Giant polymersomes from non-assisted film hydration of phosphate-based block copolymers†

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The self-assembly of amphiphilic block copolymers is a fast way to prepare chemically versatile and stable ‘protocells’ that can act as a reactor or a confinement. However, controlling their self-assembly into giant unilamellar vesicles (GUVs) with diameters of several micrometers is challenging. Electroformation has been used to generate GUVs from amphiphilic block copolymers, which can be studied by light microscopy and resemble cell-like entities. However, a mild film hydration protocol for GUV preparation would be desirable in order to prepare libraries of protocells for further applications. Here, we present the self-assembly of novel amphiphilic polybutadiene-block-polyphosphoester block copolymers into GUVs by simple film hydration. These amphiphiles are synthetic analogs of phospholipids and possess the hydrophilic poly(ethylene ethyl phosphate) (PEEP) block. The GUVs (with diameters of ca. 10–40 μm) were formed in high yields by simple non-assisted film hydration requiring no external forces and with no need of the commonly applied electroformation. PEEP-based block copolymers with a lamellar bulk morphology produced GUVs in high yields and outperformed commonly used block copolymers (e.g. with poly(ethylene oxide) as a hydrophilic segment). We quantified their respective yield (number of GUVs formed) and diameters and monitored their stability over time. In addition, we proved their encapsulation capacity and permeability to hydrophobic and hydrophilic fluorescent cargo. Due to their high performance, these phosphate-based amphiphilic block copolymers are promising candidates for the generation of protocells and self-assembled microreactors.

Introduction

Compartmentalization of cells is a key feature of life. The plasma membrane is composed of a complex, balanced ratio of lipids, proteins, and small molecules and has many functions. Cell membrane mimicking has become an important quest for simplifying the understanding of the membrane’s inherent properties, functions and behaviors as well as for using their biocompatibility to expand drugs’ bioavailability, medical imaging and diagnostics or to compartmentalize incompatible entities in the synthesis.

Cell membrane mimicking was originally achieved with liposomes, and despite their resemblance to cell membranes, these vesicles are difficult to use and specialize, as they are unstable, fluid, and permeable. More recently, polymeric vesicles (polymersomes) have gained in popularity, as block copolymers are chemically more versatile, malleable, and tougher than lipids, resulting in easily functionalizable, more stable vesicles. Classically, polymersomes are generated from commercially available block copolymers and almost always using poly(ethylene oxide) (PEO) as the hydrophilic block. A shortcoming of polymersomes is their lower bio-compatibility and mimicy of cell membranes compared to liposomes as they are constituted entirely of synthetic entities. In this study, we propose a novel block copolymer, namely polybutadiene-b-poly(ethylene ethyl phosphate) (PB-b-PEEP). The EEP block is interesting as its phosphate moiety resembles natural phospholipids and is biodegradable, bridging the gap between liposomes and polymersomes. Despite this advantage, polymersomes bearing phosphate moieties are rare.

PB is also commonly used as the hydrophobic block in polymersomes as it has a low glass transition temperature (Tg) (Tg ≈ −21 °C for Mn = 105k; Tg ≈ −77 °C for Mn = 50k). Low Tg materials are desirable, as they are flexible under the self-assembly conditions (room temperature or above) and thus are able to mimic the fluidity of biomembranes contrary to more rigid hydrophobic blocks like polystyrene.

The vast majority of studies on polymersomes focus on small vesicles of ca. 100 nm diameters, the so-called small or large unilamellar vesicles (SUVs and LUVs, respectively) as they are readily achievable by multiple methodologies. However, cells are much larger (~10–100 μm) and giant unilamellar vesicles...
cles (GUVs) (>1 µm) are thus better mimics than SUVs. As increasing evidence suggests that factors such as the membrane curvature, effective encapsulated volume, stability, and permeability differ depending on size, efficient formation of GUVs becomes necessary.

Polymeric-GUVs are more challenging to obtain than SUVs and are often generated with microfluidic devices. This controlled water-in-oil-in-water double emulsion technique selectively forms vesicles of any size in very low polydispersity. However, this solvent displacement method requires complex mixtures of additives, which can be difficult to adapt to new conditions and contaminate the vesicles (e.g. the remaining solvent, surfactants, and additives in the membrane or in the lumen), significantly changing the membrane properties.

Solvant- and additive-free methodologies generating GUVs are still desirable for the robust and high-yield formation and encapsulation. Film hydration methods are based on the initial formation of a thin layer of the amphiphile on a surface by solvent evaporation followed by hydration of this solvent-free film. It is generally accepted that simple hydration of amphiphilic block copolymer films does not result in polymersome formation, and especially no GUVs are obtained by this procedure. Water cannot penetrate the dry polymer film to induce the self-assembly. Forces are required to enhance the film hydration of amphiphilic block copolymers or lipids. Commonly, shear forces like sonicating or stirring lead to SUVs or LUVs and alternative current (AC) or the use of swelling hydrogel substrates to GUVs.

The exact mechanism behind the effect of AC on vesicle formation is not well understood. Despite the success of the so-called electroformation, that is, the AC-aided film formation, for liposomes, this technique has been used only in a few studies for assembling polymeric-GUVs. In the case of hydrogel-mediated hydration, the amphiphilic film is formed on a pre-dehydrated hydrophilic polymer or gel (such as poly(vinyl alcohol) or agarose). Hydration of the gel causes deformation of the amphiphilic film as a driving force for the formation of vesicles. Hydrogel-mediated polymersome formation has only been rarely described and can also cause undesired membrane alteration. Therefore, since the initial report of polymeric GUVs in 1999, there is a clear niche for methods to form solvent and additive-free polymeric GUVs.

In this study, we first generated a library of amphiphilic PB-b-PEEP by sequential anionic polymerization. Then, we describe how with the appropriate block ratio PB-b-PEEP can generate GUVs by electroformation and even by spontaneous non-assisted direct hydration of their film within only 1 h. We quantified their yield and mean diameter, examine their stability in terms of number and size evolution over a month, and finally their encapsulation capacity to hydrophobic and hydrophilic fluorescent dyes. All these factors proved the phosphate-based block copolymers to be efficient amphiphiles for polymersome formation and encapsulation, superior to the commonly used block copolymers.

Results and discussion

PB-b-PEEP synthesis

PB-b-PEEP block copolymers were synthesized by sequential anionic polymerization (Scheme 1). The first step was the anionic polymerization of 1,3-butadiene, initialized by organolithium reagents and end-capped with ethylene oxide (EO) to yield a hydroxyl-functionalized PB-macroinitiator (PB-OH). Preference 1,2- or 1,4-polymerization can be achieved by using THF or cyclohexane, respectively. With cyclohexane, we obtained PB-OH with 92% 1,4-microstructure (PB(1,4)-OH) (Fig. 1i) with a low molar mass dispersity (D = 1.06) (Fig. 1i). The degree of polymerization of PB-OH was determined by

![Scheme 1 Synthesis of polybutadiene-b-poly(ethylene ethyl phosphate) (PB-b-PEEP). (a) Initial living anionic polymerization of butadiene to generate hydroxyl terminated 1,4-rich polybutadiene (PB(L4)-OH). (b) Organocatalyzed anionic ring-opening polymerization of ethylene ethyl phosphate to the amphiphilic block copolymers PB-b-PEEP.](image-url)
NMR with reference to the methyl end-groups at 0.87 ppm (Fig. 1ii).

PB(1,4)-OH was then used to polymerize ethyl ethylene phosphate (EEP) in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) as a base to activate the macroinitiator and a thiourea cocatalyst (b). These additives reduce side reactions such as the transesterification of the EEP moiety. Transesterification could still be observed as a small shoulder in the GPC curves at lower elution volume than the desired block copolymer (Fig. 1i). The shoulder appeared more prominent when targeting a higher degree of polymerization. Despite the transesterification side-reaction, all block copolymers were obtained with a narrow molar mass dispersity $D$ ($D < 1.2$) (Fig. 1i).

In comparison, classically used amphiphilic block copolymers consisting of a hydrophilic PEO block are synthesized by anionic ring-opening polymerization of the gas ethylene oxide (EO) at elevated temperature, high pressure, overnight onto the hydrophobic macroinitiator, such as PB-OH for PB-b-PEO.

However, EO is a carcinogenic, colorless, flammable gas and special care has to be taken when handled in the lab. Therefore, the synthesis of PB-b-PEPs is simpler, faster, and less toxic than that of PB-b-PEOs.

We generated a library of PB-b-PEPs with a range of hydrophilic fractions $f$ ($f = \frac{M_n(\text{hydrophilic block})}{M_n(\text{block copolymer})}$) (Fig. 1i) B-F and Table 1). The degree of hydrophilicity $f$ of amphiphilic block copolymers is an important property as it determines which macromolecular self-assembly is entropically favored. The nature of the polymer blocks and $f$ determines their self-assembly morphologies (micelles, cylindrical micelles (worms), reverse micelles, lamellae, vesicles, etc.). As a general rule $0.25 < f < 0.45$ yields polymersomes and our library is well within these boundaries. The $f$ values of each block copolymer were determined by comparing their PB(1,4) signal at 5.39 ppm (Fig. 1ii) already established for the PB-OH macroinitiator with the CH$_2$-O signals at 4.34–4.10 ppm (Fig. 1ii) of the PEO block. These values were then used to determine the respective $M_n$ of each block copolymer.

**Table 1** Library of synthesized PB-b-PEPs with a hydrophilic fraction $f$, $0.13 \leq f \leq 0.54$

| Entry | Polymer$^a$ | $f^b$ | $M_n$ | $D^c$ |
|-------|-------------|------|-------|-------|
| 1     | PB(1,4)$_{21}$-b-PEEP$_7$ | 0.13 | 5000  | 1.07  |
| 2     | PB(1,4)$_{17}$-b-PEEP$_7$ | 0.21 | 5000  | 1.13  |
| 3     | PB(1,4)$_{17}$-b-PEEP$_{12}$ | 0.32 | 6000  | 1.14  |
| 4     | PB(1,4)$_{17}$-b-PEEP$_{21}$ | 0.45 | 7000  | 1.19  |
| 5     | PB(1,4)$_{17}$-b-PEEP$_{31}$ | 0.54 | 9000  | 1.17  |

$^a$ Degree of polymerization and $M_n$ were determined by NMR.

$^b$ Hydrophilic fraction defined as $f = \frac{M_n(\text{hydrophilic block})}{M_n(\text{block copolymer})}$.

$^c$ $D$, the molar mass dispersity, was determined by GPC.

PB-b-PEEP self-assembly into GUVs by electroformation

Using our homemade electro-chamber with Pt wires (Fig. 2) we tested our library of PB-b-PEPs for GUV formation by electroformation (EF) following a modified method of Discher (see ESIT pS22 for details). PB$_{73}$-b-PEEP$_{12}$ A (entry 3) and PB$_{73}$-b-PEEP$_{21}$ B (entry 4) gave GUVs in high yields. Other ratios outside these boundaries yielded no vesicles. Therefore, PB-b-PEPs behave similarly to other classical amphiphilic block copolymers, although they appear to favor slightly above average $f$ for vesicles as PB$_{73}$-b-PEEP$_7$ ($f = 0.21$) did not self-assemble into GUVs while PB$_{73}$-b-PEEP$_{21}$ B ($f = 0.45$) did.

Most surprisingly, control experiments showed that the same polymers (PB$_{73}$-b-PEEP$_{12}$ A and PB$_{73}$-b-PEEP$_{21}$ B) could also spontaneously self-assemble into GUVs in the absence of an alternating current within the same time period (1 h) on Pt-wires and on glass slides. All the other PB-b-PEPs did not self-assemble into GUVs under these conditions. Non-assisted film hydration in such a fast timescale to form GUVs has never been reported before. Even in the case of lipidic GUVs, gentle hydration has only rarely been described as it requires long swelling times (typically several hours to days), is highly sensitive to any form of agitation, is unsuccessful for many amphiphiles and forms multilamellar deformed vesicles. For polymersomes, reports even state that they have high energy requirements towards their self-assembly. Control experiments for the formation of GUVs involving electroformation have not been explicitly described in previous studies.

Dimova et al. reported that the time required to form GUVs is much longer (3 h) at lower voltage (800 mV) and this resulted in smaller vesicles than those for 15 min at 9 V yielding GUVs of 40 µm radius on average. The authors also showed that simple swelling, on Teflon surfaces, of PB-b-PEO and PEO-b-PEO took 3 days and resulted in smaller vesicles. Therefore, our fast non-assisted film hydration of PB-b-PEPs into GUVs is unprecedented.

In order to compare the GUV formation between non-assisted film hydration (na-FH) and electroformation (EF), we quantified the yield (the number of GUVs formed) and their...
mean diameters. In analogy to the well-established standard mammalian cell counting methods using a hemocytometer, we manually counted the vesicles present in a number of random locations at the bottom of the well from microscopy images at a magnification of 20×. A magnification of 20× allows the counting to be done on an area of 6.4 × 10^5 µm² (divided into 16 squares of 200 µm length to ease counting – ES† pS23) and was found to be optimal for evaluating the vesicles formed. The number of vesicles at each location was then averaged out and back calculated to the vesicular yield obtained in the electro-chamber in GUVs per µL. Similarly, to the cell counting method, our yield estimation is prone to errors. For example, despite the density difference used between the inner phase (sucrose) and the outer phase (glucose), not all vesicles settled to the bottom where we counted them. We controlled that only a small proportion of vesicles could be found floating in the wells and no significant discrepancy was observed between settling times as long as a short 10 min latent period was given. Most importantly, for polymers that showed little to no vesicles, no vesicles were also observed floating and no changes in the results were reported at later times that could account for slower settling of the vesicles. Thus, it seems that the assumption that the majority of vesicle settle at the bottom rapidly does not have a significant impact on the estimated yield. Other parameters such as the number of vesicles transferred to the well, the location analyzed in the well, and counting errors, as well as experimental parameters such as film formation, the electro-chamber used and room conditions (temperature and humidity) could also affect the number of vesicles observed. The effect of these parameters can be minimized by systematically repeating the same protocol. In order to obtain a realistic yield estimation, we counted the cells at many different locations in the well (>5), replicated the experiments at least in triplicates and calculated the standard deviation between replicates. We also measured the diameter of each vesicle in order to determine the mean diameter of the vesicles per replicate. We then calculated the average of the mean diameters over the triplicate and their respective standard deviation. Finally, in analogy to dynamic light scattering (DLS) analysis of nanosized particles, we calculated the polydispersity (PDI) as PDI = (standard deviation/mean)² for each replicate and then calculated the average PDI. The average yield and their standard deviation, the mean diameter and their standard deviation are summarized in Table 2.

We observed that PB_{73}-b-PEEP_{12} (entry 1) and PB_{73}-b-PEEP_{21} (entry 2) clearly outperform the commonly used PB_{46}-b-PEO_{23} (f = 0.29) (entry 3),55–58 PDMS_{60}-b-PEO_{48} (f = 0.30) (entry 4),59,60 PDMS_{60}-b-PMOXA_{21} (f = 0.29) (entry 5),61–63 and PMOXA_{22}-b-PDMS_{19} (f = 0.31) (entry 6)64–67 in both EF and na-FH (ESI Table S1† for details of the replicated GUV yield). PB_{73}-b-PEEP_{12} and PB_{73}-b-PEEP_{21} gave similar high yields for both na-FH and EF (400 GUVs per µL and 175 GUVs per µL, respectively), typically giving a phase contrast image as seen in Fig. 3a. On the other hand, PB_{46}-b-PEO_{23} (entry 3),

### Table 2

| Entry | Polymer                  | Yield (GUVs per µL)a | Mean Øb (µm) | PDIc |
|-------|--------------------------|----------------------|--------------|------|
| 1     | PB_{73}-b-PEEP_{12}      | 355 ± 186            | 20 ± 2       | 0.78 |
| 2     | PB_{73}-b-PEEP_{21}      | 452 ± 144            | 14 ± 2       | 0.59 |
| 3     | PB_{46}-b-PEO_{23}       | 0.00 ± 0.00          | —            | —    |
| 4     | PDMS_{60}-b-PEO_{48}     | 0.00 ± 0.00          | —            | —    |
| 5     | PDMS_{60}-b-PMOXA_{21}   | 4.77 ± 4.61          | 23 ± 4       | 0.20 |
| 6     | PMOXA_{22}-b-PDMS_{19}   | 0.00 ± 0.00          | —            | —    |
|       | -b-PMOXA_{22}           | 0.00 ± 0.00          | —            | —    |

a Determined by phase contrast optical microscopy. b The mean diameter. c Polydispersity index defined as the average of the (standard deviation/mean)². For more details, the GUV yields for each replicate can be found in ESI Table S1 and their diameter and PDI in Tables S11–25, including frequency diagrams for their size distributions (Fig. S10–12).

![Typical phase contrast microscopy image of (a) PB_{73}-b-PEEP_{12}, (b) PB_{46}-b-PEO_{23}, (c) PDMS_{60}-b-PEO_{48}, and (d) PDMS_{60}-b-PMOXA_{21} by film hydration. Scale bar: 100 µm.](Image)
(entry 5) gave a few GUVs using EF (<5 GUV per µL) contrary to PDMS$_{67}$-b-PEO$_{68}$ and PMOXA$_{32}$-b-PDMS$_{119}$-b-PMOXA$_{22}$ (Fig. 3b–d). Similar PB-b-PEOs with a variety of properties tested such as 0.2 ≤ f ≤ 0.4 and 3000 ≤ Mn ≤ 17 000 also failed to produce GUVs by EF (ESI Table S1†) despite the frequent recurrence of PB-b-PEO in the formation of SUV and even GUV by various other methods. In the same perspective, Mingotaud and coworkers also expressed difficulties in obtaining polymersomes with PB-b-PEO by EF on ITO plates and its narrow hydrophilic ratio range as well as Greene et al. by EF on Pt wires. Interestingly, despite the common assumption that EF improves the vesicular self-assembly, we did not observe such an improvement for any of the block copolymers used and na-FH performed even slightly better for PB-b-PEEPs. We hypothesized that EF results in a smaller number of GUVs than na-FH as the electrical current might catalyze the degradation of GUVs perhaps by altering the polymer structure, in parallel with the previously studied degradation of polyunsaturated phospholipids.

In terms of size, all samples had a large size distribution (PDI > 0.2); nonetheless, the replicates consistently gave the same mean diameters. The mean diameter was 20 ± 2 µm for PB$_{73}$-b-PEEP$_{12}$ A and 14 ± 2 µm for PB$_{73}$-b-PEEP$_{21}$ B during na-FH (the error representing the mean diameter uncertainty between replicates). Tuning the GUVs’ size by EF to larger monodisperse vesicles was not observed, giving identical sizes and PDI to na-FH. In the case of PB$_{94}$-b-PEO$_{23}$, the mean diameter was 37 ± 11 µm, a significantly larger diameter than that for PB-b-PEEPs and PDMS$_{60}$-b-PMOXA$_{31}$. PB-b-PEEPs gave an apparent Gaussian distribution with a maximum at 5 µm (ESI Fig. S10–12†). Smaller vesicles than 1 µm were probably also formed but cannot be accounted for on the optical microscope. Experimentally, any object below 1 µm could not be definitely distinguished between vesicles or impurities by optical microscopy or SUVs be assessed by DLS due to the presence of GUVs, altering the scattering’s statistical average.

In order to determine how long the GUVs self-assemble by na-FH, we conducted a kinetic study in triplicate with PB$_{73}$-b-PEEP$_{12}$ A (Fig. 4). We observed that the optimal vesicle number is achieved within 2 h, already achieving a large number of vesicles within 1 h (ESI Table S3†). The mean diameter of the vesicles decreased slightly over time from 23 ± 3 µm to 14 ± 1 µm, exemplifying that larger GUVs seem to be formed first (ESI Table S4†). Further na-FH experiments were thus carried out for 1 h in order to directly correlate EF and na-FH over the same timescale.

In the last decade, polymersomes have been increasingly used because of their inherent stability compared to liposomes. Block copolymers are much less prone to chemical degradation than lipids and as they are larger molecules, entanglement in the bilayer can be greater, resulting in higher mechanical stability than that of liposomes. We analyzed the size (ESI Tables S7 and S8† for more details) and yield evolution (Tables S5 and S6† for more details) of our PB-b-PEEP GUVs under no special storing conditions (kept in aqueous dispersion at room temperature). We observed for our PB-b-PEEPs that the vesicle yield slowly decreased (Fig. 5). After 1 month, 56 ± 10 GUVs per µL of vesicles were still present for PB$_{73}$-b-PEEP$_{12}$, thus effectively losing 63% in yield. In contrast, only 5% of PB$_{73}$-b-PEEP$_{21}$ remained. In terms of size, the mean diameter and size distribution of PB$_{73}$-b-PEEP$_{12}$ polymersomes over one month were similar to the freshly prepared GUVs, while the vast majority of PB$_{73}$-b-PEEP$_{21}$ were much smaller. For PB$_{73}$-b-PEEP$_{21}$, >80% of vesicle size was between 1 and 10 µm compared to 50% at the formation and with a mean diameter dropping to 6 ± 1 µm. Thus, it appears that PB$_{73}$-b-PEEP$_{12}$ GUVs are more stable than PB$_{73}$-b-PEEP$_{21}$ GUVs, influenced by a favored hydrophilic/hydrophobic block ratio.

Scaling up the film hydration protocol in a round bottom flask using 4 mg of polymer in 5 mL of aqueous sucrose solution (100 mM) was also successful. A similarly high number of GUVs for both PB-b-PEEP A and B were obtained in a round bottom flask, even whilst vigorously stirring, than in our small 350 µL-capacity reactors. These agitated film hydration protocols are most frequently used to obtain a large amount of poly-
dispersed multilamellar vesicles (MLV), usually <1 μm, which are then extruded through a polycarbonate membrane with small pores to obtain a homogeneous SUV population. In the case of PB-b-PEEps, many GUVs were obtained with diameters >25 μm, whilst PDMS-b-PEO and PMOXA-b-PDMS-b-PMOXA did not yield any GUVs, PB-b-PEO formed only a few GUVs (with smaller diameters of ca. 5 μm) and PDMS-b-PMOXA formed a small number of GUVs (20 μm).

The polymers' physical properties

We wanted to determine why the PB-b-PEEP block copolymers formed a much higher number of GUVs than the classically used block copolymers. By analysis of the block themselves, we formed a much higher number of GUVs than the classically used block copolymers. By analysis of the block themselves, we formed a much higher number of GUVs than the classically used block copolymers. By analysis of the block themselves, we formed a much higher number of GUVs than the classically used block copolymers. By analysis of the block themselves, we formed a much higher number of GUVs than the classically used block copolymers. By analysis of the block themselves, we formed a much higher number of GUVs than the classically used block copolymers. By analysis of the block themselves, we formed a much higher number of GUVs than the classically used block copolymers. By analysis of the block themselves, we formed a much higher number of GUVs than the classically used block copolymers. By analysis of the block themselves, we formed a much higher number of GUVs than the classically used block copolymers. By analysis of the block themselves, we formed a much higher number of GUVs than the classically used block copolymers. By analysis of the block themselves, we formed a much higher number of GUVs than the classically used block copolymers. By analysis of the block themselves, we formed a much higher number of GUVs than the classically used block copolymers. By analysis of the block themselves, we formed a much higher number of GUVs than the classically used block copolymers. By analysis of the block themselves, we formed a much higher number of GUVs than the classically used block copolymers. By analysis of the block themselves, we formed a much higher number of GUVs than the classically used block copolymers. By analysis of the block themselves, we formed a much higher number of GUVs than the classically used block copolymers. By analysis of the block themselves, we formed a much higher number of GUVs than the classically used block copolymers. By analysis of the block themselves, we formed a much higher number of GUVs than the classically used block copolymers. By analysis of the block themselves, we formed a much higher number of GUVs than the classically used block copolymers. By analysis of the block themselves, we formed a much higher number of GUVs than the classically used block copolymers. By analysis of the block themselves, we formed a much higher number of GUVs than the classically used block copolymers. By analysis of the block themselves, we formed a much higher number of GUVs than the classically used block copolymers. By analysis of the block themselves, we formed a much higher number of GUVs than the classically used block copolymers. By analysis of the block themselves, we formed a much higher number of GUVs than the classically used block copolymers. By analysis of the block themselves, we formed a much higher number of GUVs than the classically used block copolymers. By analysis of the block themselves, we formed a much higher number of GUVs than the classically used block copolymers. By analysis of the block themselves, we formed a much higher number of GUVs than the classically used block copolymers. By analysis of the block themselves, we formed a much higher number of GUVs than the classically used block copolymers. By analysis of the block themselves, we formed a much higher number of GUVs than the classically used block copolymers. By analysis of the block themselves, we formed a much higher number of GUVs than the classically used block copolymers.

During EF and na-FH, one of the crucial steps is polymeric film formation on Pt-electrodes; thus the block copolymers are further analyzed as such for simplification.

Table 3 Comparison of the physical properties of block copolymers

| Block copolymer     | f  | σθ (mN m⁻¹) | m (g mol⁻¹) | Tg (°C) | Tm (°C) |
|---------------------|----|-------------|-------------|---------|---------|
| PB₇₃-b-PEEP₁₂       | 0.32 | 8.96 ± 0.34 | 6,000       | -97     | -59     |
| PB₄₆-b-PEO₂₃        | 0.29 | 19.82 ± 0.49| 6,000       | -60     | -76     |
| PDMS₆₀-b-PEO₄₈     | 0.30 | 19.80 ± 0.70| 6,000       | -50     | -70     |

- Interfacial tension σ measured by spinning drop tensiometry between CHCl₃ and H₂O at a concentration of 1.0 mg mL⁻¹. Measured by differential scanning calorimetry (DSC) between -100 °C and 100 °C.
- σθ = 0.4 nm. Filter prior to measurement did not limit that effect. The DSC curve for the thermal analysis can be found in ESI Fig. S4-9.
Encapsulation of hydrophilic and hydrophobic dyes

Ultimately, vesicles are interesting because of their compartmentalization, whether for chemical synthesis or biological mimicking. They are especially versatile compared to other carriers as they can encapsulate both hydrophilic and hydrophobic cargos with increasing complexity such as transmembrane proteins and even living cells.

Table 4: The yield of electroformation (EF) and non-assisted film hydration (na-FH) in the presence of Nile Red (NR) and the hydrophilic cargo Alexa Fluor 647 (AF)

| Entry | Polymer | Additive | Yield (GUVs per µL) | ee (%) |
|-------|---------|----------|---------------------|--------|
| 1     | PB73-b-PEEP12 | NR       | 583 ± 101           | >99    |
| 2     | PB73-b-PEEP21 | NR       | 145 ± 128           | 8 ± 3  |

*Encapsulation efficiency. Defined as the number of vesicles expressing fluorescence over the total number of vesicles observed by phase contrast. Details of the GUV yields and ee for each replicate can be found in ESI Table S2.*
diluted by a factor of five and allows a contrast to be observed if AF647 is encapsulated (Fig. 7 – right). When using AF647 for passive encapsulation into PB-b-PEEP GUVs during na-FH, the yield significantly decreased: PB73-b-PEEP12 A produced only 49 ± 11 GUVs per µL and PB73-b-PEEP21 B 74 ± 36 GUVs per µL compared to typically 400 GUVs per µL (Table 4 and ESI Table S2† for more details). Thus hydrophilic cargo can have a strong negative influence on the self-assembly of the GUVs themselves. Small quantities of additives such as ions or salts have been shown to affect the self-assembly processes of SUVs to yield aggregates of various morphologies.82,83 Thus it is not surprising that charged dyes like Af647 would disturb GUV formation.

For EF, the yield of PB-b-PEEP GUVs in the presence of AF647 was two times higher than for na-FH, giving similar values to the standard experiments in the absence of hydrophobic cargo (∼100 GUVs per µL). In other EF studies, large frequencies (500 Hz) have been reported to compensate for ionic strength, such as charged lipids36 or physiological buffers.84,85 It is thus reasonable to conclude that the use of a moderate frequency (10 Hz) in our system is enough to compensate for the ionic strength of AF647, impairing the spontaneous swelling of the polymeric films. This observation can also be correlated to the encapsulation of hydrophilic cargos by electroformation, a technique first tested on living cells.51 We thus observed for the first time the beneficial effect of EF compared to na-FH when using PB-b-PEEPs.

The ee was higher than expected for PB73-b-PEEP12 A with a decent ∼50% ee for both EF and na-FH, although generally, the fluorescence was weak. In the case of PB73-b-PEEP21 B, only low encapsulation (8 ± 3%) was obtained by na-FH. EF slightly improved the encapsulation to 23 ± 5%. Therefore, hydrophilic dyes are indeed harder to encapsulate than hydrophobic dyes; nonetheless, we were able to obtain a decent encapsulation efficiency when using PB73-b-PEEP12 A.

Polymersomes have been described to be less permeable than liposomes due to a much lower membrane fluidity.24,86–88 This allows polymersomes to retain hydrophilic cargo and thus makes them promising candidates for protocells. In order to assess the permeability of the PB-b-PEEP vesicles, we analyzed the evolution over time of the intravesicular Af647 fluorescence (Fig. 8, ESI Tables S9 and S10†). Vesicles of PB73-b-PEEP12 A appeared to be relatively hermetic with their fluorescence oscillating around 100% over 100 min, retaining Af647 in the polymersome’s lumen. PB73-b-PEEP21 B GUVs are more permeable, losing 85% fluorescence during the first 100 min. These results might also explain why the ee of AF647 in GUVs of B was significantly lower compared to GUVs prepared from A, rendering A a promising candidate for generating protocells.

**Summary**

We successfully synthesized a library of novel amphiphilic block copolymers (polybutadiene-block-poly(ethyl ethylene phosphate) (PB-b-PEEP)), with a polyphosphoester as the hydrophilic segment, resembling phospholipid-like structures for protocell assembly. PB-b-PEEPs with hydrophilic ratios of 0.32 and 0.45 successfully self-assembled into solvent- and additive-free GUVs with high yields by electroformation and non-assisted direct film hydration, i.e. in the absence of an alternating current or any other energy forces. In contrast to classically used block copolymers for polymersome formation, which are PB-b-PEO, PDMS-b-PEO, and PDMS-b-POMOA block copolymers, we observed that polyphosphoester-based amphiphiles produced GUVs by spontaneous film hydration or electroformation very efficiently. Stability experiments proved that PB-b-PEEP GUVs could be stored at room temperature for several weeks. Furthermore, we proved that hydrophobic and hydrophilic cargos were encapsulated into the GUVs. Hydrophobic dyes were efficiently encapsulated by non-assisted film hydration. Hydrophilic dyes tested with AF647 were more challenging to encapsulate into the GUVs. Nevertheless, 50% encapsulation efficiency could be achieved in PB73-b-PEEP12 GUVs and could be efficiently retained in the polymersomes for at least 2 h.
The straightforward synthesis of well-defined PB-b-PEEP block copolymers, their structural similarities to phospholipids, and the ease of producing loaded GUVs by simple film hydration make them promising new materials for the generation of protocells and microreactors.

Conflicts of interest
There are no conflicts to declare.

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