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Self-Directed Reconstitution of Proteorhodopsin with Amphiphilic Block Copolymers Induces the Formation of Hierarchically Ordered Proteopolymer Membrane Arrays

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Supporting Information

ABSTRACT: Manipulating recognition and transport at the nanoscale holds great promise for technological breakthroughs in energy conversion, catalysis, and information processing. Living systems evolve specialized membrane proteins (MPs) embedded in lipid bilayers to exquisitely control communications across the insulating membrane boundaries. Harnessing MP functions directly in synthetic systems opens up enormous opportunities for nanotechnology, but there exist fundamental challenges of how to address the labile nature of lipid bilayers that renders them of inadequate value under a broad range of harsh nonbiological conditions, and how to reconstitute MPs coherently in two or three dimensions into non-lipid-based artificial membranes. Here we show that amphiphilic block copolymers can be designed to direct proteorhodopsin reconstitution and formation of hierarchically ordered proteopolymer membrane arrays spontaneously, even when the membrane-forming polymer blocks are in entangled states. These findings unfold a viable approach for the development of robust and chemically versatile nanomembranes with MP-regulated recognition and transport performance.

Engineered materials rivaling the inherent specificity and efficiency of membrane proteins (MPs) at the nanoscale have been enthusiastically pursued with only limited success.[1] Since most amphiphilic MPs are purified by detergents from their native environment,[2] harnessing MPs' recognition and transport properties directly in synthetic systems[3] faces a great challenge in how to direct coherent reconstitution of detergent-bound MPs into robust proteomembrane arrays. Conventional approaches such as mechanical means or detergent-assisted reconstitution rely on hydrophobic and van der Waals interactions to strip one type of amphiphile (e.g., detergent) from MPs while encouraging the embedding of another type (e.g., lipid); this often demands external help to drive selective detergent removal while enduring slow assembly kinetics (e.g., days to weeks), poor control on MP orientation, and easily trapped states of randomized proteoliposome−detergent mixed assemblies that are of limited practical value.[4] Besides the two-dimensional (2-D) MP reconstitution into proteoliposomes with isolated internal volumes typically seen in conventional approaches, planar proteomembrane arrays with externally addressable MP extramembrane domains are highly desired for engineering purposes.[5] Three-dimensional (3-D) stacked proteomembranes offer additional advantages to harvest MP functions collectively. For instance, the stacked electrocytes in electric eels amplify the transmembrane potential of a single electrocyte membrane (150 mV) to an amazingly high voltage (600 V) to stun prey and ward off predators.[6] Obtaining coherently assembled proteoliposome membranes at 2-D in synthetic systems is possible by tethering tagged MPs unidirectionally on supporting substrates before reconstitution,[7] but this becomes extremely difficult at 3-D via conventional approaches.[4] Using proteorhodopsin (PR), a light-driven proton pump capable of converting solar energy into chemical energy,[8] as a prototype, Liang et al. recently identified a charge density matching between one PR extramembrane domain and lipid membranes that drove coherent PR reconstitution spontaneously; asymmetrically charged PR extramembrane domains resting on the opposite surfaces of the resultant proteoliposome membranes played a decisive role in directing polarized stacking of these membranes at 3-D with a tunable packing density.[9]

Despite progress on proteoliposome assembly, practical applications involving liposomes have been hindered by their lack of stability.[5,10] Little is known about how to design synthetic membranes in lieu of the labile lipid bilayers to direct spontaneous MP reconstitution and support MP performance with unsurpassed stability. We suggest that a good membrane candidate needs to have at least the following features: (i) amphiphilicity and nanoscale thickness commensurate with MPs' structures; (ii) good insulating properties to preclude random transport across the membrane; (iii) sufficient area elastic modulus to supply appropriate levels of lateral pressure needed to keep MPs from denaturation; and (iv) sufficiently high bending modulus to minimize membrane rupture under environmental disturbance. Polymersome membranes fulfill all the requirement put forth,[11] but rational design of amphiphilic block copolymers to serve as structural materials supporting MP functions has not been widely examined. Previous pioneering studies[12] focused exclusively on a neutral triblock copolymer system with poly(dimethylsiloxane) (PDMS) as the hydrophobic membrane-forming block and either poly(2-methyloxazoline) or a slightly modified poly(2-ethyl-2-oxazoline) as the hydrophilic membrane-surface block. The resultant proteopolymersomes demonstrated undisturbed MP activities with an improved stability, but the reconstitution had to follow conventional approaches[4,12] and hence suffered the same limitations. Furthermore, the PDMS chosen as the membrane-forming block is one of the most flexible polymers, with a glass transition temperature (T_g) as low as 150 K.[13] It is not clear whether conventional approaches could help MP reconstitution when more robust polymer membranes that strongly resist detergent destabilization are used.

We hypothesize that a generic charge-interaction-directed MP reconstitution paradigm applies to amphiphilic block copolymer membranes. It works synergistically with van der Waals forces

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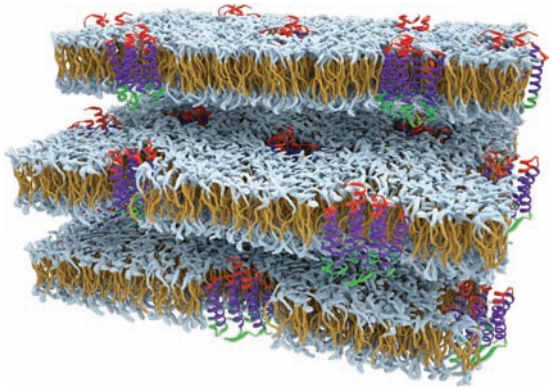

Figure 1. Self-directed MP reconstitution induces the spontaneous formation of hierarchically ordered proteopolymer membrane arrays. PR molecules embedded in each nanometer-thick polymer membrane are packed into a 2-D hexagonal lattice, and different proteopolymer layers are stacked into a multilamellar structure.

and hydrophobic interactions at the onset of detergent decortication and MP embedment and directs spontaneous reconstitution of MP, even when the charge density of MP does not match that of the polymer membranes. We use PR as a prototypical MP and express a recombinant PR strain (*BAC31A08*) that has an isoelectric point of 4.5.[9] A neutral pH (7.4) is chosen to examine the directed reconstitution of PR into oppositely charged polymer membranes. At this pH, both PR extramembrane domains are anionically and asymmetrically charged. The block copolymers are prepared via controlled/living polymerization methods to yield nearly monodisperse size distributions and uniform membrane structures. We show here that the self-directed MP reconstitution induces spontaneous formation of hierarchically ordered proteopolymer membrane arrays (Figure 1).

The first block copolymer we examined is poly(4-vinylpyridine)-*block*-polybutadiene-*block*-poly(4-vinylpyridine) ($P4VP_{28}$-*b*-PBD_{22}-*b*-$P4VP_{28}$, Supporting Information (SI)). NMR and GPC studies confirm the successful synthesis and show that both the copolymer and individual blocks have polydispersity indices (PDIs) near unity (Figure 2a,c). To examine its charge-interaction-directed assembly behavior with PR, the P4VP blocks were rendered cationically charged to poly(4-vinyl-*N*-methylpyridine iodide) (P4MVP) by a quaternization reaction; ~100% quaternization is shown in the NMR results (Figure 2b). The amphiphilic block copolymer self-assembles readily in aqueous solution into spherical particles with a relatively focused size distribution, as observed by TEM and DLS (Figure 2e,d). High-resolution TEM studies of individual particles stained by OsO_4 (which preferentially binds to the unsaturated PBD blocks) reveal their circular-ring membranes, suggesting they are polymersomes (Figure 2e, inset).

One distinct difference between charged polymersomes and liposomes is that each phospholipid molecule often has only one charged headgroup, so lipid bilayers have a relatively small surface charge density (typically ~1/100 $Å^2$).[14] In contrast, each block copolymer chain usually has many charged repeating units. For instance, the P4MVP block here has 28 charged units. Considering a typical interfacial area per polymer chain of ~250 $Å^2$,[11] this polymersome membrane has a surface charge density ~10 times higher than that of a charged lipid bilayer and is also an order of magnitude higher than the extramembrane domains of PR at neutral pH.[9] Interestingly, charge-interaction-directed reconstitution still drives the formation of condensed proteopolymer complexes spontaneous (Figure 3a, inset), which show invariant chromophore absorption, indicative of unaffected PR tertiary structure and proton-pumping

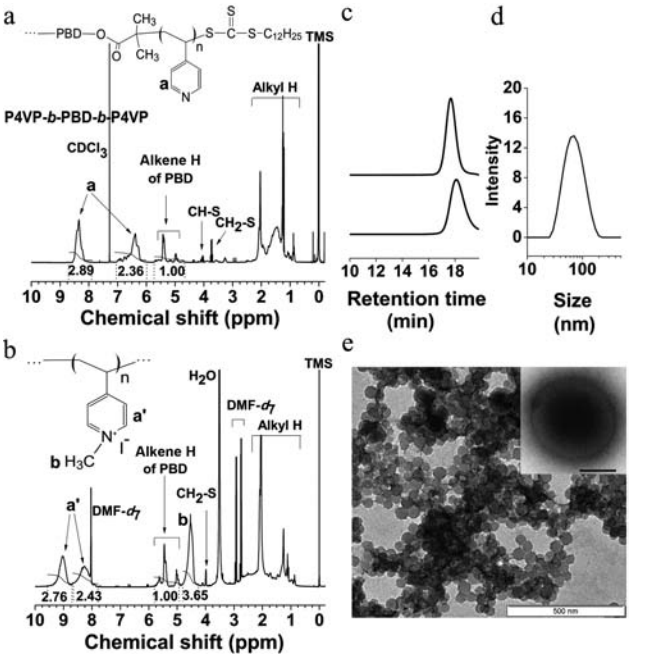

Figure 2. Charged amphiphilic triblock copolymer $P4MVP_{28}$-*b*-PBD_{22}-*b*-$P4MVP_{28}$ and its self-assembly. (a) ^{1}H NMR of $P4VP_{28}$-*b*-PBD_{22}-*b*-$P4VP_{28}$ (300 MHz, $CDCl_3$). δ(ppm): 8.15−8.65 and 7.00−6.25 (pyridine), 5.75−4.75 (alkene H of PBD), 4.35 (CH_2−O at the ends of PBD), 4.10 (CH−S), 3.70 (CH_2−S), and 2.25−0.50 (alkyl H); trace solvent impurities (3.60−2.80) from THF, methanol, and DMF[23] are also observed. On the basis of the size of PBD (1200 Da, assuming 100% 1,4-addition), the block copolymer is estimated to be 7200 Da. (b) ^{1}H NMR of $P4MVP_{28}$-*b*-PBD_{22}-*b*-$P4MVP_{28}$ (300 MHz, DMF-d_7). Note: ~100% shift of the pyridine peaks in panel a to the quanternized equivalencies, accompanied by the appearance of the strong CH_3−N peak. (c) GPC of nearly monodisperse P4VP block (lower trace, M_n = 2900 Da, PDI = 1.08) and $P4VP_{28}$-*b*-PBD_{22}-*b*-$P4VP_{28}$ copolymer (upper trace, M_n = 6600 Da, PDI = 1.19). (d) DLS and (e) TEM of the polymersomes (scale bar 500 nm). Inset: An individual polymersome stained with 4% OsO_4 and imaged at 120 kV (scale bar 100 nm).

function (SI, Figure S2).[22] Confocal microscopy studies of fluorescently labeled PR and polymer membranes show nicely the presence of PR (Figure 3d), block copolymer membrane (Figure 3e), and their co-localization (Figure 3f) in the proteopolymer complexes. SAXS studies of the complexes reveal two strong harmonics, indicative of a multilamellar structure (Figure 3a), which is further confirmed by TEM (Figure 3b). The first harmonic is centered at ~0.103 $Å^{-1}$, indicating a lamellar periodicity of 61 Å that is just big enough to accommodate the transmembrane dimension of PR after detergent micelle decortication (~55 Å).[9] Note that the overall size of detergent-solubilized PR with the associated detergent micelle is much larger (~16 ± 3 nm at pH 7.4).[9] Despite a highly mismatched charge density, the nanometer-thick polymer membranes directed the spontaneous reconstitution of PR into a simple multilamellar structure, in sharp contrast to the self-assembly of liposomes and uniformly charged biopolymers, where charge density mismatching has been shown to drive the transition from a simple multilamellar structure[14] to a "missing-layer" lamellar superlattice structure.[15] It is also different from the directed reconstitution of detergent-solubilized PR with liposomes, where a superlattice-like expanded lamellar structure was observed when both PR extramembrane domains were anionically and asymmetrically charged at neutral pH.[9] The results here suggest that van der Waals attraction between the dangling membrane−surface blocks protruding from adjacent proteopolymer layers plays an

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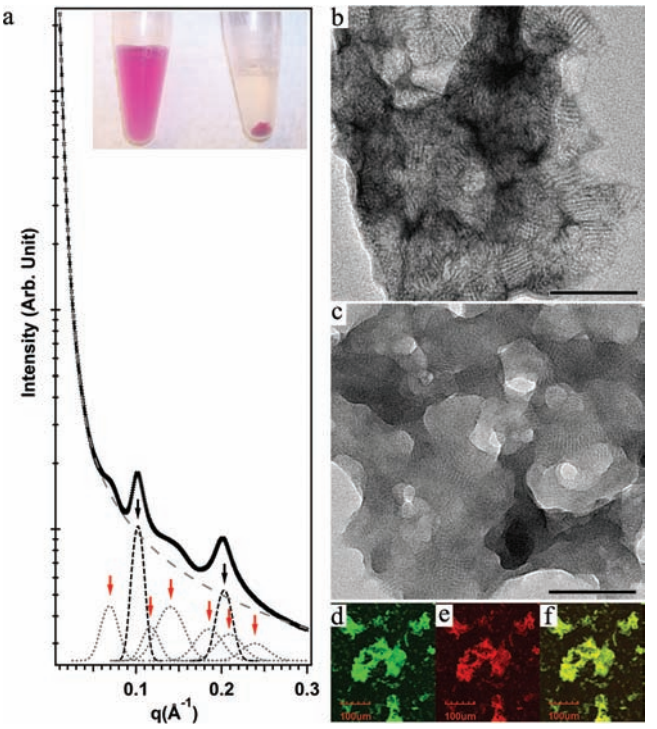

Figure 3. Amphiphilic block copolymer P4MVP-*b*-PBD-*b*-P4MVP directs spontaneous reconstitution of oppositely charged PR into hierarchically ordered proteopolymer membrane arrays. (a) Synchrotron SAXS of proteopolymer complexes when the PBD block is 1200 Da. The scattering data (×) are fitted to resolve a background (dashed line) and two sets of structural features: a multilamellar proteopolymer membrane (dotted peaks marked by black arrows, $q_{001} = 0.102$ Å^{-1}) and a 2-D hexagonal PR lattice in individual membrane layers (dotted peaks marked by red arrows at 0.069, 0.117, 0.141, 0.185, 0.207, and 0.238 Å^{-1}, corresponding to q_{01}, q_{11}, q_{02}, q_{12}, q_{03}, and q_{22} of the PR lattice, respectively). The summation of these contributions (black line) overlaps nicely with the scattering data. Inset: PR solution (left) and spontaneously condensed proteopolymer complexes (right) in PBS buffer at pH 7.4. (b,c) TEM of the multilamellar proteopolymer complexes when the PBD block is 1200 and 5000 Da, respectively (scale bar 100 nm). (d−f) Confocal microscopy pictures of PR (d, labeled with FITC), block copolymer membranes (e, labeled with Rhodamin B), and their coexistence (f) in the proteopolymer complexes when the PBD block is 1200 Da (scale bar 100 μm).

important role in their 3-D stacking. Although the P4MVP block has a hydrophobic tail (Figure 2a) introduced by the controlled/living polymerization process, it is easily removable (SI, Figures S1 and S3). We did not find any different behavior in the self-directed reconstitution of PR incurred by the presence of this hydrophobic tail (SI, Figure S4).

Another distinct difference between polymersomes and liposomes lies in their hydrophobic membranes. Amphiphilic block copolymers assume more complex membrane structures than lipid bilayers made up of two small-amphiphile leaflets. It has been shown that the membrane-forming blocks of amphiphilic block copolymer membranes adopt an unperturbed state.[11] The mean square end-to-end distance of monodisperse PBD under θ conditions was determined experimentally as $\langle r^2 \rangle_0^{1/2} = 0.892 M^{1/2}$ (in Å), where M is its molar mass.[16] The membrane thickness of polymersomes with PBD as the membrane-forming block calculated from this equation is almost identical to that measured by cryo-TEM;[11] thus, we estimate the polymer membrane thickness here as 31 Å (M = 1200 Da). Considering the proteopolymer membrane lamellar periodicity of 61 Å, this leaves ∼30 Å intermembrane spacing, sufficient to accommodate the extramembrane domains of PR and the P4MVP blocks on the membrane surface. The lamellar harmonics are noticeably asymmetric, and the second harmonic (q_{002} = 0.201 Å^{-1}) deviates from its expected position (∼0.206 Å^{-1}), indicating the presence of shoulder peaks. There are also clearly identifiable correlations centered at 0.070 and 0.142 Å^{-1}, (Figure 3a). In PR−liposome systems, Liang et al.[9] and Klyszejko et al.[17] observed a well-ordered 2-D hexagonal PR lattice when different liposomes were used, and its first scattering peak ($q_{01} = 0.071$ Å^{-1})[9] appeared at nearly the same position as the first correlation peak observed here. Using a 2-D hexagonal PR lattice model for each proteopolymer membrane layer, we fit the SAXS data and show a series of 2-D PR correlations (peaks marked by red arrows) superimposed on the 3-D stacked proteopolymer membrane harmonics (peaks marked by black arrows) (Figure 3a). The PR correlation peaks are more diffusive than observed previously in proteoliposome systems,[9] which could be explained by the long-chain nature of block copolymers that do not respond to in-plane PR packing as readily as short-chain lipids. The highly mismatched charge density may also restrict in-plane PR assembly to avoid the rearrangement of unbalanced counterions on P4MVP backbones. Nevertheless, these data unambiguously demonstrate, for the first time, that reconstitution of MPs into hierarchically ordered proteopolymer membranes occurs spontaneously via a generic charge-interaction-directed reconstitution paradigm without the need for charge density matching or poorly controllable external means for selective detergent removal.

Although amphiphilic block polymer membranes have oil−water interfacial energies (γ) similar to those of lipid bilayers,[11] and hence similar membrane area elastic moduli ($K_a \approx 4\gamma$)[18] exerting the same levels of lateral pressure to reconstituted MPs, they could have much higher bending moduli (K_d) to withstand deformation, which explains their often superior stability.[11] In previous studies of MP reconstitution in polymersomes via conventional approaches,[12] the PDMS membrane-forming block was chosen to have a size (∼5400 Da) far smaller than the onset of its entanglement molecular weight (∼9600 Da),[19] and the polymersome membranes were actually in a viscous flow stage at room temperature that offered only limited enhancement to membrane stability in engineered systems. Although PBD has a T_g (218 K[13]) much higher than that of PDMS, the size of PBD used here is still below its entanglement molecular weight (∼1500 Da).[19] A larger membrane-forming block not only increases membrane thickness to yield a higher bending modulus ($K_d \propto d^\alpha$, where d is the membrane thickness and α is a scaling factor)[20] but also drives the transition of polymer chain motion from Rouse dynamics to an entanglement release regime[19] that further reinforces membrane integrity from environmental disturbance. It is not clear how closely the membrane thickness has to be commensurate to the hydrophobic domains of MPs, but mean-field analysis suggested that MPs can be reconstituted into amphiphilic membranes with considerable thickness mismatching.[21] To examine whether this charge-interaction-directed MP reconstitution paradigm works for more robust polymer membranes with entangled membrane-forming blocks, we prepared another triblock copolymer, $P4MVP_{18}$-*b*-PBD_{93}-*b*-$P4MVP_{18}$, with a PBD block size of 5000 Da. NMR and GPC studies confirm the successful synthesis, and both the copolymer and individual blocks have PDIs near unity (SI, Figure S5). At neutral pH, spontaneous reconstitution of PR into the oppositely charged block copolymer membranes still occurs, and condensed proteopolymer complexes form. TEM studies of the complexes reveal a multilamellar structure ($d \approx 61$ Å, Figure 3c), in agreement with synchrotron SAXS studies (q_{001} = 0.102 Å^{-1}, Figure S5). Interestingly, although the PBD

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membrane thickness is expanded by 32 Å via increasing its size from 1200 to 5000 Da,[16] the lamellar periodicity stays the same with respect to the transmembrane dimension of PR, and the in-plane hexagonal PR lattice is still resolvable (Figure S5). This result not only affirms PR reconstitution within block copolymer membranes but also confirms the simulations[21] in which, unlike lipid bilayers, polymeric membranes are highly compressible to accommodate MP reconstitution, even when their hydrophobic domain sizes are not physically matched with each other initially. It also demonstrates that charge-interaction-directed reconstitution of MPs into polymeric membranes occurs readily, even when the membranes are in entangled states with significantly restricted chain motion and remarkably enhanced bending moduli.

In summary, we investigated how to rationally design amphiphilic block copolymers to direct spontaneous MP reconstitution and formation of robust nanometer-thick proteopolymer membrane arrays. These membranes may serve as structural materials to support MP-regulated recognition and transport performance in lieu of lipid bilayers known for their poor stability in synthetic systems. We identified a generic charge-interaction-directed MP reconstitution paradigm that guides MP reconstitution into polymeric membranes spontaneously. Significantly, the self-directed reconstitution was accomplished without the time-consuming and poorly controllable external means for selective detergent removal that are conventionally needed.[4,12] It proceeded even when the membrane-forming polymer blocks were in entangled states with excellent stability, resisting membrane disintegration. We also demonstrated, for the first time, that 3-D hierarchically ordered proteopolymer membrane arrays can be obtained spontaneously, with the MPs crystallized in individual polymeric membrane layers. Combined with methods to tether MPs unidirectionally on 2-D substrates,[7] these findings open the possibilities for future development of abiotic−biotic hybrid nanomembranes in robust and chemically versatile polymeric thin-film formats in both two and three dimensions, which may be used to harvest the unique functions of MPs in synthetic systems with unsurpassed stabilities.

■ ASSOCIATED CONTENT

Supporting Information. Experimental details and characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

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