Lipid exchange enhances geometric pinning in multicomponent membranes on patterned substrates

Multicomponent supported lipid bilayer on a substrate patterned with colloidal spheres. In lipid membranes, the alternation of highly and gently curved regions favours the segregation of phase-separated domains that are more or less compliant to bending. By studying this phenomenon experimentally and theoretically, we find estimates of the material properties of the lipid phases.

See Daniela J. Kraft et al., Soft Matter, 2020, 16, 4932.
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Experiments on supported lipid bilayers featuring liquid ordered/disordered domains have shown that the spatial arrangement of the lipid domains and their chemical composition are strongly affected by the curvature of the substrate. Furthermore, theoretical predictions suggest that both these effects are intimately related with the closed topology of the bilayer. In this work, we test this hypothesis by fabricating supported membranes consisting of colloidal particles of various shapes lying on a flat substrate. A single lipid bilayer coats both colloids and substrate, allowing local lipid exchange between them, thus rendering the system thermodynamically open, i.e. able to exchange heat and molecules with an external reservoir.

Multicomponent artificial lipid membranes, consisting of a ternary mixture of cholesterol and phospholipids, undergo liquid–liquid phase separation at specific temperature and lipid chemical composition.1–4 When they assemble into a bilayer, at high temperatures, the lipids form a uniformly mixed membrane, while at lower temperatures, they spontaneously separate into two different liquid phases, known as liquid ordered (LO) and liquid disordered (LD). The LO phase is rich in saturated lipids and cholesterol while the LD phase is rich in unsaturated lipids.5–9

The LO phase is more packed and thicker than the LD phase, implying that LO domains are less prone to bending and splay deformations. These domains are preferentially localised in regions of relatively low mean curvature and avoid regions of high negative Gaussian curvature. Experimental evidence of this phenomenon has been obtained in experiments with giant unilamellar vesicles (GUVs),10–15 with supported lipid bilayers (SLBs) on patterned substrates16–18 and, most recently, on scalfolded lipid vesicles (SLVs), i.e. membrane-coated colloidal particles.19 While GUVs can change shape during the phase separation process, SLBs and SLVs are scaffolded. Therefore, they can mimic the supporting property of the actin cytoskeleton in cells and allow for the fabrication of stable bilayers for atomic force microscopy (AFM) studies,20–24 for applications in biosensing,25 in cell biology,26 and in drug delivery.27

Experiments with GUVs have shown that there is a correlation between positioning of soft domains and bilayer curvature. However, the large variety of possible shapes and the fact that vesicles change their shape dynamically while phase-separating has prevented a quantitative characterisation of this phenomenon, owing to the scarce reproducibility. Conversely, experiments with SLBs on corrugated glass16 and flat substrates with hemispherical asperities17,18 have shown that the LD domains are consistently geometrically pinned to regions of high mean curvature. This pinning was also observed in experiments with SLVs of symmetric and asymmetric dumbbells19 albeit only for LD domains relatively small compared to the total vesicle size. Even more remarkably, the most common configurations observed in anisotropic SLVs, featured antimixing: i.e. a state in which the lipids are mixed and yet strongly partitioned on regions with different curvature.19,28

Further numerical
We emphasize that, while at the scale of a single colloid there is exchange of lipids with the substrate, at the scale of the entire system the total amount of lipids is fixed. This allows us to sample the Gibbs phase triangle of the lipid mixture by varying the total membrane composition, while rendering the colloid a thermodynamically open system, that is able to exchange lipids with the substrate, which, in turn, acts as a reservoir. Furthermore by comparing experimental and numerical data, we are able to estimate the material properties of the membrane and show that these are consistent with previous measurements made on multicomponent GUVs of similar compositions. Finally, we forecast the outcome of possible experiments on catenoidal and conical necks and show that these geometries could greatly improve the precision of the current estimates of the bending moduli.

II. Experimental setup

A. Reagents

The lipids 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), porcine brain sphingomyelin (BSM), oxine wool cholesterol (choi), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-lissamine rhodamine B sulfonyl 18 : 1 (Liss Rhod PE), N[11-(dipyrrometheneboron difluoride)undecanoyl]derythro-sphingosylphosphorylchoine, and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DOPE–PEG2000), were purchased by Avanti Polar Lipids and stored at −20 °C.

B. Substrates and colloids

Borosite glass coverslips 1 mm thick were purchased by VWR International. Silica spheres were purchased from Microparticles GmbH ((2.06 ± 0.05) μm and (7.00 ± 0.29) μm in diameter) and Fluka ((3.00 ± 0.25) μm in diameter). Polystyrene-3-(triethoxysilyl)propyl methacrylate (PS-TPM) isotropic dumbbell particles with long axis of (5.23 ± 0.05) μm and ratio of the diameters of the two lobes of (0.98 ± 0.04) and asymmetric dumbbell-shaped particles with long axis of (4.01 ± 0.04) μm and ratio of the diameters of the two lobes of (0.57 ± 0.02) were synthesized by making a protrusion from swollen PS-TPM particles30 and coated with silica.31 Hematite cubic particles were made following,32 coated with silica33 and treated with HCl to remove the hematite core to obtain cubic shells with a resulting corner-to-corner distance of 1.76 μm and m-value of 3.3 ± 0.6. Hepes buffer was made with 115 mM NaCl, 1.2 mM CaCl₂, 1.2 mM MgCl₂, 2.4 mM K₂HPO₄ and 20 mM Hepes, all purchased from Sigma Aldrich. All chemicals were used as delivered.

C. Sample preparation

1. Substrates. Particular attention was given to the cleaning of the substrates since it strongly influences the formation of the bilayer. Glass coverslips were cleaned while magnetic stirring in a solution of 0.2% Hellmanex, ethanol and milliQ, and washed three times in milliQ after each step. The coverslips were kept in milliQ for a maximum of three days, in order to keep them hydrophilic.
All particles were washed three times in milliQ before being used in lipid coating experiments. To attach the colloids to a substrate, a dispersion of particles was then dried on the glass coverslip. Since the particles experience strong capillary forces during the drying process, they often form clusters. To suppress aggregation, we used a small quantity of particles, namely $\approx 50 \mu$L of 5 g L$^{-1}$ colloid dispersion for each coverslip. For the experiments with cubic particles, we heated the sample to 450 °C to burn the polymers used for the stabilisation of the cubic colloids from the surface, as shown in ref. 34. In some experiments with symmetric and asymmetric dumbbells and spheres, we also burnt the particles at 450 °C to remove the inner polymer network of polystyrene and TPM. We observed occasional breaking of the silica shells of calcinated silica-coated PS-TPM spheres and dumbbell-shaped colloids.

2. Supported lipid bilayers. A mixture of 500 µg of POPC, BSM and Chol in different mole ratios with 0.2% M/M DOPE Lissamine Rhodamine, 0.2% M/M Topfluor Sphingomyelin and 5% M/M DOPE–PEG in chloroform was prepared and the chloroform was evaporated in a vacuum chamber for 2 hours. The lipids were dispersed in HEPES buffer in 250 g L$^{-1}$ concentration, and let to self-assemble into multilamellar vesicles during 30 minutes of vortexing. The dispersion was heated in an oven to 70 °C, and then extruded 21 times with a mini-extruder (Avanti Polar Lipids) placed on a heating plate set at 70 °C, equipped with two 250 µL gas-tight syringes (Hamilton), four drain discs, and one nucleopore track-etch membrane with pore size of 50 nm (Whatman). The resulting SUVs are expected to have a diameter of 114 ± 1 nm. Then, 50 µL of SUVs were added to a holder with the colloidal particles on the substrate and 1 mL of HEPES buffer and put in the oven at 70 °C for one hour. After that, the sample was rinsed three times with HEPES buffer, in order to remove excess SUVs in dispersion. During each step described above, the SUVs were covered by aluminium foil to prevent bleaching of the dye and oxidation of unsaturated lipids.

D. Sample characterisation

The samples were imaged at room temperature with an inverted confocal microscope (Nikon Eclipse Ti-E) equipped with a Nikon A1R confocal scan head with Galvano and resonant scanning mirrors. A 100× oil immersion objective (NA = 1.4) was used for 488 nm and 561 nm lasers. These lasers were used to excite Top Fluor and Lissamine Rhodamine dyes, respectively. Lasers were passed through a quarter wave plate to avoid polarisation of the dyes and the emitted light was separated using 500–550 nm and 565–625 nm filters. The mobility of the bilayer on the colloids, on the substrates and in the contact region was checked by fluorescence recovery after photobleaching (FRAP) experiments (see Fig. S2 in the ESI†). 3D image stacks were acquired by scanning the sample in the $z$-direction with an MCL Nano-drive stage and reconstructed with Nikon AR software.

III. Results and discussion

In previous work we showed how the organisation of LO and LD domains in SLVs is determined the interplay between their rigidity upon bending – which in turn results from the chemical composition of the LO and LD phases – and the curvature of the underlying substrate. To study how these equilibrium configurations are affected by lipid exchange, we present here an experimental set-up in which the lipid composition is not conserved in the neighborhood of the colloid. To do so, we fabricate substrates patterned with colloidal particles and then coat them with a multicomponent lipid bilayer (see Fig. 2a). In this way, there is a continuous exchange of lipids between the portion of the membrane adhering to the colloid and the membrane lying on the flat substrate, which thus acts as a reservoir. We stress that, while there is lipid exchange between the portion of the membrane coating the colloid and the one lying on the substrate, the total lipid composition of the whole...
SLB is fixed and, as we will detail in the next section, its location in the Gibbs phase triangle determines the chances of phase separation occurring on the colloids.

A. The ternary phase diagram of a SLB patterned with colloids

We studied the phase behavior of different SLBs by varying the concentration of POPC, BSM, and cholesterol on various substrates patterned with colloidal spheres, dumbbells, and asymmetric dumbbells (referred to as “snowman particles” in the following). A homogeneous and mobile lipid coating is achieved by deposition of small unilamellar vesicles (SUVs) at 70 °C. The PEGylated phospholipid DOPE–PEG2000 was included in the mixture to increase the water layer between the surface of the particle and the colloids, hence improving the mobility of the bilayer on the rough silica surface. Upon lowering temperature to room temperature, the bilayer separates into LD and LO phases. From Förster resonance energy transfer (FRET) and small-angle neutron scattering (SANS) data on data on multimellar vesicles consisting of SM/PC/Chol mixtures we expect that the transition temperature is between 35 °C and 45 °C. We perform all our experiments at room temperature which is below the expected transition temperature. The fluorescent lipids DOPE–rhodamine and TopFluor cholesterol were used to image the LD and the LO phases, respectively. In all images, these phases are shown in magenta (LD phase) and green (LO phase).

We note that preference of the LD phase for locally spherical regions has also been observed by Subramaniam et al., where SLBs on patterned half-spherical PDMS caps were employed. In our case, however, we did not observe LD domains forming on the flat substrate for any composition, for waiting times of up to five days (see Fig. S3 in the ESI†). Furthermore, we also did not observe gel domains which are expected for binary mixtures of SM/Chol. This could be ascribed to the following two possible mechanisms. First, as it was shown by Goodchild et al., nanoscopic gel domains cannot fuse on a glass surface because of the roughness of the glass thereby hindering detection by confocal microscopy. Second, the mixtures lying on the SM/Chol axis are not “pure” in our experiment because of the inclusion of DOPE–PEG and rhodamine–PE necessary for the coating and identification of the LD phase, respectively. This may result in preference for the mixed configuration, even in the absence of POPC. The use of MICA substrates in place of glass can alleviate this effect and allow for the formation of LD domains of circular shape, see also ESI,† Fig. S3. While the substrate might hinder domain fusion, we have never observed hindered diffusion on the colloidal scaffolds, which instead enhance macroscopic phase-separation. Furthermore, non-zero mean curvature can promote phase separation, as the stability landscape of the mixture is shape-dependent. We further note that although we expected LD geometric pinning in the contact region between colloids and substrate, we have rarely observed localization of domains at such edges (see ESI,† Fig. S3). We hypothesize that this is due to partial detachment of the membrane from the colloid, allowing for a local decrease (in modulus) of the membrane curvature.

Next, we studied how the size of the colloidal spheres affects the performance of geometric pinning. To this end, we prepared a patterned substrate consisting of multiple spheres of different radii, namely (2.06 ± 0.05) μm, (3.00 ± 0.25) μm and (7.00 ± 0.29) μm. Interestingly, the sphere size did influence the partitioning of the lipids: Fig. 3b shows that only the smaller spheres are covered by LD domains, while the larger ones tend to have a composition similar to the surrounding substrate. Moreover, a few of the larger spheres have an irregular, bumpy, surface, and we observed POPC-rich domains on these dimples (indicated by yellow arrows in Fig. 3b). This is a further manifestation of geometric pinning, in which the lipids going into the LD phase preferentially localize to regions of small radii of curvature.

To investigate the effect of high mean curvature and negative Gaussian curvature on the Gibbs triangle, we have repeated the same analysis for substrates patterned with dumbbell-shaped particles. The dumbbells were obtained by inducing protrusions on PS–TPM spheres, which were subsequently coated with silica (as in Fig. 2b) for SLB formation. The colloids were calculated at 450 °C, such that they consist of a single shell of silica. In some cases, this shell can break and indent the surface. We determined the ternary phase diagram for substrates patterned both with symmetric and asymmetric dumbbell particles and plot our results in a Gibbs phase triangle in Fig. 3c. Similarly to the case of substrates patterned with spherical colloids, we first...
distinguish between mixed and demixed configurations. The former state is indicated by red squares in the diagram. However, different with respect to spheres (Fig. 3a), we can further differentiate phase-separated states depending on whether LD domains wrap the entire colloid (blue circles) or whether they localize only along the neck region of the dumbbell (yellow triangles). We label these two phase-separated states respectively as type I and II. Thus, spheres exhibit only type I demixed configurations.

Comparing Fig. 3c with Fig. 3a, we observe that the binodal line, separating mixed form demixed configurations, is rather insensitive, within the resolution of our sampling, to the geometry of the colloids. In the phase-separated region of the phase diagram of the dumbbell particles, it appears that cholesterol is the main discriminant between type I and type II states. Since cholesterol has also been previously found to influence domain size distributions,40 we conjecture that Fig. 3c shows a similar effect. Furthermore, we observe that symmetric and asymmetric dumbbells exhibit a very similar behaviour that is dominated by the highly curved neck region, as opposed to what we observed in SLVs.19 Therefore, we conclude that for open membranes, the relative curvature difference between the two dumbbell lobes is not as important as for closed SLVs. Finally, in line with the theoretical predictions,28,29 we have never found any signs of antimixing on any of the membrane shapes in contact with a reservoir, emphasizing the significant difference induced by opening up the membrane.

Dumbbells and spheres were not the only shapes that were considered: in Fig. 4 we show a representative image of a substrate patterned with cuboidal particles. Here, the inhomogeneous curvature of the colloids induces lipid segregation as

![Fig. 3](a) Ternary phase diagram of a lipid bilayer on a substrate patterned with colloidal spheres. Depending on the overall composition, the membrane can be either in the mixed state (red squares) or in the phase separated state (blue circles). In the insets, we show 3D representative reconstructions of the bilayer in the mixed/phase separated states on a spherical substrate. LD and LO phases are represented in magenta and green, respectively. Single channel images of the insets are reported in the ESI,† Fig. S1. (b) Equatorial view of a SLB with spherical colloidal particles of three different sizes. LD domains clearly prefer regions of higher curvature, i.e. spheres of smaller radii and dimples (indicated with yellow arrows). (c) Ternary phase diagram of a lipid bilayer on a substrate patterned with dumbbell-shaped particles. In the insets we show 3D representative reconstructions of the bilayer in the mixed and phase separated state. (d) Equatorial view of a lipid bilayer supported on a plane with both symmetric and asymmetric dumbbell-shaped colloids. The LD phase is preferentially localized on the neck of the dumbbells. In this image the scaffold was first calcinated at 450 °C so that only the colloidal silica shell survives, which in some cases is broken. For these particles, LD domains locate along broken edges, as shown by the light blue arrows. The lipid composition of the bilayer in (b) and (d) is BSM : POPC : SM = 50 : 25 : 25.
observed similar to the case of spheres and dumbbells. However, we did not observe configurations in which the LD phase was consistently pinned to the region of highest curvature, i.e. either the edges and corners or the entire cubic particles. We hypothesise that this is due to the small curvature differences of the surfaces of the cube which hinder a pinning of the softer phase on the whole surface of the colloid. This result is similar to what we previously observed in SLVs in which there was no significant correlation between the position of the LD domains and the curvature of the cubic supports.

B. Quantification of geometric pinning in dumbbell-shaped colloids

The image in Fig. 3d suggests that the size and localisation of the LD domains along neck regions is rather constant. To further investigate this, we measured the area fraction occupied by each domain on a given colloid. We collected data for 200 distinct colloidal particles of each kind taken from a SLB with composition of 50% BSM, 25% POPC and 25% cholesterol, see Fig. 3c. The area fraction was computed from measurements of the normalised fluorescence intensity of the mid-plane profile, Fig. 3c. The area fraction was computed from measurements of composition of 50% BSM, 25% POPC and 25% cholesterol, see distinct colloidal particles of each kind taken from a SLB with by each domain on a given colloid. We collected data for 200 further investigate this, we measured the area fraction occupied of the bilayer for which we have observed the softer phase at the neck. These are BSM:POPC:Chol equal to 40:40:20, 20:50:30, 60:20:20, and 40:30:30. We analysed 50 colloidal symmetric dumbbell-shaped particles and found that the area fractions of the pinned domains are 26 ± 6% 26 ± 4%, 30 ± 9%, 25 ± 7%, respectively. We thus conclude that the global composition of the membrane also does not significantly affect the size of the LD domains.

The data presented above can be used to estimate the difference in the bending moduli of the LO and LD phases. For this purpose, we model the membrane using the Jülicher–Lipowsky free energy for multi-component bilayers, which describes the membrane as a two-dimensional multi-phase stationary fluid, whose free-energy depends upon the local mean curvature $H$ and Gaussian curvature $K$, namely:

$$F = \sum_{i=LD,LO} \int_{S_i} \left( k_i H^2 + \tilde{k}_i K \right) + \sigma \int ds,$$  

where $S_i$ denotes the, possibly disconnected, portion of the membrane occupied by the LO and LD phases and $\Gamma$ their interface. The constants $k_i, \tilde{k}_i$ and $\sigma$ embody, respectively, the stiffness of the lipid phases with respect to bending and Gaussian splay, while $\sigma$ indicates the tension of the LO/LD interface. We neglect contributions to the free energy due to spontaneous curvature by assuming a unilamellar bilayer with two identical leaflets. Our assumption is based on the fact that we use PEGylated lipids to decrease the interaction of the lower interface in an open membrane to be at equilibrium is that

$$\kappa_g = \frac{\Delta k}{\sigma} H^2 + \frac{\Delta \tilde{k}}{\sigma} K,$$  

where $\kappa_g$ is the interface’s geodesic curvature. Applying eqn (2) to the case of an axisymmetric membrane is a rather straightforward task as described in the ESL. The final equation can be
solved numerically for a given profile shape, such as the ones of Fig. 5c. These profiles are constructed by joining two spherical caps via a smooth polygonal neck. Their absolute size is fixed by the experimental system (Fig. 5a) leading to areas of 44.6 \( \mu \text{m}^2 \) (symmetric dumbbell, orange) and 28.5 \( \mu \text{m}^2 \) (asymmetric dumbbell, black) and with minimal neck radius of 650 nm and 580 nm, respectively.

By comparing the area fraction histograms of Fig. 5b with the solutions of eqn (2), it is possible to estimate the two parameters, the absolute size is fixed by the experimental system (Fig. 5a) leading to areas of 44.6 \( \mu \text{m}^2 \) (symmetric dumbbell, orange) and 28.5 \( \mu \text{m}^2 \) (asymmetric dumbbell, black) and with minimal neck radius of 650 nm and 580 nm, respectively.

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C. Forecast of experiments on catenoidal and conical necks

The umbilical nature of spherical caps prevents a separate quantification of the two parameters. Such a measurement could be achieved only for interfaces lying on non-spherical, curved parts of the patterned substrate. Therefore, we here propose shapes and sizes which could provide a more precise and independent estimation of \( \Delta k/\sigma \) and \( \Delta \kappa/\sigma \). One approach would be to have an interface on a portion of a surface where either of the two terms in the right hand side of eqn (2) vanishes, i.e. when either \( H = 0 \) or \( K = 0 \). In the former case, the surface must be minimal, while in the latter it must be developable. Surfaces with a neck that have these properties and retain the axisymmetric structure are uniquely determined: they must be a catenoid or a circular cone, respectively. Since none of these are compact surfaces, we can smoothly close them by gluing them to spherical end caps. The resulting geometry for the patterned feature is either a catenoidal or a conical dumbbell, depicted in Fig. 6a and b.

The geometry of these surfaces allows us to solve eqn (2) analytically under the assumption that the interface is parallel, see the ESI.‡ Both shapes depend on just one free parameter, respectively the catenoid radius and the cone slope. We can
IV. Conclusions

In our previous work, we have shown that closed lipid membranes supported on colloidal particles (so-called scaffolded lipid vesicles, or, SLVs) feature domains whose position and composition are strongly affected by curvature and the overall membrane composition. In spherical SLVs of constant curvature, we observed the coexistence of two domains as result of minimisation of line tension. In dumbbell-shaped SLVs we have previously identified the presence of geometric pinning and antimixing, i.e. a state in which lipids are mixed and yet organized in domains with strikingly different compositions. As we have shown in theoretical analyses, these states are a consequence of the fact that the membrane is at the same time closed and scaffolded.

In this paper, we test this hypothesis experimentally by relieving the constraint of membrane closeness by connecting SLVs to a reservoir of lipids. To this end, we deposited colloidal particles on a flat substrate and covered both particles and substrate with a continuous multicomponent lipid bilayer. In this setup, the lipid membrane region on the colloid is open in the sense that it can continuously exchange lipids with the membrane on the flat substrate. The latter can be considered as a reservoir of lipids for the bilayer on the colloids. Using spherical particles of different radii as well as symmetric and asymmetric dumbbell-shaped particles, we showed that the equilibrium landscape changes dramatically when the membrane is opened up. Instead of the composition dependency which was found for SLVs, we here observed a highly regular localisation of the softer LD phase in regions of higher mean curvature and negative Gaussian curvature for a range of compositions. The lipid exchange enhanced the geometric pinning of the LD phase to small spherical and dumbbell-shaped membrane regions as well as on the neck of the dumbbell-shaped membranes. For spheres, our results are in agreement with previous observations on substrates with half-spherical asperities. However, we here have also reported how this behaviour depends on the total lipid composition of the supported lipid bilayer in Gibbs phase triangles for both spheres and dumbbells.

The regular pinning of the lipid domains on the neck of the dumbbells furthermore allowed us to measure the size of the disordered domains with higher precision than previously. By combining the experimental results with numerical simulations, we were able to obtain a precise estimation of the combination of the elastic material parameters \((\Delta k + \bar{\Delta}k)/\sigma\), which was found to be \(0.47 \pm 0.38\) \(\text{nm}^{-1}\). Interestingly, our result is compatible with the two other measurements available in the literature obtained from free-standing GUVs. This agreement indicates that our measurements are related to true membrane properties rather than being linked to the specific experimental set-up. In particular, the role of the adhesion energy to the scaffold, which might be relevant in our experiments, seems to be negligible.

Finally, we have proposed membrane shapes that would allow a more precise and independent measurement of these material parameters by disentangling mean and Gaussian curvatures, i.e. dumbbell-shaped membranes with either catenoidal or conical necks. We have furthermore determined the size and curvature that are necessary to observe disordered domains and enable the experimental determination of material parameters. These geometries are not just hypothetical, since substrates and hence membranes of designed 3D shape can in principle be obtained by a combination of micropointing and replica-molding. Our work offers a collection of results to guide the design of experiments for the experimental determination of material parameters and the exploration of new membrane phases.
of experimental and theoretical tools as well as new insights into the effect of lipid exchange in multicomponent bilayers.

Conflicts of interest
There are no conflicts of interest to declare.

Acknowledgements
This work was supported by the Netherlands Organisation for Scientific Research (NWO/OCW), as part of the Frontiers of Nanoscience program (MR), the VIDI scheme (LG, PF) and by the European Research Council (ERC) under the EU Horizon 2020 research and innovation program (DJK, grant agreement no. 758383). We thank Vera Meester and Rachel Doherty for help with particle synthesis and electron microscopy imaging.

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