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Templated formation of giant polymer vesicles with controlled size distributions

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Unilamellar polymer vesicles are formed when a block copolymer self-assembles to form a single bilayer structure, with a hydrophobic core and hydrophilic surfaces, and the resulting membrane folds over and rearranges by connecting its edges to enclose a space. The physics of self-assembly tightly specifies the wall thickness of the resulting vesicle, but, both for polymer vesicles and phospholipids, no mechanism strongly selects for the overall size, so the size distribution of vesicles tends to be very polydisperse. We report a method for the production of controlled size distributions of micrometre-sized (that is, giant) vesicles combining the 'top-down' control of micrometre-sized features (vesicle diameter) by photolithography and dewetting with the 'bottom-up' control of nanometre-sized features (membrane thickness) by molecular self-assembly. It enables the spontaneous creation of unilamellar vesicles with a narrow size distribution that could find applications in drug and gene delivery, nano- and micro-reactors, substrates for macromolecular crystallography and model systems for studies of membrane function.

A vesicle is a small, enclosed liquid compartment, separated from its surroundings by at least one thin membrane consisting of a bilayer (unilamellar) or several layers (multilamellar) of amphiphilic molecules. Biological vesicles are generally formed from heterogeneous mixtures of amphiphiles, predominantly phospholipids—small-molecule amphiphiles with a charged head group and two hydrocarbon tails, and the latter molecules are used for the synthetic vesicles—liposomes—that find extensive applications in drug delivery and the cosmetics industry. It is now routine[1] to produce vesicles using amphiphilic block copolymers resulting in structures know as polymersomes[2], where the copolymers consist of covalently linked hydrophobic and hydrophilic chains of which the molecular architecture is such that in solution they form lamellar phases. These lamellar phases can be coerced into vesicle structures through a variety of energy-intensive protocols such as electroformation, extrusion and sonication[3,4]. Different processes for the formation of vesicles lead to ensembles of structures with a wide range of sizes distributed around some mean characteristic of the process. These characteristically wide size distributions are an inherent result of the physics of self-assembly, which does not give rise to a strong size selection mechanism for the vesicle radius. This is because the energetic penalty for the formation of spherical vesicles from a membrane with zero natural net curvature is independent of the radius, being the sum of the mean and Gaussian curvature.

A polymersome-forming polymer must be amphiphilic in nature, having both hydrophobic and hydrophilic domains. Their volume fraction, in aqueous solution, should be roughly symmetrical, such that in microphase-separated regimes the polymer domains self-assemble into a lamellae structure. The full range of structures expressed by bulk diblock copolymers includes cubic spheres, hexagonally packed cylinders, bicontinuous networks (typically the gyroid) and lamellar morphologies[5]. These structures can also be observed in aqueous solution; however, at high dilution the equilibrium structures formed are spherical micelles, cylindrical micelles and vesicles[6] corresponding to the unbound structural units.

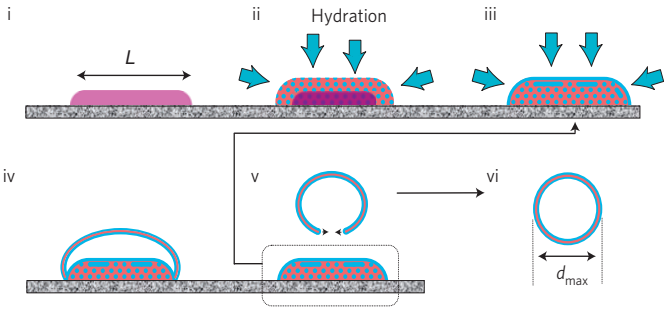

Figure 1 | Schematic representation of the controlled formation of vesicles. (i) Resulting drop profile following dewetting. (ii) Hydration resulting in microphase separation—hexagonal rod phase (blue: hydrophilic, red: hydrophobic). (iii) Further hydration at the surface resulting in surface lamellae and further internal phase separation. (iv) Expansion of exterior bilayer. (v) Detachment. (vi) Surface minimization leading to closure and vesicle formation.

The potential advantages of vesicles made from copolymers rather than phospholipids stem from the ability to optimize the physical and biological properties through polymer engineering; this has enabled increased colloidal stability, enhanced mechanical properties and an ability to 'tune' the membrane thickness[4] and hence the membrane permeability[7]. Polymeric membranes also enable efficient control of the surface functionalities[8–10].

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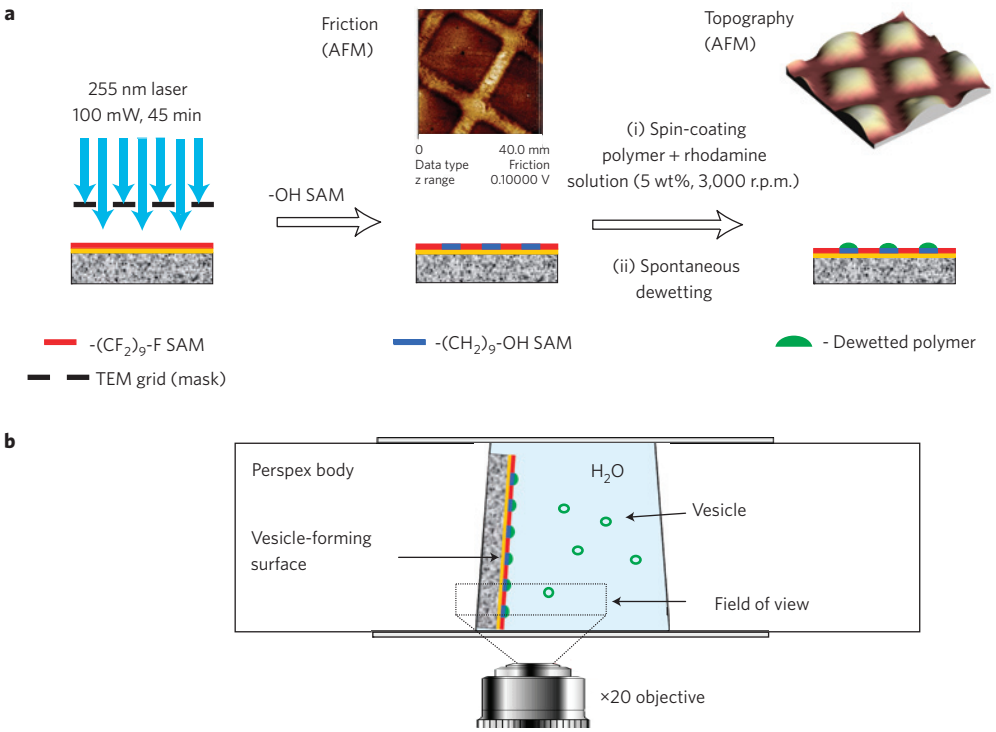

Figure 2 | Schematic representations of the polymer island formation and the confocal microscopy set-up. a, Procedure for the formation of patterned hydrophilic, fluorocarbon-decorated self-assembled monolayers (SAMs) and the spontaneous dewetting of a hydrophobic–hydrophilic block copolymer resulting in micrometre-sized domains of polymer. Friction atomic force microscopy (AFM) was used to characterize the fluorocarbon (light) and hydrophilic (dark) domains. **b**, Experimental set-up for the confocal microscopy of vesicle formation.

Poly(ethylene glycol)-coated phospholipid vesicles are sometimes referred to as 'stealth vesicles' and have been shown to have a very long circulation time *in vivo*[11], and these advantages are likely to be retained by polymer vesicles in which the hydrophilic block is similar[12].

The principal methods for vesicle production bring the amphiphilic molecules into contact with water either through phase inversion or an organic-solvent-free technique. In the first instance, the amphiphile is dissolved in a suitable organic solvent, and then this solvent is slowly exchanged by water, either by the evaporation of the volatile organic solvent in a water/solvent mixture[13,14] or through dialysis[15–17]. These methods rely on alteration of the packing parameters for the amphiphile and therefore the optimal surface area per molecule and it is possible to follow the transition from a micellar solution to a solution containing vesicles as the water content increases[18,19]. The size and the morphologies of the final vesicles cannot be efficiently controlled and the final membrane will always contain an amount of organic solvent, limiting their application in biomedical processes. In organic-solvent-free conditions, the amphiphile is deposited onto a surface, the solvent removed and then the amphiphile hydrated in the presence of an external energy source such as mixing[20] or an a.c. electrical field[21,22]. All of these different techniques have shown the formation of unilamellar and multilamellar vesicles with sizes that change considerably from one technique to another. Electroformation of vesicles from polymeric film deposited onto electrodes, and phase inversion from chloroform amphiphile solutions, for example, results in the formation of micrometre-sized vesicles, whereas rehydration techniques such as vigorous mixing with water, sonication or extrusion[23], have all been reported to give more or less polydisperse nanosize vesicles. To achieve a narrower size distribution, the vesicular solution is routinely sonicated or extruded to achieve relatively monodisperse ~100 nm sized vesicles[1].

Although electroformation can be used to form larger structures (greater than tens of micrometres), it is restricted to the use of low-molecular-weight copolymers and it exhibits poor control over the particle size distribution as well as the nature and geometry of the membrane structure formed. Rehydration and solvent exchange gives a broad distribution of vesicle size and topology, and energy input through sonication or extrusion results in the formation of smaller, stable vesicles and can be used to break up multilamellar vesicles, leading to the formation of stable distributions of smaller vesicles. In an electron microscopy study, Bates and co-workers clearly demonstrated[24] the non-ergodic nature of block copolymer assembly. For large amphiphiles, owing to the high energy barrier opposing redissolution of a molecule from an aggregate, the structures that are initially formed on the introduction of water have a fixed number of molecules, and without an extra energy input (greater than kT) the only reorganization that can take place to minimize their energy is within the aggregate itself.

At present no method for producing micrometre-sized polymer vesicles has been reported that will spontaneously make micrometre-sized vesicles in large numbers, of a controlled size. Paunov and co-workers[25] demonstrated the formation of liposomes using a polydimethylsiloxane stamp to control liposome size; however, a combination of electroformation and ultrasound was required to detach the lipid bilayers to form liposomes. Here, we present the spontaneous and energetically unassisted controlled formation of unilamellar, micrometre-sized polymer vesicles from a self-assembled surface. The polymer was a diblock of poly(ethylene oxide)-co-poly(butylene oxide) (PEO–PBO) copolymer ($E_{16}B_{22}$ $M_W = 2{,}300$), which in the bulk shows only limited microphase separation. On contact with water (<10% hydration), the interaction between the partially swollen hydrophilic and hydrophobic domains results in microphase separation into hexagonally packed rods, with further hydration leading to the formation of lamellae. Previous vesicle-forming studies have shown

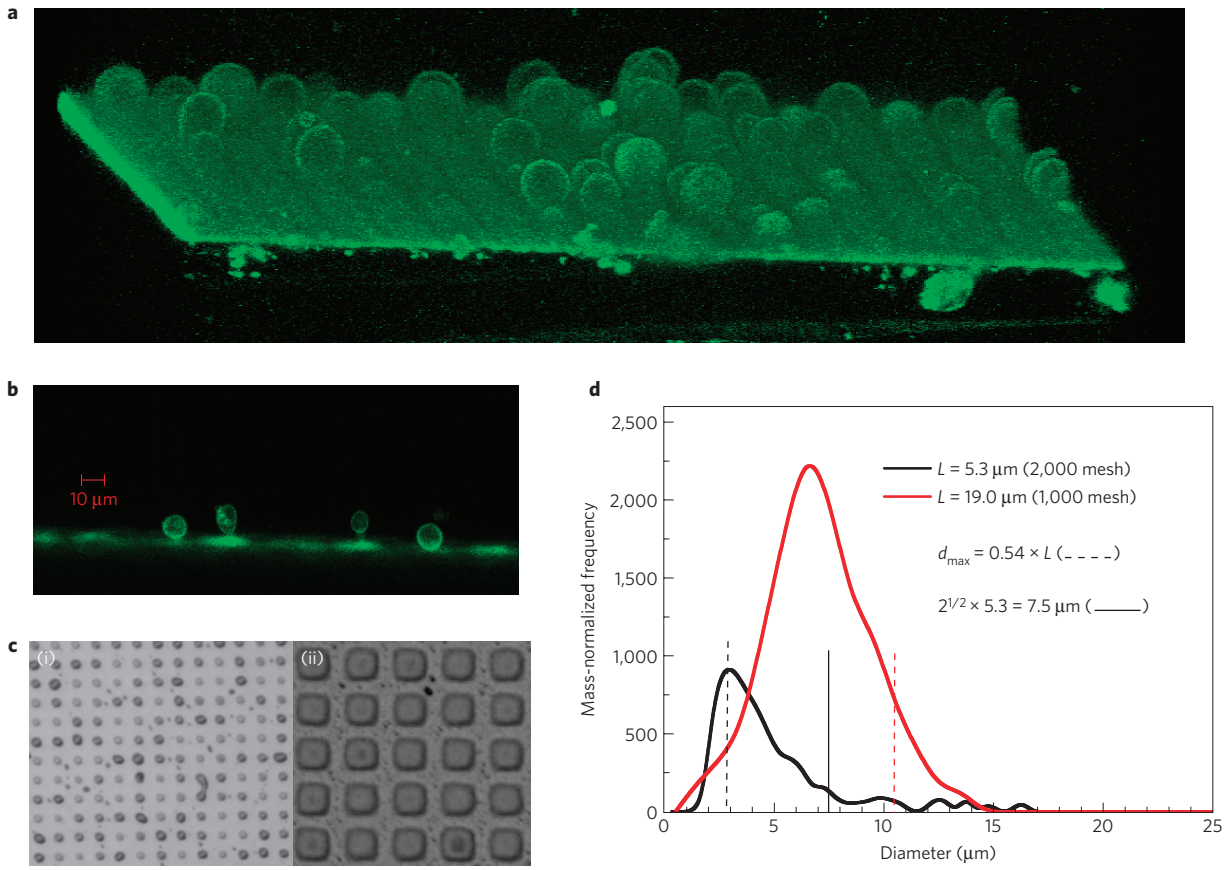

Figure 3 | Images of the polymer islands, the vesicle formation process and vesicle size distributions. a, 3D image (generated from a series of vertical slices) of the vesicle-forming surface showing the swollen exterior bilayer before detachment. **b**, A single vertical slice showing a series of vesicles 'budding' from the surface. **c**, Differential interference contrast optical microscopy of the dewetted surfaces following spin-coating using 2,000 mesh (i) and 1,000 mesh (ii) TEM grids. **d**, Mass-normalized frequency of vesicle size distribution for the patterned surfaces shown in **c**.

that a thin film of this material forms an aqueous solution containing a wide variety of membrane structures (unilamellar and multilamellar) that range in size from tens of micrometres to sub-micrometre sizes[26]. The method we describe here generates micrometre-sized, unilamellar spherical vesicles of a controlled diameter, the formation of which is characterized in real-time. It relies on the vesicle-forming polymer first microphase-separating into a lamellar structure on a laterally patterned surface. These squares are surrounded by fluorocarbon regions that are both hydrophobic and oleophobic, from which the block copolymer completely dewets. This restricts the lateral, in-plane, size of the individual lamella bilayers that form on the surface of the polymer island such that they have a finite area. When these bilayer squares are detached from the polymer island surface they form vesicles of which the size is specified by the conservation of bilayer area (Fig. 1). Ultraviolet photolithography of a self-assembled perfluoroalkanethiol monolayer was used to produce a patterned surface decorated with hydrophilic squares surrounded by a continuous fluorocarbon surface (Fig. 2). Spin-casting a dilute solution of vesicle-forming polymer produces a thin, continuous film that covers the entire surface and that spontaneously dewets, resulting in a patterned surface (Fig. 2a, upper right) of well-defined islands of polymer.

The composition of the block copolymer is such that on hydration it initially forms hexagonal rods of the hydrophilic polymer in a matrix of hydrophobic polymer, and this structure has been observed in an equilibrated 9:1 polymer/water mixture[26]. On contact with water, there is a gradient of water concentration and each individual island consists of a partially hydrated lamellae outer surface with a phase-separated (hexagonal rods) internal structure. Moreover, the lowest-surface-energy hydrophilic polymer also self-assembles such that it covers the exterior of the domains, which is both the upper surface and the perimeter. A benefit of this arrangement is that the water is able to swell the hydrophilic domains within the film much faster than with a continuous film as studied previously[26].

On contact with water, the hydrophilic domains swell whereas the hydrophobic domains remain unaffected, although still flexible. The steric repulsion between facing hydrophilic layers increases, resulting in increased separation of the surface lamellae. At the surface of the polymer island, the upper-most bilayer is free to expand from the surface, resulting in a curved bilayer that ultimately unbinds. The line tension in the perimeter causes the detached sheet to minimize its surface energy and form a spherical vesicle. The process then repeats as the exposed surface of the polymer island then continues to swell and results in a fresh upper lamellar layer that again detaches. The surface area of the vesicle, and thereby its size, is dictated by the surface area of the lamellae sheet from which it was formed. If we assume that the pattern comprises stacks of polymer bilayer sheets (lamellae), the relationship between the vesicle diameter and the size of the polymer island is governed by the surface area of each, such that $L^2 = 4\pi r^2$, or $d = 0.54 \times L$, where r and d are the radii and diameter of the vesicle and L is the length of one side of the square feature. By controlling the size of the parent polymer island, we show that we are able to control the size of the vesicle formed.

There have been few previous reports of processing methods to produce narrow-size-distribution vesicles. Förster and co-workers

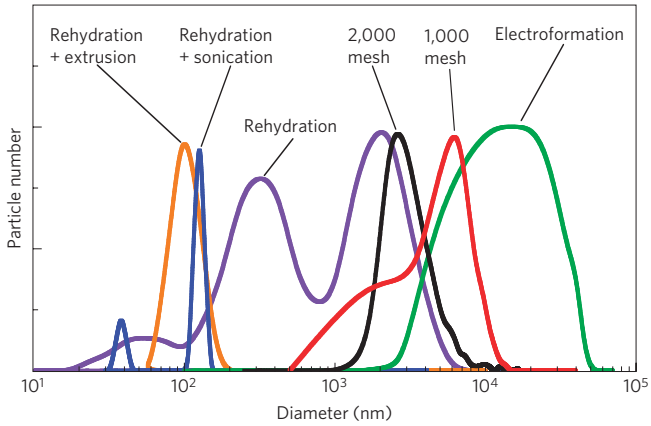

Figure 4 | Comparison of vesicle-forming procedures. Size distribution of PEO–PBO vesicles formed through rehydration, rehydration + extrusion, rehydration + sonication, electroformation, and the surface-directed templating described here. Data taken from LoPresti *et al.*[28].

used an inkjet printer to introduce well-controlled, nanolitre droplets of amphiphiles in solvent to a large volume of water[27]. This gives some control over vesicle size, setting an upper limit, but is essentially an extension of the solvent exchange method and there is not a direct correlation between the droplet size and the vesicle size as multiple vesicles are produced by each droplet. The extrusion process also produces a distribution of vesicles sizes constrained by an upper limit related to the size of the holes in the extrusion membrane. Neither of the techniques described above takes the approach of repeatedly detaching a single lamellar sheet from a multiple stack and folding them up to form single vesicles and it is this supramolecular control that is demonstrated here by this unique combination of top-down patterning by lithography coupled with the spontaneous dewetting (that ultimately sets the size) and molecular amphiphilic self-assembly that closes the structure.

Patterned hydrophilic/fluorocarbon surfaces were generated using 1,000 and 2,000 mesh transmission electron microscopy (TEM) grids. These gave square hydrophilic domains, which were $19 \times 19\,\mu m$ and $5.3 \times 5.3\,\mu m$, respectively. The polymer solution was spin-coated, generating polymer islands as shown in Figs 2a and 3c that have a height of ~500 nm. The formation of vesicles from these surfaces was studied using confocal microscopy and the diameters of the vesicles produced were determined, and the rate of vesicle formation was determined to be approximately 2×10^3 vesicles $cm^{-2}\,s^{-1}$. Figure 3a and b show a three-dimensional (3D) computer-generated surface produced from a series of 2D slices and the vesicle-forming surface in 2D (a single optical slice), respectively, clearly showing the hydration and expansion of the upper surface layers and the formation of unilamellar membranes and vesicles. The unilamellar nature cannot explicitly be determined using confocal microscopy but the uniform fluorescence from different vesicles indicates a uniform membrane thickness for the vesicles formed.

From the relationship between the dewetted domain size and the theoretical vesicle diameter for our samples, we expect to see vesicles that are limited in size to diameters of 10.3 μm (1,000 mesh) and 2.9 μm (2,000 mesh). Figure 3d shows the mass-normalized frequency of the diameter for these two samples with an indication for the predicted vesicle diameter limit (vertical dashed line of corresponding colour). What can clearly be seen is that there is a limit to the size of vesicles formed, which goes beyond the theoretical limit of $d = 0.54 \times L$. There is, however, a correlation with the predicted size—smaller domains give smaller vesicles, larger domains result in larger vesicles and in the instance of only a single bilayer of polymer the predicted vesicle size would be recovered. We attribute this underestimation to the 'loading' of the polymer on its hydrophilic island as it dewets from the fluorinated areas. For the smaller islands (2,000 mesh substrate), the ratio between these two areas is significantly less, meaning more polymer had to dewet onto smaller hydrophilic islands. Although the concentration of the polymer solution was reduced, it creates a curved exterior surface having a larger surface area than the square island on which it sits. If we consider an extreme example, where we have a hemisphere of polymer sitting on the hydrophilic island, the size of the vesicle produced then becomes $d = 2^{1/2}L$. This upper limit is also marked and correlates well with the observed data. The data set for the 1,000 mesh sample behaves as described by theory and is to be expected as the optical microscopy image of the surface indicated a very flat surface having a surface area closely matching that of the underlying pattern.

In comparing our results to standard methods of vesicle formation[28] (Fig. 4) we see a size distribution narrower than normal rehydration and smaller in size than standard electroformation with the limiting size of the template clearly limiting the maximum size of the vesicles formed. In principle, there is no lower-limiting size to which this method may be used. However, the patterning of the thiol surface uses ultraviolet lithography and any pattern is therefore diffraction limited, resulting in vesicles of ~500 nm in diameter. To achieve the more biologically active sized vesicles of diameters ~100 nm, then extra extrusion would be required. Studies were undertaken to asses the suitability of this technique to chemically similar (PEO–PBO), higher-molecular-weight vesicle-forming polymers. Dewetting, and the formation of the polymer islands was achievable at T > 70 °C, (T_m (PEO) ~65 °C, T_g (PBO) −88 °C). However, undertaking the vesicle formation step in water, at elevated temperatures, resulted only in the detachment of the polymer island from the surface. We suspect this was due to degradation of the thiol surface layer and increased wetting, by the solvent, of the silicon surface. Therefore, we expect the method described here requires a liquid-like mobility of both phases of the block copolymer at room temperature (if thiol substrates are used).

The fluorescence images were obtained using amphiphilic Rhodamine-B octadecyl ester dye that is trapped within the hydrophobic domain of the bilayer. If a hydrophilic cargo was evenly loaded into the polymer island before their formation then as the vesicles bud from the surface, the outer-most bilayer would prevent loss of the precious cargo into the bulk, resulting in a highly efficient encapsulation route.

We have shown that we are able to control the size of vesicles produced for a single polymer spontaneously, without the need for energy input, and that the vesicles produced are of a constant wall thickness. This process will be a useful technique for the study of vesicle dynamics, membrane behaviour, bilayer hydration and the basics of vesicle formation. It is also predicted that this will provide a favourable thermodynamic pathway for materials that are reluctant to form vesicles as long as the amphiphilic polymers are able to form lamellar structures and be made suitably mobile. The ability to make vesicles of controlled bilayer thickness and diameter opens many opportunities for both picolitre-dosage drug delivery and studies of membrane function.

Methods

Patterned substrates were prepared as follows. Plasma-cleaned gold-coated silicon wafers were placed in a 10 mM solution of 1H, 1H, 2H, 2H-perfluorodecanethiol (Aldrich) in dry, degassed ethanol for 18 h to create a self-assembled monolayer of the perfluoroalkane. Photolithography was carried out by photo-oxidation of the perlfluoroalkanethiol to an alklysulphonate[29] self-assembled monolayer and was conducted using an argon ion laser (Coherent) operating at 244 nm and 100 mW. The beam was de-focused to produce a beam spot diameter of 15 mm with a power density of approximately 1,500 W m^{-2}. Nickel TEM grids

(AGAR scientific) 25 mm × 25 mm with mesh sizes of 1,000 and 2,000 lines per inch were used as masks. Square features with sides of 19 and 5.3 μm, respectively were generated. The photo-oxidized substrates were placed in a solution of 10 mM 11-mercapto-1-undecanol (Aldrich) in ethanol for 18 h to generate square, hydrophilic domains surrounded by a perfluoronated surface (Fig. 2a). To form the vesicles, a PEO–PBO copolymer ($E_{16}B_{22}$ $M_W = 2{,}300$) was prepared by sequential anionic copolymerization and the polymer's hydrophobic domains were labelled using Rhodamine-B octadecyl ester perchlorate (Aldrich), solubilized within the hydrophobic domains. A solution of the polymer (5 wt%), in a dilute (0.05 wt%) Rhodamine-B in chloroform solution was spin-cast to generate films several hundred nanometres thick. The solution was diluted for 2,000 mesh samples, as the ratio between hydrophilic and fluorophilic domains is significantly less. The thin polymer film spontaneously dewetted from the perfluoronated areas to result in a series of square islands of solvent-free polymer with sizes corresponding to the original TEM grid used.

A polymer-decorated surface was placed in a 5-mm-thick Perspex sample holder and fixed in place, at a slight incline, using vacuum grease. A glass coverslip was fixed to the base of the cell (grease) and the cell was filled with filtered (4.5 μm polytetrafluoroethylene) water at 18.2 $M\Omega^{-1}$. A second coverslip was fixed to the upper surface to prevent evaporation.

Confocal laser microscopy was carried out using a Zeiss LSM 510 M microscope. Excitation was at 543 nm and fluorescence was collected at >620 nm. Several hundred images were collected over a 3 h period for each sample using a narrow vertical slice (~50 μm). The 3D projection was obtained from a series of 100 images. Image analysis was conducted using Labview.

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