability. The lipid/PDA aggregates are stable for long time periods, and can be stored for weeks without degradation. Overall, the results presented herein demonstrate that the phospholipid/PDA vesicles can be employed for detection and visualization of a wide range of membrane processes using conventional spectroscopic and microscopic techniques.

4. Experimental

**Materials:** 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-dimyristoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] (DMPG), and 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine (DMPPE), nortriptyline hydrochloride (C₂₀H₂₁N·HCl), and 5-doxyl-stearic acid (5-DS) were purchased from Sigma. The diacetylenic monomer 10,12-tricosadiynoic acid was purchased from Alfa Aesar (Karlsruhe, Germany), and was washed in chloroform, and passed through a nylon 0.45 μm filter (Whatman) before use.

**Methods: Vesicle Preparation:** Phospholipid/polydiacetylene (PDA) giant vesicles were prepared by the rapid evaporation method [13]. Briefly, the constituents were separately dissolved in chloroform/ethanol (1:1 volume ratio) and subsequently added to a 250 mL round-bottom flask containing 1 mL of chloroform. The aqueous phase (5 mL of distilled water) was then carefully added along the flask walls. The organic solvent was removed in a rotary evaporator under reduced pressure (final pressure 40 mbar) at 65 °C and 40 rpm. After evaporation for 2 min, an opalescent fluid was obtained with a volume of approximately 2 mL. The vesicle solution was cooled to room temperature and kept at 4 °C overnight. The solution was then irradiated at 254 nm for 30 s, resulting in intense blue color appearance due to polymerization of the diacetylene units. All ratios specified in the text refer to molar ratios.

**Sample Preparation:** Samples for evaluating vesicle interactions of externally added compounds were prepared by adding aqueous solutions of the test compounds to 90 μL vesicle solutions, followed by addition of 30 μL Tris-base 50 mM, pH = 8. The spectroscopic and microscopic experiments were subsequently carried out after equilibration for few minutes.

**Scanning Electron Microscopy (SEM):** For the SEM experiments, a drop of the giant vesicle solution to be tested was applied to a 0.2 μm filter (Whatman). Heating scans were run at a rate of 1.5 °C min⁻¹. Data analysis was performed using Microcal Origin 6.0 software.

**Electron Spin Resonance (ESR):** Samples for the ESR experiments were prepared using the spin probe 5-doxyl-stearic acid (5-DS) which was added to the vesicle samples after the polymerization step, at a concentration of 25 μM. Distilled water served as a blank.

**Processing temperature.** Amplitudes of 12.5 kHz and 100 kHz, modulation, and microwave power levels were selected at sub-critical values (0.5 G and 20 mW, respectively) to obtain the best signal-to-noise ratio. Processing of the ESR spectra was carried out using Bruker WIN-ESR software.

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