Hybrid Vesicles

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Controlling Molecular Recognition with Lipid/Polymer Domains in Vesicle Membranes**

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Biological membranes[1] and their synthetic analogues are formed by the self-assembly of amphiphilic molecules, either of low molecular weight (phospholipids) or high molecular weight (block copolymers).[2] Both types of membranes (liposomes or polymersomes)[3,4] can undergo lateral phase segregation processes,[5,6] which especially in lipid membranes comply with their biological functions in, for example, receptor clustering, viral recognition, and other specific protein binding in model as well as living cell membranes. So-called "lipid rafts" or domains[7] are the consequence of the lateral phase segregation of complex lipid mixtures within membranes,[2] which can be induced by molecular effects such as mismatches of the lengths of their lipid hydrophobic chains and by the interaction of cholesterol and sphingolipids (so-called "detergent-resistant membranes", DRMs).[8] In contrast, vesicles composed of block copolymers ("polymersomes") can display either horizontal or lateral phase segregation through demixing processes caused by the immiscibility of their different polymer building blocks,[9,10] or by the action of ions[11] or nanoparticles.[12] The recent trend in blending phospholipids and block copolymers to generate hybrid membranes,[13–15] which combine the biofunctionality of liposomes with the remarkable mechanical stability and functional variability of polymersomal membranes, has further shown the formation of phase-separated membrane morphologies (domains), either as a result of immiscibility between the lipid and polymer component[15,16] or induced by a clustering agent as an external stimulus.[14]

It is of course tempting to speculate about the biological function of recognition processes in giant unilamellar vesicles (GUVs) composed of both lipids and polymers, in particular in addressing the location and assembly of glycosphingolipids (gangliosides) active in protein recognition processes. Can lateral domains within a hybrid vesicle prevent the recognition of a protein by blocking the function of a membrane receptor? Or is it possible to induce additional lateral phase separation by receptor–ligand interactions in a specially designed hybrid membrane?

Herein we report our study of the binding of a biological protein to receptor-functionalized hybrid membranes, which consist of an amphiphilic block copolymer (BCP) and a natural lipid as the major components, and demonstrate the biofunctionality of such designed hybrid lipid/BCP membranes. In our current approach for studying receptor recognition on such a hybrid vesicle membrane we prepared hybrid membranes composed of the biocompatible polyisobutylene-*block*-polyethyleneoxide copolymer (PIB_{87}-*b*-PEO_{17}; BCP **1**) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC; **2**) that were functionalized with ganglioside GM1 (**3**); we then studied the binding of cholera toxin B (CTB) to these hybrid membranes either in the laterally mixed or demixed phase state (Figure 1).

When the designed amphiphilic block copolymer **1** is incorporated into gel-phase vesicles of DPPC, it induces phase heterogeneities within the "hybrid vesicle membrane"[15] that can display a two-dimensional, lateral domain structure, as illustrated in Figure 1. The membrane morphology of these vesicles appears uniform at the resolution of confocal microscopy (roughly 200–300 nm) when prepared from mixtures containing less than 20 mol% and more than 28 mol% of BCP. In contrast, it is phase-separated into a patched membrane in the narrow compositional range between 20 and 28 mol% of BCP. Figure 1B shows a confocal microscopy image of such a phase-separated GUV (see also Figure 2), prepared from a mixture with 20 mol% of BCP (**1**) and 80 mol% of DPPC (**2**), where individual domains were visualized by the incorporation of the fluorescently labeled BCP **4**, which was selectively excited at $\lambda_{exc} = 488$ nm (blue region in Figure 1B), and the lipid-analogue membrane dye $DiDC_{18}$ ($\lambda_{exc} = 633$ nm; green region in Figure 1B). We found that the phase separation process leads to the formation of a less-ordered polymer-enriched phase (see Figure 2 and the Supporting Information) and a lipid-enriched domain (black patch in Figure 2A,C), the latter being depleted of polymer molecules.

As reported in the literature, the lipid dye Rh-DHPE ($\lambda_{exc} = 561$ nm) is largely excluded from ordered phases in mono- and bilayer membranes (e.g., liquid condensed phases[15,17]). In Figure 2B, Rh-DHPE is preferentially incorporated into the polymer-enriched phase (compare with Figure 2A) which indicates a less-ordered phase state,

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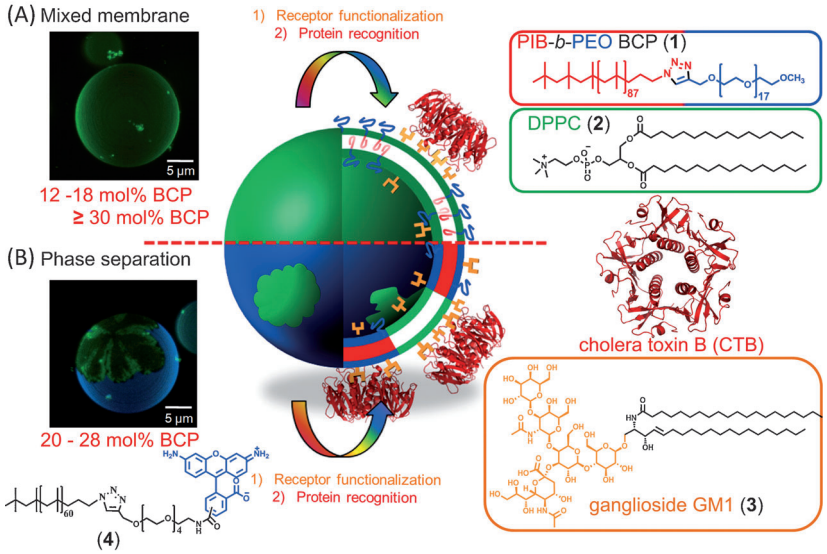

Figure 1. Binding of cholera toxin B to either mixed or phase-separated hybrid vesicles composed of DPPC (**2**) and the biocompatible PIB_{87}-*b*-PEO_{17} block copolymer (**1**) at various lipid/BCP mixing ratios and containing ganglioside GM1 (**3**). A) Confocal microscopy image of a hybrid vesicle typical for compositions of 12 to 18 mol% and above 30 mol% of BCP **1**; Rh-DHPE as the fluorescent lipidic tracer. B) Image of a phase-separated hybrid vesicle typically obtained for compositions containing 20 to 28 mol% of BCP **1,** fluorescently labeled BCP **4** (blue), and a lipid-enriched domain ($DiDC_{18}$ (green)).

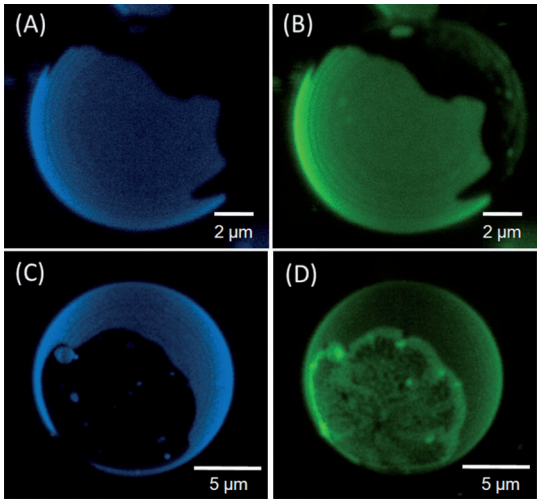

Figure 2. Confocal microscopy images of phase-separated hybrid DPPC/PIB_{87}-*b*-PEO_{17} vesicles (20 mol% BCP **1**) visualizing phase inhomogeneities by different phase-labeling behavior of a rhodamine-labeled lipid (Rh-DHPE, panel B), the fluorescently labeled BCP **4** (panels A and C), and $DiDC_{18}$ (panel D). Panels A and C: three-dimensional reconstruction from axial stacks of a hybrid GUV using compound **4** (excited at 488 nm, in blue). Panel B: same hybrid GUV as in (A) using the Rh-DHPE dye (excited at 561 nm, in green) (preferential incorporation of the dye into the polymer-enriched phase). Panel D: same hybrid GUV as in (C) using $DiDC_{18}$ as membrane dye (excited at 633 nm, in green).

whereas the black patches in Figure 2 A,B consist of a more-ordered DPPC-enriched phase. Additional experiments using $DiDC_{18}$ ($\lambda_{exc} = 633$ nm) to visualize membrane heterogeneities in hybrid GUVs (20 mol% of BCP **1**) showed a variable degree of enrichment of $DiDC_{18}$ in the lipid-enriched phase (compare black patch in Figure 2 C with 2 D) supporting the assumption that this particular phase consists of a gel-like DPPC domain (ordered lipid phase).[18a,b]

Functionalization of the hybrid vesicles by the addition of ganglioside GM1 (**3**) to the mixtures (e.g. DPPC/BCP/GM1 80:20:0.1) did not lead to changes in the respective phase state, proving that the incorporation of roughly 0.1 mol% of GM1 does not perturb the phases. Continuously varying the lipid/BCP composition resulted in the formation of different GUV types with stable phase states. At increasing concentrations of compound **1** the lipid/BPC mixtures first formed a mixed state (12–18 mol%) , then a phase-separated state (20–28 mol%), and finally another mixed state (≥30 mol%). These vesicles were stable over time (monitored over several hours). The thermally driven phase separation leads to macroscopic domain structures, which are stable over time, also suggesting a thermodynamically stable state.

Subsequently, the bacterial protein cholera toxin B (CTB) binds specifically to GM1 (**3**) in a highly cooperative process, as the multivalent toxin contains five equal binding sites, each able to interact with one GM1 lipid. The binding affinity of the pentameric toxin has been reported to be about $5 \times 10^9\,M^{-1}$.[19] Upon addition of CTB (5.3 µM) to the vesicle solution (see the Supporting Information), selective binding of the fluorescently labeled CTB ($\lambda_{exc} = 488$ nm, colored in red) to the functionalized vesicle membrane occurred rapidly.

As a result, we were able to monitor the lateral distribution of the CTB-bound GM1 within the hybrid vesicle membrane (see Figure 3) depending on the lipid/BCP membrane composition. It should be noted that gel-phase vesicles composed of DPPC with 0.1 mol% of GM1 (**3**) did not exhibit the protein binding (CTB) under our experimental conditions, whereas a fluid membrane system composed of DOPC lipids ($T_m = -20$ °C) containing 0.1 mol% of GM1 showed uniform CTB binding over the entire GUV surface (see the Supporting Information). A comparison between the membrane morphology before (visualized with Rh-DHPE; green) and after the CTB binding (Figure 3 A) indicated that hybrid GUVs composed of lipid/BCP mixtures containing 12 to 18 mol% BCP display CTB binding over the entire GUV surface. In contrast, with phase-separated GUVs (DPPC/BCP/GM1 = 80:20:0.1) CTB incubation results in the recognition of both domain types by the protein, proving that the GM1 lipids are incorporated into the polymer-enriched phase as well as into the lipid-enriched domains (Figure 3 B and the Supporting Information).

When a mixture of DPPC/BCP/GM1 (70:30:0.1) was used, which generated GUVs with a mixed membrane morphology, the morphology changed significantly upon

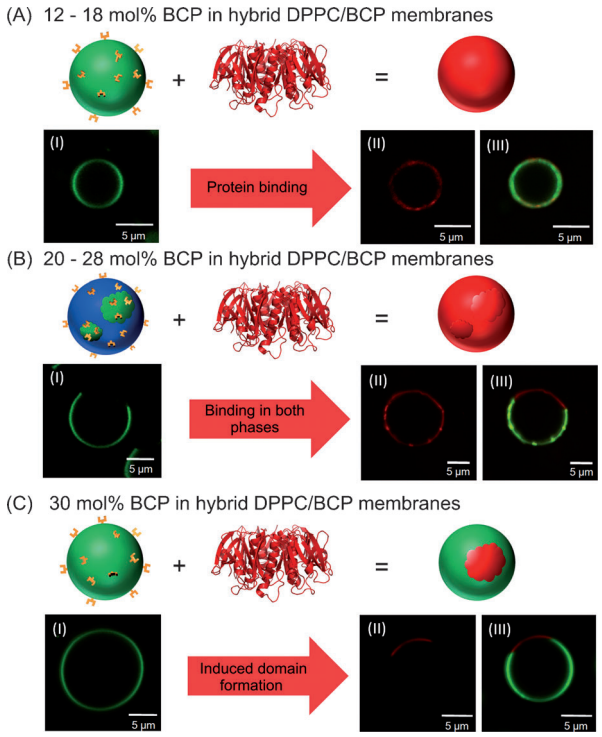

Figure 3. Effect of the hybrid membrane morphology on the protein (CTB) binding behavior; Rh-DHPE (green; excited at 561 nm) Alexa 488-labeled CTB (red; excited at 488 nm). A) Mixed DPPC/BCP membranes (12 to 18 mol% BCP **1**), before (I) and after (II, III) CTB binding . B) Mixed DPPC/BCP (**1**) membranes (20 mol% BCP **1**) before (compare B/I) and after CTB-binding (see B/II). C) At more than 28 mol% BCP (**1**): mixed membrane morphology (C/I) after CTB binding forms large GM1-containing lipid domains (red; see C/II). Panels A/III, B/III, and C/III are the corresponding overlay images of both dyes.

addition of CTB. Within minutes after protein incubation, protein binding and the formation of large GM1-enriched lipid domains was observed (Figure 3 C/I before and 3 C/II after addition of CTB), indicating lateral reorganization during the binding process between CTB and GM1. The kinetics of this phenomenon was fast, taking place within minutes. Thus, we conclude that the highly cooperative binding of the multivalent CTB in the case of hybrid GUVs with 30 mol% BCP leads to the formation of a more ordered GM1-enriched lipid domain, which segregates from the surrounding hybrid bilayer.[20] The fact that we observed only one or at most a few macroscopic domains per vesicle over several hours rather than many small domains reflects the thermodynamic stability of the vesicles.

At a vesicle composition of around 40 mol% of BCP and above in mixtures with DPPC, the obtained hybrid vesicles containing 0.1 mol% of GM1 (**3**) showed no protein binding to the receptor-functionalized GUV membrane. This is probably due to steric hindrance caused by the long PEO chains of the BCPs (17 ethylene oxide units per BCP). At higher BCP content, the PEO chains appear to form a polymer brush, which blocks the receptor from binding CTB.

Next, we tried to determine the effect of the lipid/BCP composition and the resulting lateral mobility in the membrane on the recognition between CTB and GM1. These vesicles showed different lateral distributions of the membrane-bound CTB when the lipid/BCP composition was varied. To this end, lateral diffusion processes within unmodified hybrid DPPC/PIB_{87}-*b*-PEO_{17} membranes were investigated by FRAP (fluorescence recovery after photobleaching) and FCS (fluorescence correlation spectroscopy) measurements.[21,22] Rh-DHPE served as a membrane dye since it should be localized in the more disordered phases in the hybrid membranes. These techniques (see the Supporting Information) provided information about the dynamics in DPPC as well as in hybrid membranes and revealed the effect of incorporated BCP molecules on the organization of the lipid membrane (see Figure 4).

For a better understanding of the membrane dynamics in hybrid membranes, we first performed the lateral diffusion analysis of pure DPPC membranes by FRAP (Figure 4 A). An irreversible bleaching of the irradiated membrane region was observed, confirming the gel-phase state of the liposomal bilayer at room temperature (which is below the T_m of 41.6 °C). In contrast to the high rigidity of pure DPPC bilayers

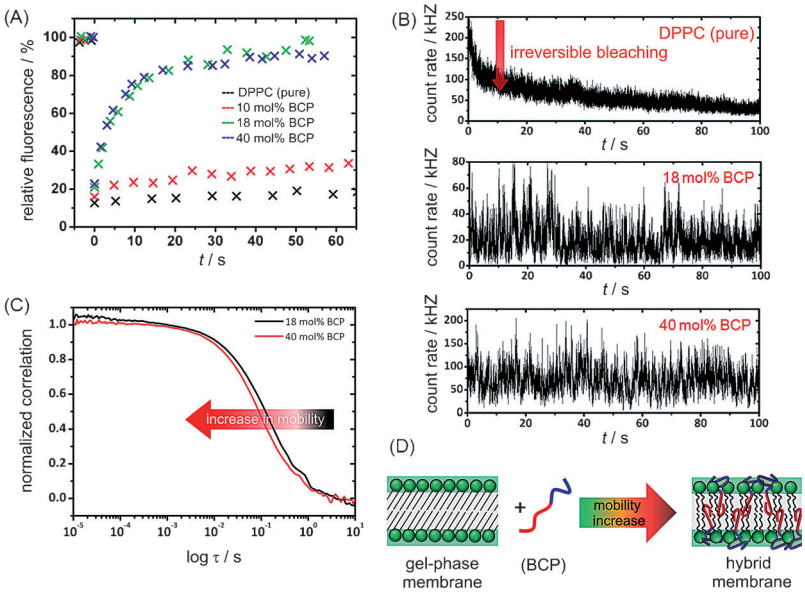

Figure 4. Membrane mobility analysis of Rh-DHPE-labeled DPPC and hybrid membranes by FRAP and FCS measurements for BCP contents ranging from 0 to 40 mol%. A) Normalized fluorescence versus time, recorded during FRAP experiments. At low or zero BCP content (black curve), little fluorescence recovery has occurred in the bleached area after 1 min. At 18 mol% (green curve) and 40 mol% of BCP (blue curve), the recovery has a half-time of roughly 4 s and is almost complete after 1 min. B) Fluorescence intensity traces obtained on pure DPPC and hybrid vesicle membranes with 18 mol% and 40 mol% BCP **1**. C) Fluorescence autocorrelation functions for selected mixtures using 18 mol% (black) and 40 mol% BCP (red). The lateral mobility increases with increasing polymer content because of the incorporated BCP molecules breaking up the rigid lipid packing in the DPPC membranes, as depicted schematically in (D).

at room temperature, hybrid membranes exhibited a clear increase in the lateral diffusion, which was evident from the faster recovery rates with increasing polymer amounts (see Figure 4A and the Supporting Information). Indeed, our experimental FRAP results show that the incorporation of amphiphilic BCPs into gel-phase DPPC membranes strongly affects the lipid organization, shifting it to a more mobile system.

A more detailed picture of membrane dynamics and organization is given by FCS.[22,23] In an FCS experiment, fluctuations in fluorescence intensity observed for a defined detection volume report on the diffusion of labeled membrane molecules within the plane of the membrane. Autocorrelation analysis of the monitored fluctuations in the fluorescence time traces (Figure 4B) yields information about the diffusion times (τ_D) and the corresponding diffusion coefficients (D), which are characteristic of each selected membrane composition.

FCS analysis of pure DPPC membranes confirms the results obtained by FRAP. Figure 4B (top) presents a typical fluorescence intensity trace from pure DPPC membranes; a rapid decrease in the fluorescent intensity due to bleaching is observed, indicating a very low lateral mobility of the fluorescent dye which cannot be analyzed by standard FCS models.

Significantly different results were obtained with the hybrid membranes. The fluctuating signal in the fluorescence traces obtained from samples prepared with 18 mol% and 40 mol% BCP (Figure 4B) indicated that the molecular mobility in the mixed membranes is significantly higher and enabled the analysis of the autocorrelation curves (see Figure 4C). Differences between the correlation curves are significant and prove that the hybrid membrane sample with 40 mol% BCP (red curve) has shorter diffusion times ($\tau_D = (89.5 \pm 2.5)$ ms; $D = 1.6 \times 10^{-13}\,m^2 s^{-1}$), corresponding to a higher lateral mobility of the membrane components. For the sample with 18 mol% BCP, we determined a diffusion time of (120 ± 10) ms ($D = 1.2 \times 10^{-13}\,m^2 s^{-1}$). As depicted schematically in Figure 4D, the incorporation of amphiphilic PIB_{87}-*b*-PEO_{17} (**1**) into gel-phase membranes of DPPC leads to a disorder of the lipid bilayer caused by the BCP molecules. As a consequence, the lateral mobility is much greater than that in pure DPPC bilayers.

In conclusion, the lipid/BCP composition of hybrid GUVs and their resulting morphologies play a significant role in the successful binding of cholera toxin B to the receptor-functionalized vesicle surfaces. The interplay between CTB binding and reorganization of the hybrid membrane is highlighted by the observation of GM1-enriched lipid domains in hybrid vesicles containing 30 mol% BCP. This lipid/BCP composition is particularly interesting in view of the phase-specific binding of membrane proteins to receptor functionalized hybrid vesicle surfaces, which combine the features of polymer membranes and liposomes. Furthermore, the successful protein recognition on mixed as well as phase-separated membrane morphologies demonstrates the high biofunctionality of our system. Such hybrid membrane systems can serve as biomimetic models to understand biological receptor/ligand recognition on hybrid vesicles, which can be fine-tuned in their lateral organization and mobility by varying the lipid/BCP composition.

Experimental Section

Materials and methods used for this study are given in the Supporting Information. Furthermore, the novel synthesis of the fluorescently labeled PIB-*b*-PEO BCP **4** and the procedure of the protein binding studies are given in the Supporting Information.

Hybrid DPPC (**2**)/PIB-PEO BCP (**1**) giant unilamellar vesicles (GUVs) were prepared as described previously[15] using an electroformation method.[24] For the visualization of the resulting hybrid GUVs and their membrane heterogeneities, different membrane dyes ($DiDC_{18}$, Rh-DHPE, fluorescently labeled diblock copolymer **4**) were added to the initial mixture at a total amount of 0.5 mol%.

For the surface functionalization of mixed DPPC/PIB-PEO BCP (**1**) membranes with ganglioside GM1 molecules, all lipid/polymer mixtures of various compositions were additionally mixed with 0.1 mol% of GM1. The lipid/polymer mixtures containing 0.1 mol% of GM1 were prepared in chloroform, dried under a continuous N_2 stream, and dissolved in a defined solvent volume to reach a total concentration of 10 mg mL^{-1}. The final mixtures were used to generate a homogenous thin film on optically transparent indium–tin–oxide (ITO)-coated coverslips using a spin-coating method. After the preparation of the thin films on two coverslips (electrodes), the coverslips were placed in a capacitor-type configuration with a separation of 2 mm using a home-built flow-chamber. The flow chamber was filled with a sucrose solution (96 mosmol L^{-1}). The conditions for the electroformation process were applied as reported previously.[15]

All binding studies between cholera toxin B and GM1-modified liposomal (DPPC or DOPC) or hybrid membranes composed of DPPC and BCP **1** were conducted at room temperature (20 °C) using CTB (5 μg) dissolved in a sucrose solution (ca. 100 μL). The dilute solutions of CTB were prepared immediately prior to use. After the electroformation process, the prepared GUVs with incorporated GM1 receptor molecules were first cooled down to room temperature and then monitored by laser scanning microscopy, which revealed changes in the membrane morphologies. Subsequently, GUVs were treated with the protein solution. The CTB solutions were injected into the flow chamber, which contained the freshly prepared GUVs, using a microsyringe. All experiments were performed with the fluorescently labeled cholera toxin.

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