

Membrane Engineering: Phase Separation in Polymeric Giant Vesicles

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Cell membranes exhibit elaborate lipidic patterning to carry out a myriad of functions such as signaling and trafficking. Domain formation in giant unilamellar vesicles (GUVs) is thus of interest for understanding fundamental biological processes and to provide new prospects for biocompatible soft materials. Lipid rearrangements in lipidic GUVs and lipid/polymer GUVs are extensively studied whereas polymer/polymer hybrid GUVs remain elusive. Here, the focus is on the thermodynamically driven phase separation of amphiphilic polymers in GUVs. It is demonstrated that polymer phase separation is entropically dictated by hydrophobic block incompatibilities and that films topology can help to determine the outcome of polymeric phase separation in GUVs. Lastly, Janus-GUVs are obtained and GUVs exhibit a single large domain by using a compatibilizing hydrophobic block copolymer.

1. Introduction

The compartmentalization effect of the cellular membrane is undeniably at the heart of all life forms. The plasma membrane is, however, more than a simple shell to protect biomolecules from the extracellular environment. In particular, the cell membrane exhibits a lateral heterogeneity composed of domains rich in glycolipids and cholesterol.^[1] These lipid rafts were determined to have many biological purposes such as intracellular signaling, membrane trafficking and to be essential for protein function. Consequently, mimicking membranes' lateral homogeneity has become an important quest in understanding membrane's inherent properties and behaviors.^[2]

In lipidic giant unilamellar vesicles (GUVs), microdomains formation can be obtained at a given temperature by mixing a lipid in the gel state with a fluid lipid often with cholesterol.^[3] The state of the lipids is determined by their gel transition temperature (T_m). The T_m is mostly dependent on the lipids' aliphatic chain length and their saturation and not on their head groups.^[4] High T_m lipids are commonly saturated, while low T_m lipids are short and/or unsaturated with double bonds, which

prevent them from tight packing. In the presence of cholesterol, the cholesterol itself can phase separate from the lipid phases, creating its own cholesterol-rich liquid-ordered phase.^[2a]


Further studies of phase separation within GUVs have been carried out in lipid/polymer hybrid vesicles. Here, the physical basis for domain formation is the chemical and geometrical incompatibility between lipids and polymers.^[2b] If the hydrodynamic radius difference between polymer and lipid is large, the formation of domains would be entropically favored in order to minimize exposure of hydrophobic moieties to water. Nonetheless, due to lipid and polymer incompatibility, these

vesicles also suffer from their kinetic tendency to undergo fission forming distinct liposomes and polymersomes rather than phase separating within a single GUV.^[5]

Despite the success of lipid/lipid and lipid/polymer phase separation in GUVs, few examples of polymer/polymer phase separation in vesicles of all size have been reported.^[2d,6] In contrast to lipids, which mostly rely on acyl chain variation for phase separation, polymersomes' studies used polymers with an identical hydrophobic block and different hydrophilic blocks. In 2009, Discher and co-workers described one of the rare compelling example of microdomains in mixed polymer GUVs.^[6b] These domains were induced by cations (Ca^{2+} or Cu^{2+}) cross-bridging of poly(butadiene)-*block*-poly(acrylic acid) (PB-*b*-PAA) and poly(butadiene)-*block*-poly(ethylene oxide) (PB-*b*-PEO). As acrylic acid has a pK_a value of 4.25, PAA is anionic at this pH value, chelating to the Ca^{2+} cation, phase separating from the neutral PB-*b*-PEO.

Here, we want to explore the formation of microdomains in GUVs by using thermodynamic polymer immiscibility^[6c,7] rather than demixing from a chemical or thermal stimulus.^[2a,4,6b,8] Immiscible polymer blends based on the Flory–Huggins parameter χ (degree of block incompatibility) and their compatibilizing have been extensively studied.^[7] In particular, the driving force behind the self-assembly of amphiphilic block copolymer into vesicles originate from the hydrophobic and hydrophilic block immiscibility and their rearrangement into an ordered entropically favored structure in water.^[9] Immiscibility is however not limited to the hydrophobicity differences in polymers and hydrophobic block such as PB, polydimethylsiloxane (PDMS), and polystyrene (PS) have been shown to be immiscible in blends.^[10] The χ parameter of for PB are under-reported but can be correlated to values obtained for polyisoprene (PI). Thus the χ value of PB-*b*-PDMS is

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estimated to be $\chi \approx 0.07$ at 25 °C like PI-*b*-PDMS,^[11] PB-*b*-PS to be $\chi \approx 0.088$ at 25 °C like PI-*b*-PS,^[12] and PS-*b*-PDMS was reported to be $\chi = 0.26$ at 25 °C.^[13] Based on their χ values, all our hydrophobic blocks are incompatible to varying degrees and thus in this study, we will examine the effect of polymer incompatibility in the hydrophobic and hydrophilic blocks on the overall GUV's surface topology. As such, we would like to understand polymer blends in vesicles and control the engineering of membrane domains.

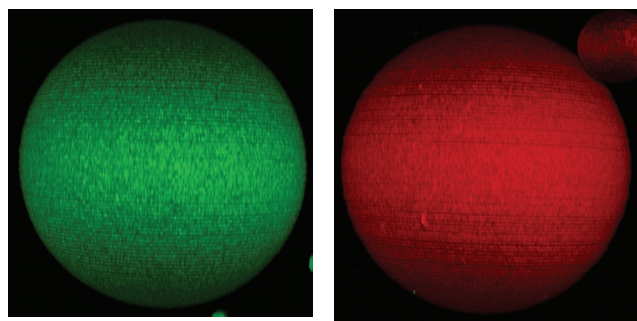
2. Results and Discussion

2.1. Polymer Synthesis and Unipolymeric GUVs

We recently developed a novel block copolymer, polybutadiene-*block*-poly(ethylene ethyl phosphate) (PB-*b*-PEEP), which hydrophilic block PEEP resembles natural phospholipids.^[14] This novel polymer reproducibly and rapidly self-assembles into a large number of GUVs with 20 μm in diameter by spontaneous hydration of its dry film (Table 1, entries 1 and 2). In the past it was thought that polymers always require a large input of energy to force their self-assembly into GUVs by electroformation^[15] or by coswelling with a hydrophilic polymer^[16] or by double emulsion techniques.^[17] We showed that PB-*b*-PEEP behave differently to the traditionally used polymeric amphiphiles because they are entropically favored to form GUVs in aqueous media similarly to lipids. We thus decided to synthesize other PEEP-based amphiphilic block copolymers, which had not been reported (entries 3 and 4).^[18]

Table 1. Block copolymers' characteristics.

Entry	Polymer	M_n^a	f^b	\mathcal{D}^c	GUV ^d
1	PB ₇₃ - <i>b</i> -PEEP ₁₂	6000	0.32	1.14	Yes
2	PB ₇₃ - <i>b</i> -PEEP ₂₁	7000	0.45	1.19	Yes
3	PDMS ₆₁ - <i>b</i> -PEEP ₁₂	7000	0.35	1.29	Yes
4	PS ₄₃ - <i>b</i> -PEEP ₁₇	7000	0.37	1.16	No
5 ^e	PB ₄₆ - <i>b</i> -PEO ₂₃	3000	0.29	1.11	No



^aThe degree of polymerization and M_n was determined by NMR; ^bHydrophilic fraction defined as $f = M_n(\text{hydrophilic block})/M_n(\text{block copolymer})$; ^c \mathcal{D} , the molar mass dispersity, was determined by GPC; ^dObserved by confocal laser scanning microscopy (CLSM); ^eCommercially available. Below the table, 3D rendering of typical GUV using BODIPY-FL (503/512 nm) on the left and BODIPY 630 (630/650 nm) on the right is shown. Abbreviations: PB: polybutadiene; PEEP: poly(ethylene ethyl phosphate); PDMS: polydimethylsiloxane; PS: polystyrene; PEO: poly(ethylene oxide); *b*: block.

The direct polymerization of ethylene ethyl phosphate (EEP) onto hydroxyl-terminated PDMS (PDMS-OH) was impaired by the proximity of the anionic polymerization initiation site to Si. The simple lengthening of the end group on PDMS to PDMS-(CH₂)₃-O-(CH₂)₂-OH resulted in the successful polymerization of EEP to form the block copolymer PDMS-*b*-PEEP (entry 3, see the Supporting Information). Although the starting PDMS-(CH₂)₃-O-(CH₂)₂-OH polymer has a molar mass dispersity (\mathcal{D}) of 1.13 and ¹H NMR analysis suggests a high level of terminal functionalization, we obtained PDMS-*b*-PEEP with slightly higher dispersity ($\mathcal{D} = 1.29$) than for other hydrophobic macroinitiators for the polymerization of ethylene ethyl phosphate (EEP) despite multiple synthesis attempts. PS-*b*-PEEP (entry 4) was also successfully synthesized from hydroxyl-terminated PS (PS-OH) in low $\mathcal{D} = 1.16$. PB-*b*-PEO (entry 5) was obtained commercially as a mean to compare our method and polymers to typically used polymer in vesicle self-assembly.^[19]

Subsequently, we proceeded in labeling the amphiphilic polymers described in Table 1. The fluorescent tagging of these polymers was carried out by Steglich esterification between the hydroxyl-terminated amphiphilic block copolymers and carboxylic acid-terminated boron-dipyrromethene (4,4-difluoro-4-bora-3a,4a-diaza-s-indacene (BODIPY)) dyes (see the Supporting Information).^[20] We also covalently attached BODIPY dyes on the hydrophobic homopolymers PB-OH, PDMS-(CH₂)_n-OH and PS-OH to respectively labeled PB-*b*-PEEP, PDMS-*b*-PEEP, and PS-*b*-PEEP GUVs in order to control for any artefact in the vesicles patterning arising from the capping of the hydrophilic end of amphiphilic polymers with a small hydrophobic moiety. The end group functionalization of our polymers and their purity was determined by high-performance liquid chromatography (HPLC). The high purity of the fluorescent polymers from the free BODIPY dye was found to be imperative for the correct visualization in GUV of polymer phase separation. Any remaining free BODIPY dye resulted in the apparent compatibility of all polymers. For PDMS-*b*-PEEP, a fraction of our labeled polymers was unfunctionalized as the esterification did not undergo full conversion. This only meant that higher concentrations of the partially functionalized polymer were used for better fluorescent resolution (5 mol% rather than 0.5 mol% for PB-*b*-PEEP).

We then proceeded in forming GUVs with our polymer library by nonassisted film hydration. This mild technique helps to minimize phase separation artefacts caused by the method of self-assembly rather than pure physicochemical immiscibility. Indeed, other techniques such as electroformation, gel-assisted hydration and double emulsion might force the mixing of the polymers as it has been shown for hybrid liposomes.^[21] We modified our original procedure¹⁴ by letting the film hydrate overnight rather than 1 h in order to maximize the number of vesicles, to diminish artefacts and variability caused by short hydration times and to observe the GUVs in their thermodynamic state.

Both PB-*b*-PEEP (entries 1 and 2) and PDMS-*b*-PEEP (entry 3) formed vesicles in high yield. PS-*b*-PEEP (entry 4) did not self-assemble into GUV, despite a similar hydrophilic ratio to PB-*b*-PEEP and PDMS-*b*-PEEP, which empirically should form vesicles.^[22] Similar failure to form GUVs by nonassisted film hydration was also observed with a commercially available PS-*b*-PEO ($M_n = 5000$, $f = 0.30$). The high glass transition temperature (T_g) of PS₄₃ (86 °C) compared to the low T_g of PB₇₃ (-97 °C) and

PDMS₆₁ ($T_g = -126$ °C, $T_m = -42$ °C) (differential scanning calorimetry (DSC) measurements in the Supporting Information) seemingly affects the formation of GUVs by this mild hydration method. Presumably because of the glassy aspect of PS, the thin films of PS-*b*-PEEP require a larger input of energy to swell than PDMS-*b*-PEEP and PB-*b*-PEEP similarly to other commonly used polymers as previously discussed and remain attached to the glass surface. The commonly used PB-*b*-PEO (entry 5) also gave no vesicles or very few numbers (2–3 per samples) using this mild hydration technique, as reported previously.^[14]

We did not observe any difference in terms of vesicle formation and appearance when using the amphiphilic or the corresponding hydrophobic homopolymer fluorescent tags. This observation suggests that neither the incorporation of hydrophobic polymers into membranes nor the hydrophobic capping of amphiphilic polymers disrupts the self-assembly process of polymers into GUVs.

2.2. Binary Polymer Mixture in GUVs

We then investigated polymer mixing in GUVs using our polymer library from Table 1. Each combination of two polymers was tested at three different ratios: 30:70, 50:50, and 70:30. The polymers were mixed in CHCl₃ prior to film formation and subsequent hydration. In order to differentiate the polymers in the GUVs, we elected to use BODIPY-FL ($\lambda_{Ex/Em}$ 503/512 nm) and BODIPY 630 ($\lambda_{Ex/Em}$ 630/650 nm). Using unipolymeric GUVs, we checked that no cross-talk could be observed between the channels on the confocal laser scanning microscopy (CLSM) therefore demonstrating that each channel corresponds to a uniquely labeled polymer (see the Supporting Information). If the polymers are colocalized, the vesicles would appear yellow as a result of the overlapping green and red channels.

When mixing equal amounts of PB-*b*-PEEP **A** and **B** of different M_n (A 6000 g mol⁻¹ (Table 1, entry 1) in green and B 7000 g mol⁻¹ (entry 2) in red) with identical PB₇₃ block, we obtained only GUVs composed of both polymers (Figure 1A, GUV). Therefore, even though both PB-*b*-PEEP **A** and **B** can separately yield a large number of GUVs, when hydrating the polymers together, solely homogeneously mixed GUVs are formed. We do not observe any domain formation at the micrometer scale. At nonequal ratios of polymers (30:70 and 70:30), the GUV were still composed of both PB-*b*-PEEP **A** and **B** but color gradients (green to orange) between GUVs could be observed (Figure S4, A). These color moduli are due to different composition of the GUVs in terms of polymeric ratios and highlight the challenge to obtain uniform GUV population when raising the complexity of these micrometer-sized soft materials even when swelled from the same film.

When mixing PB-*b*-PEEP (green) with PB-*b*-PEO (red) we also obtained fully mixed (yellow) GUVs in good numbers (Figure 1B). In this case, despite the use of different hydrophilic blocks PEEP and PEO, the PB block dictates the favorable mixing of the two polymers during self-assembly. In correlation to results obtained with PDMS-*b*-PEEP/PB-*b*-PEEP mixtures discussed below, the homogeneity of PB-*b*-PEEP/PB-*b*-PEO GUVs emphasizes that membrane demixing is governed by the favored or unfavored interactions of the hydrophobic blocks

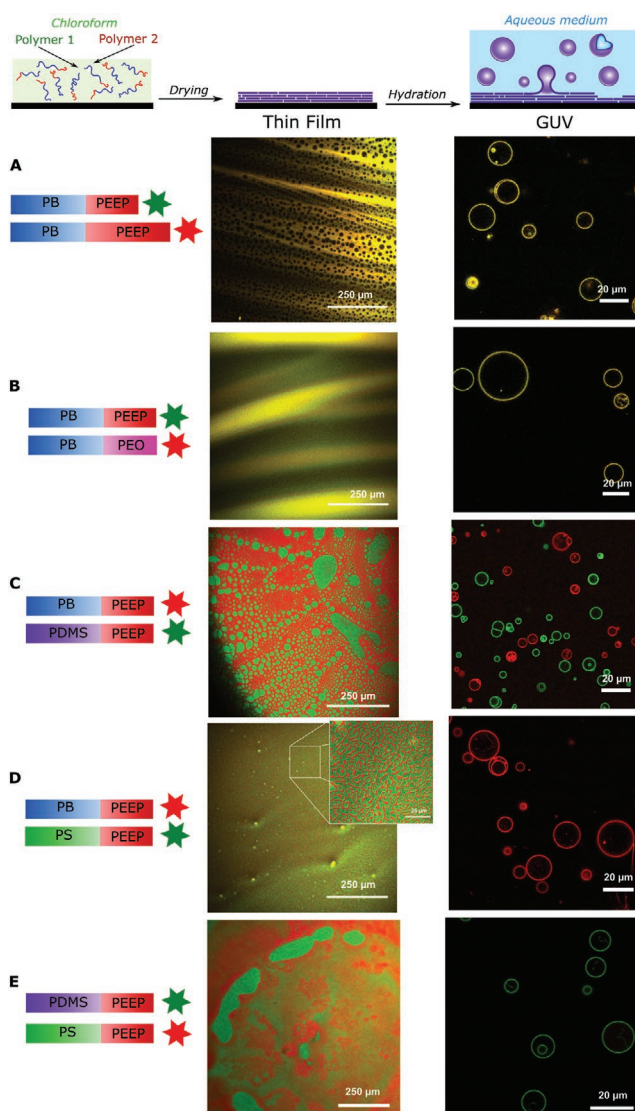


Figure 1. Polymer films on glass slides and hybrid GUVs observed under confocal laser scanning microscopy in their overlaying channels. The CLSM images are shown as the overlay channel of the BODIPY-FL fluorescence, excited at 496 nm and detected at 520–540 nm and BODIPY-630/650 fluorescence, excited at 633 nm and detected at 650–700 nm.

of the amphiphiles rather than their hydrophilic blocks. Interestingly, this example also shows that despite the poor self-assembly of PB-*b*-PEO alone, the co-self-assembly of PB-*b*-PEEP and PB-*b*-PEO promotes the incorporation of the challenging polymer in GUVs. As such, we have shown that polymers can be expressed in membranes by nonassisted film hydration even if they themselves would not form vesicles. The diversification of membranes by incorporating various polymers was also proven by doping the GUVs with the tagged homopolymer. We observed no disruption in the self-assembly in the same way that small hydrophobic molecules can be added to membranes.^[14,23] In membrane tagging, we added only 1 mol% of the hydrophobic homopolymer. Further studies showed that larger quantities of hydrophobic polymers can be used notably as described later with hydrophobic PDMS-*b*-PB as long as the

polymers used are of similar length (in this case ≈ 5 kDa). PB-*b*-PEEP vesicles are thus robust enough to express other entities with minimal disruption to their self-assembly process. This observation is particularly advantageous for future works that aim to explore more complex systems toward cell mimicry.

In the case of PDMS-*b*-PEEP (green) and PB-*b*-PEEP (red), we obtained two cohabitating monochromatic GUV populations (Figure 1C). The physical incompatibility of the hydrophobic blocks^[10a,b] resulted in a full phase separation of the polymers in GUVs regardless of the ratio used. Neither the identical hydrophilic PEEP block nor the common entropically driven unfavorable interaction of the hydrophobic blocks with the aqueous solvent suffices to assemble both PDMS-*b*-PEEP and PB-*b*-PEEP in a single GUV.

When mixing PB-*b*-PEEP (red) and PS-*b*-PEEP (green), we obtained monochromatic red GUVs thus comprising of PB-*b*-PEEP GUVs only (Figure 1D). Similarly, the mixing of PDMS-*b*-PEEP (green) and PS-*b*-PEEP (red) gave PDMS-*b*-PEEP GUVs only (Figure 1E). As PS-*b*-PEEP cannot form GUVs by this hydration method, presumably due to its high T_g (Table 1, entry 4), only the polymer which can self-assemble into GUVs are obtained. It appears that the thermodynamic immiscibility of PS with PB and PDMS^[24] did not lead to the incorporation of PS-*b*-PEEP into the PB-*b*-PEEP or PDMS-*b*-PEEP GUVs. Our study however showed that the integration of troublesome polymers into GUVs is possible by coswelling with the appropriate block copolymers if the hydrophobic blocks are miscible, even if the polymers themselves might not be prone to self-assembly due to a poor physicochemical profile as we observed for PB-*b*-PEO and hydrophobic polymers (PB and PDMS). In contrast, PS-*b*-PEEP could not be integrated into GUVs due its high T_g and incompatibilities with block copolymers which can form GUVs by mild film hydration.

Finally, we tested the hydration of PB-*b*-PEEP A films in the presence of PB-*b*-PEEP B preformed GUVs in the aqueous medium and vice versa (Figure S5, Supporting Information). Here, despite the already established miscibility of PB-*b*-PEEP A and PB-*b*-PEEP B when hydrating the mixed polymeric films (Figure 1A), we observed cohabitating populations of green (PB-*b*-PEEP A) and red (PB-*b*-PEEP B) GUVs and no mixed (yellow) GUVs. This experiment shows the underlying principle behind GUV formation. Even if the mixing of PB-*b*-PEEP A and PB-*b*-PEEP B is thermodynamically allowed when both polymers are coswelling to form vesicles, once formed, GUVs do not interact with the self-assembly of other amphiphiles. As such, polymeric mixed GUVs can only be obtained when mixing polymers prior to their self-assembly.

We observed no discrepancy in results when using the homopolymer or amphiphile tags in binary polymeric mixtures. Therefore, we have demonstrated that the hydrophobic capping of amphiphilic polymers by hydrophobic fluorescent tag like BODIPY does not cause artefacts in the GUV membranes patterning and polymer miscibility. Both the hydrophobic homopolymer and the amphiphile appear equally adequate for GUV membranes labeling.

In summary, by mixing our library of amphiphilic block copolymers described in Table 1, we have shown that polymer mixing in membrane is entropically dictated by hydrophobic block incompatibility only and that PB-*b*-PEEPs can be used as

a self-assembly platform for other polymers with any desired functionality.

2.3. Polymer Film Topology

We then studied the topological aspect of the dry drop-casted film on glass as generated in the prehydration step of GUV formation in order to try to rationalize the phase separation behavior of the polymers in GUVs. We observed that the polymers mixing/demixing behavior in the assembled state generally correlates to the mixing and demixing behavior of the polymers in their dry state (Figure 1, thin film).

For PB-*b*-PEEP A/PB-*b*-PEEP B and PB-*b*-PEEP/PB-*b*-PEO (Figure 1A,B), we observed the formation of homogeneously mixed films (Figure 1A,B). The polymers appear to be miscible in their neat state as both fluorescent tags overlap even at higher resolution (up to 40 \times). The swelling of these well-blended polymer films yielded homogeneously mixed GUVs.

In the case of the PB-*b*-PEEP/PDMS-*b*-PEEP film (Figure 1C), this film clearly exhibits a phase separation of the two polymers. The films are patterned with PDMS-*b*-PEEP domain of typically 50 μm within a PB-*b*-PEEP continuous phase. In the center of the film, the PDMS-*b*-PEEP polymer constitutes the continuous phase with some PB-*b*-PEEP domains. In the PB/PDMS homopolymer mixtures, we observed an even stronger phase separation where both polymers almost completely segregated forming large continuous domains on the opposite pole of the film (Figure S8, Supporting Information). Therefore, the hydrophilic PEEP block present in each amphiphile provides a compatibilizing effect on the polymer films resulting in improving polymer miscibility. However, we know that the GUVs formed from this film result in two distinct populations of GUVs rather than domain formation. The weak compatibilizing effect of PEEP is thus not strong enough to form patterned GUV but shows that a degree of miscibility (larger interacting surface) is allowed between PDMS and PB. The polymer domains in the films are larger than the diameters of the GUVs and thus the two distinct GUV populations can swell from their mixed films entropically favoring unipolymeric populations, reducing entropically unfavored immiscibility interactions.

The analysis of the PB-*b*-PEEP/PS-*b*-PEEP film (Figure 1D) also exhibited phase separation of the polymers. However, the PB-*b*-PEEP/PS-*b*-PEEP demixing was on a smaller scale than the PDMS-*b*-PEEP/PS-*b*-PEEP mixtures as observed when increasing the resolution scale. Indeed, most domains had a diameter of < 5 μm . However, we know that PB-*b*-PEEP/PS-*b*-PEEP films form PB-*b*-PEEP GUVs only. Thus, although the apparent higher miscibility of PB-*b*-PEEP/PS-*b*-PEEP in comparison to PB-*b*-PEEP/PDMS-*b*-PEEP in the film state, the entropic self-assembly of these amphiphilic polymers still plays a primary role in polymer demixing in membranes.

The mixing of PDMS-*b*-PEEP and PS-*b*-PEEP exhibited a significant demixing in the film state (Figure 1E). A PDMS-based core was surrounded by a PS-*b*-PEEP corona. This film morphology is coherent with the formation of PDMS-*b*-PEEP only GUVs. These results however clearly contrast with the film behavior of PB-*b*-PEEP/PS-*b*-PEEP mixture despite a similar outcome in the GUVs.

Therefore, film morphology can help to assess GUV demixing behavior rapidly. However, the degree of polymer demixing does not directly correlate to the patterning of GUVs.

Analysis of film topology across different ratios similarly to the ratio used for the GUV formation (30/70, 50/50, and 70/30) did not exhibit any trend in the phase separation behavior of the polymers based on the polymeric ratio (Figure S7, Supporting Information). The exact topology of the films is affected by experimental variability (order of mixing, surface coverage, drying speed, etc.). These variabilities in the film only have a low impact on the polymers mixing/demixing outcomes in the GUVs as we reproducibly obtained the same GUVs populations. We also noticed that when using polymer mixtures comprising of PDMS-*b*-PEEP, the CHCl₃ droplet dried in a smaller surface area (2 mm in diameter) compared to other mixtures (8 mm in diameter) regardless of the ratio used (Figure S9, Supporting Information).

2.4. Ternary Polymer Mixed GUVs

Based on our comparison of mixed polymer GUV and film, we wanted to investigate whether immiscible polymers could be compatibilized. Using the mixture of PB-*b*-PEEP/PDMS-*b*-PEEP, we doped our mixture with the hydrophobic block copolymer PDMS-*b*-PB ($M_n = 3.3$ kDa) in various ratios (Figure 2).

The vast majority of these mixtures gave monochromatic PDMS-*b*-PEEP and PB-*b*-PEEP GUVs (blue in Figure 2) as observed in the absence of a compatibilizer (Figure 1C). Below a 10 mol% threshold, single population of GUVs (PDMS-*b*-PEEP only or PB-*b*-PEEP only) could be observed; presumably as statistically, the number of GUVs formed with the other polymer is very low. Additionally, even when using large quantities of the hydrophobic copolymer PDMS-*b*-PB, we never observed black domains within GUVs. Presumably, as PDMS-*b*-PB hydrophobicity and similarity to both amphiphiles dictates a statistical distribution of the polymer within the bilayer. For some ternary mixtures (orange in Figure 2), we observed the formation of GUVs comprising both amphiphilic polymers. In general, a threshold of 10 mol% of PDMS-*b*-PB seemed to be required for compatibilizing the otherwise immiscible polymers. The GUVs population in these complex ternary polymeric mixtures was not uniform and included both monochromatic GUVs and GUVs expressing domains of different sizes. This heterogeneity was not surprising as the thin film of PDMS-*b*-PEEP and PB-*b*-PEEP mixtures already showed heterogeneous demixing of the polymers across the film (Figure 1C). Moreover, as discussed previously even when using a simpler system with the miscible PB₇₃-*b*-PEEP₁₂ and PB₇₃-*b*-PEEP₂₁ we observed that GUVs differ in polymeric ratios.

In terms of aspect, these GUVs were mostly Janus-type GUVs (Figure 3A,B), where the vesicles exhibit two large domains of similar size. Here the surface functionality difference is fluorescent tagging; however, we could consider simply modifying the tags to chemically relevant moieties such as alkyne or azide for asymmetrical click chemistry and obtained GUV asymmetrically functionalized. Other GUVs exhibited a single domain (green in red (Figure 3C) or red in green (Figure 3D)).

When checking the films of mixtures yielding hybrid vesicles (Figure S10, Supporting Information), we did not detect

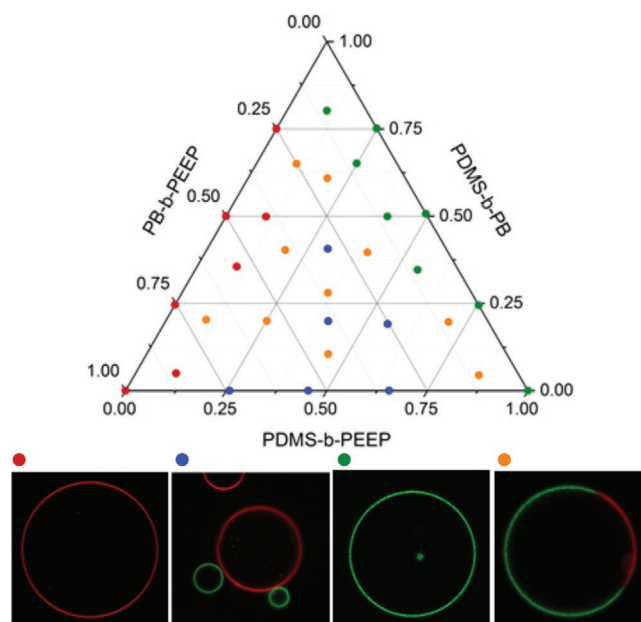


Figure 2. Ternary phase diagram. Diagram of PB-*b*-PEEP/PDMS-*b*-PEEP/PDMS-*b*-PB polymeric mixtures with corresponding CLSM typical images. PB-*b*-PEEP was labeled green with PB-BDPFL and PDMS-*b*-PEEP was labeled red with PDMS-BDP630. PDMS-*b*-PB was left unlabeled. Red data points represent the observation of PDMS-*b*-PEEP GUV only, green points to PB-*b*-PEEP GUV only, blue to a mixture of PDMS-*b*-PEEP GUV and PB-*b*-PEEP GUV, and orange to the observation of hybrid PDMS-*b*-PEEP/PB-*b*-PEEP GUV.

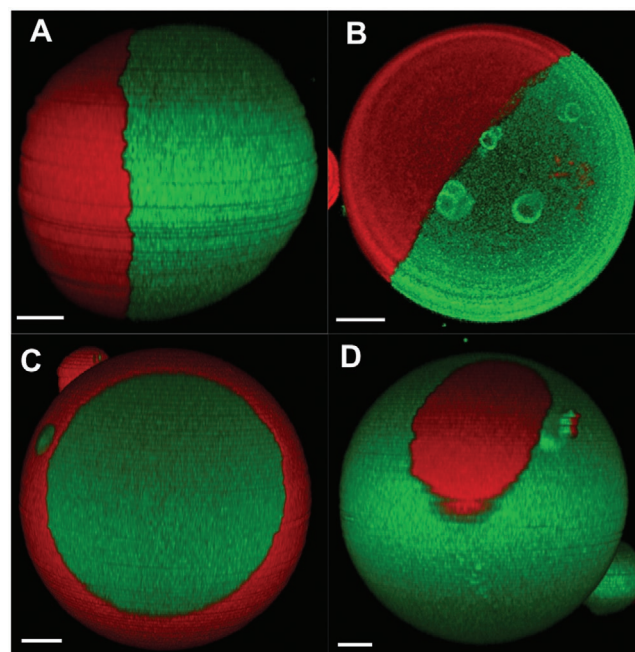


Figure 3. 3D projections of PB-*b*-PEEP/PDMS-*b*-PEEP/PDMS-*b*-PB hybrid GUV. These 3D projections were compiled from Z-stack measurements on the CLSM. (A,C,D) show the GUV as observed from the side and (B) is the seen from above with PB-*b*-PEEP/PDMS-*b*-PEEP/PDMS-*b*-PB ratio of 40/40/20 mol% for (A,B) and 36/36/28 mol% for (C,D). Scale bars: 5 μ m.

any significant difference in the film topology. The number and size of domains were similar to the compatibilizer-free PDMS-*b*-PEEP/PB-*b*-PEEP films. Only when using >60 mol% of PDMS-*b*-PB, we could observe complex patterning with different degrees of miscibility across the film (Figure S10, top left). Here we could witness the compatibilizing effect of PDMS-*b*-PB as the PDMS-*b*-PEEP and PB-*b*-PEEP domains were smaller (<10 μm) in diameter and interconnected with nonfluorescent PDMS-*b*-PB domains.

Contrary to incompatibility issues in lipid/polymer hybrid GUVs,^[2b] which are commonly only observable for a short period of time prior to fission, our hybrid polymer/polymer GUVs did not undergo fission. PDMS-*b*-PB therefore acts as a true compatibilizer of PDMS-*b*-PEEP and PB-*b*-PEEP despite the strong immiscibility of those polymers. This compatibilizing strategy could be used in lipid/polymer GUVs to obtain stable hybrid vesicles and limit their fission.

3. Conclusion

In this study, we showed that novel PEEP-based block copolymers PDMS-*b*-PEEP and PS-*b*-PEEP could be synthesized and that PDMS-*b*-PEEP could readily self-assemble into GUVs in a similar fashion to PB-*b*-PEEP by simple hydration (no external forces). We also successfully fluorescently tagged amphiphilic polymers by end group functionalization. Then when mixing different block copolymers we observed the complete phase separation of hydrophobically incompatible amphiphiles (PB/PDMS, PB/PS, and PDMS/PS) into distinct monochromatic GUV population. In contrast, identical hydrophobic block based amphiphiles (PB) formed homogeneously mixed GUVs regardless of the hydrophilic block used (PEEP or PEO). Thus, we have shown that polymer mixing in membrane is entropically dictated by hydrophobic block incompatibility. Furthermore, we analyzed the aspect of the films of binary polymeric mixtures prior to self-assembly. We observed that the film morphology grossly corresponds to the obtained phase separation in GUVs. Finally, due to the high immiscibility of our polymer hydrophobic blocks, we used a PDMS-*b*-PB compatibilizer to form GUVs comprising both immiscible PDMS-*b*-PEEP and PB-*b*-PEEP polymers. At certain ratios, we obtained Janus vesicles where each GUV half comprised of a different polymer or vesicles with a single circular domain of the immiscible polymer.

4. Experimental Section

Amphiphilic Block Copolymer Synthesis: The hydroxy-terminated hydrophobic polymer (P-OH) and N-[3,5-bis(trifluoromethyl)phenyl]-N'-cyclohexyl-thiourea (TU) were dried under reduced pressure from toluene. EEP and 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU) were distilled and stored under inert atmosphere. To a solution of P-OH (100 mmol, 1.0 eq.) and TU (500 mmol, 5.0 eq.) in dry THF (2.0 mL) under inert atmosphere, EEP (*n* mmol, *n* eq.) was added. After cooling the solution to 0 °C, DBU (75 μL, 500 mmol, 5.0 eq.) was added. The polymerization was carried out for 30 min and terminated using acetic acid in THF (300 μL, 5.0 mmol, 1.0 M, 50 eq.). The colorless solution mixture was dialyzed against H₂O in a 1000 molecular weight cut-off (MWCO) regenerated cellulose dialysis tube for 24 h. The resulting white suspension was dissolved in THF and dried under reduced pressure to obtain the desired polymer.

Block Copolymer Fluorescent Labeling: According to a modified procedure,^[20] the polymer (10 μmol, 1.0 eq.) was dissolved in anhydrous CH₂Cl₂ (100 μL). The carboxylic acid terminated BODIPY fluorescent dye (BDP630 and BDPFL) (12 μmol, 1.2 eq.) was then added to the polymeric solution and rinsed with further CH₂Cl₂ (100 μL), followed by the addition of 4-dimethylaminopyridine (DMAP) (10 μmol, 1.0 eq.). After cooling to 0 °C, N,N'-dicyclohexylcarbodiimide (DCC) (20 μmol, 2.0 eq.) was added and stirred for a further 15 min at this temperature. The reaction mixture was then stirred at room temperature overnight. The resulting solution was washed with THF/acetic acid and dialyzed against EtOH with acetic acid multiple times (regenerated cellulose MWCO 1 kDa) for 72 h to yield the desired labeled title polymer.

GUV Film Hydration: Form a modified nonassisted film hydration procedure,^[14] a solution of block copolymer in CHCl₃ (20 μL, 4.0 mg mL⁻¹) was loaded in a glass vial and dried at reduced pressure (10⁻³ mbar) for 15 min to form a thin film at the bottom of the vial. The vial was filled with a sucrose solution (200 μL, 100 × 10⁻³ M in MilliQ H₂O) and let to hydrate overnight. The sucrose solution (50 μL) was then diluted into an equiosmotic glucose solution (100 × 10⁻³ M in MilliQ H₂O, 200 μL) for facilitating visualization of the vesicles at the bottom of the well due to density differences. The fluorescently labeled block copolymers were mixed at 0.5–5 mol% into the nonlabeled corresponding polymer prior to loading. The polymer–polymer hybrid GUVs were obtained by mixing the block copolymer in the desired ratio preloading in CHCl₃.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

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- [1] a) K. Simons, E. Ikonen, *Nature* **1997**, *387*, 569; b) K. Gaus, E. Gratton, E. P. W. Kable, A. S. Jones, I. Gelissen, L. Kritharides, W. Jessup, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 15554; c) L. Bagatolli, P. B. Sunil Kumar, *Soft Matter* **2009**, *5*, 3234.
- [2] a) S. L. Veatch, S. L. Keller, *Biochim. Biophys. Acta, Mol. Cell Res.* **2005**, *1746*, 172; b) J. F. Le Meins, C. Schatz, S. Lecommandoux, O. Sandre, *Mater. Today* **2013**, *16*, 397; c) T. P. T. Dao, F. Fernandes, M. Er-Rafik, R. Salva, M. Schmutz, A. Brulet, M. Prieto, O. Sandre, J. F. Le Meins, *ACS Macro Lett.* **2015**, *4*, 182; d) L. Ruiz-Perez, L. Messenger, J. Gaitzsch, A. Joseph, L. Sutto, F. L. Gervasio, G. Battaglia, *Sci. Adv.* **2016**, *2*, e1500948; e) E. Rideau, R. Dimova,

- P. Schwillle, F. R. Wurm, K. Landfester, *Chem. Soc. Rev.* **2018**, *47*, 8572.
- [3] a) J. Zhao, J. Wu, F. A. Heberle, T. T. Mills, P. Klawitter, G. Huang, G. Costanza, G. W. Feigenson, *Biochim. Biophys. Acta, Biomembr.* **2007**, *1768*, 2764; b) T. M. Konyakhina, J. Wu, J. D. Mastroianni, F. A. Heberle, G. W. Feigenson, *Biochim. Biophys. Acta, Biomembr.* **2013**, *1828*, 2204; c) H. Umakoshi, K. Suga, *Solvent Extr. Res. Dev., Jpn.* **2013**, *20*, 1.
- [4] S. L. Veatch, S. L. Keller, *Biophys. J.* **2003**, *85*, 3074.
- [5] a) J. Nam, P. A. Beales, T. K. Vanderlick, *Langmuir* **2011**, *27*, 1; b) M. Chemin, P. M. Brun, S. Lecommandoux, O. Sandre, J. F. Le Meins, *Soft Matter* **2012**, *8*, 2867; c) T. P. T. Dao, F. Fernandes, E. Ibarboure, K. Ferji, M. Prieto, O. Sandre, J. F. Le Meins, *Soft Matter* **2017**, *13*, 627.
- [6] a) M. Massignani, C. LoPresti, A. Blanazs, J. Madsen, S. P. Armes, A. L. Lewis, G. Battaglia, *Small* **2009**, *5*, 2424; b) D. A. Christian, A. Tian, W. G. Ellenbroek, I. Levental, K. Rajagopal, P. A. Janmey, A. J. Liu, T. Baumgart, D. E. Discher, *Nat. Mater.* **2009**, *8*, 843; c) C. LoPresti, M. Massignani, C. Fernyhough, A. Blanazs, A. J. Ryan, J. Madsen, N. J. Warren, S. P. Armes, A. L. Lewis, S. Chirasatitsin, A. J. Engler, G. Battaglia, *ACS Nano* **2011**, *5*, 1775.
- [7] a) G. H. Fredrickson, A. J. Liu, F. S. Bates, *Macromolecules* **1994**, *27*, 2503; b) D. R. Paul, *Polymer Blends*, Elsevier Science, New York **1978**.
- [8] S. Patarai, Y. G. Liu, R. Lipowsky, R. Dimova, *Biochim. Biophys. Acta, Biomembr.* **2014**, *1838*, 2036.
- [9] a) F. S. Bates, G. H. Fredrickson, *Phys. Today* **1999**, *52*, 32; b) Y. Y. Mai, A. Eisenberg, *Chem. Soc. Rev.* **2012**, *41*, 5969.
- [10] a) C. A. Fleischer, J. T. Koberstein, V. Krukoni, P. A. Wetmore, *Macromolecules* **1993**, *26*, 4172; b) Z. M. Zou, Z. Y. Sun, L. J. An, *Chin. J. Polym. Sci.* **2014**, *32*, 255; c) C. D. Han, S. B. Chun, S. F. Hahn, S. Q. Harper, P. J. Savickas, D. M. Meunier, L. Li, T. Yalcin, *Macromolecules* **1998**, *31*, 394.
- [11] A. Ciolino, G. Sakellariou, D. Pantazis, M. A. Villar, E. Valles, N. Hadjichristidis, *J. Polym. Sci., Part A: Polym. Chem.* **2006**, *44*, 1579.
- [12] Y. Ren, T. P. Lodge, M. A. Hillmyer, *Macromolecules* **2000**, *33*, 866.
- [13] I. F. Hsieh, H. J. Sun, Q. Fu, B. Lotz, K. A. Cavicchi, S. Z. D. Cheng, *Soft Matter* **2012**, *8*, 7937.
- [14] E. Rideau, F. R. Wurm, K. Landfester, *Polym. Chem.* **2018**, *9*, 5385.
- [15] B. M. Discher, Y. Y. Won, D. S. Ege, J. C. M. Lee, F. S. Bates, D. E. Discher, D. A. Hammer, *Science* **1999**, *284*, 1143.
- [16] A. C. Greene, I. M. Henderson, A. Gomez, W. F. Paxton, V. VanDelinder, G. D. Bachand, *PLoS One* **2016**, *11*, e0158729.
- [17] J. Petit, I. Polenz, J. C. Baret, S. Herminghaus, O. Baumchen, *Eur. Phys. J. E: Soft Matter Biol. Phys.* **2016**, *39*, 59.
- [18] K. N. Bauer, H. T. Tee, M. M. Velencoso, F. R. Wurm, *Prog. Polym. Sci.* **2017**, *73*, 61.
- [19] a) J. Gaspard, L. M. Casey, M. Rozin, D. J. Munoz-Pinto, J. A. Silas, M. S. Hahn, *Sensors* **2016**, *16*, 390; b) H. L. Che, B. C. Buddingh, J. C. M. van Hest, *Angew. Chem., Int. Ed.* **2017**, *56*, 12581; c) A. Peyret, E. Ibarboure, J. F. Le Meins, S. Lecommandoux, *Adv. Sci.* **2018**, *5*, 1700453.
- [20] B. Neises, W. Steglich, *Angew. Chem., Int. Ed. Engl.* **1978**, *17*, 522.
- [21] N. F. Morales-Pennington, J. Wu, E. R. Farkas, S. L. Goh, T. M. Konyakhina, J. Y. Zheng, W. W. Webb, G. W. Feigenson, *Biochim. Biophys. Acta, Biomembr.* **2010**, *1798*, 1324.
- [22] a) D. E. Discher, A. Eisenberg, *Science* **2002**, *297*, 967; b) D. E. Discher, F. Ahmed, *Annu. Rev. Biomed. Eng.* **2006**, *8*, 323.
- [23] a) A. M. Eissa, M. J. P. Smith, A. Kubilis, J. A. Mosely, N. R. Cameron, *J. Polym. Sci., Part A: Polym. Chem.* **2013**, *51*, 5184; b) L. Messenger, J. Gaitzsch, L. Chierico, G. Battaglia, *Curr. Opin. Pharmacol.* **2014**, *18*, 104.
- [24] a) M. Baer, *J. Appl. Polym. Sci.* **1972**, *16*, 1109; b) L. del Valle-Carrandi, A. Alegria, J. Colmenero, *Eur. Phys. J.* **2010**, *189*, 257.