Pathway for Insertion of Amphiphilic Nanoparticles into Defect-Free Lipid Bilayers from Atomistic Molecular Dynamics Simulations: Supplementary Information

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Details of nanoparticle parameterization

Following previous work\(^1,2\), the GROMOS 54a7 united atom force field was used to model the lipids, ions, and nanoparticle (NP) in conjunction with the SPC water model\(^3\). The GROMOS force fields were parameterized to reproduce free enthalpies of solvation and are well-suited to model spontaneous NP insertion, with the latest 54a7 version reparameterized to reproduce lipid structural observables\(^3–5\). Several modifications were made to the standard GROMOS 54a7 force field to model the NP as detailed in previous work\(^1\). Lennard-Jones (LJ) parameters for the sulfonate end groups used standard GROMOS values\(^6\) while the partial charges were adapted from a parameterization of ionic liquids\(^7\). All sulfonate groups bore a net -1 charge under the assumption of complete dissociation given the low pKa of sulfonate. LJ parameters for interactions between gold and alkane beads were taken from the Hautman-Klein model using a parameterization fit to the GROMOS 12-6 LJ form\(^8,9\). Similar treatments of the interactions between gold and hydrocarbons have been used in several previous studies\(^2,10–12\). All other LJ interactions with gold were calculated using the parameters for gold from the Universal Force Field and standard GROMOS combination rules\(^13\). However, in practice only gold-hydrocarbon interactions were relevant due to the steric barrier imposed by the protecting monolayer. Gold atoms were treated as uncharged and no polarization effects were included under the assumption that surface properties are dominated by the protecting monolayer. While a polarizable force field for gold surfaces has recently been developed\(^14\), we believe that including polarization charges would have minimal significance as previous studies have suggested that image charge effects largely cancel out in water\(^15\). Additional discussion justifying the neglect of image charges is included elsewhere\(^8\).

The NP was represented as a hollow, perfectly spherical gold shell with the mass of missing core atoms redistributed to the surface atoms. Constraints were placed between near-neighbor gold atoms to maintain a rigid spherical structure. In this work, all gold core diameters were set to a diameter of 2.0 nm. The sulfur head groups of the grafted monolayer were distributed uniformly across the surface at a grafting density of 4.62 ligands/nm\(^2\) yielding 58 ligands for the 2.0 nm gold core diameter. The sulfur head groups were rigidly grafted to the gold core using constraints to the nearest gold atoms, eliminating any diffusion of ligands on the surface. For both 1:1 MUS:OT and 1:1 MUS:HDT surface compositions described in the main text the two different ligand species were distributed in a “checkerboard”-like morphology as in previous work\(^1,2,16\). Following the Hautman-Klein model, no bond angle or dihedral constraints were placed on the carbon atom bonded to the thiol group grafted to the NP surface allowing ligand flexibility at the gold interface\(^8\).

Method for inducing lipid protrusions

During the “searching” simulations described in the main text, three protrusions were introduced in the top monolayer of the ribbon by pulling lipid tail atoms to a distance of 0.3 nm along the z-axis beyond the plane formed by the phosphorus atoms in the upper monolayer. Pulling was achieved by applying an umbrella potential to the last atom in the sn-1 tail of each lipid with a force constant of 500 kJ/mol/nm\(^2\). The phosphorus atoms of all of the DOPC lipids in the bottom monolayer were restrained to prevent motion in the z-direction to minimize ribbon curvature induced by the applied pulling force. With the pulling potential still enforced to prevent protrusions from relaxing, the NP was allowed to diffuse freely for 40 ns. No potential was applied to the NP during this process as the electrostatic attraction between the charged MUS end groups and the zwiterionic head groups of the DOPC bilayer was sufficient to adsorb the NP to the surface of the bilayer. Three protrusions were induced simultaneously only to increase the likelihood that the NP would stochastically contact a protruding tail; all extracted configurations involved contact with only one protrusion. After NP-protrusion contact was obtained, all restraints on the pulled lipids and the bottom monolayer were removed to allow for unbiased lipid/NP dynamics with the exception of restraining the z-motion of two phosphorus atoms in the bottom monolayer to prevent ribbon rotation.

Importance of free boundary conditions

One of the important aspects of the workflow described in the main text is the use of a bilayer ribbon with free, water-exposed boundaries rather than a more typical bilayer that
spans the x-y plane of the simulation box. In previous work, the same structure was exploited to test interactions with the high-curvature ribbon edge to mimic the approach of NPs to bilayers with large defects. In this work, however, the use of a ribbon was continued due to preliminary findings that a box-spanning bilayer inhibited NP insertion. To test whether a fully-periodic bilayer would permit NP insertion, a 392 lipid box-spanning DOPC bilayer was equilibrated for 100 ns in a square box with semi-isotropic pressure coupling following typical techniques. The same workflow used with the ribbons was attempted - a single protrusion was induced to a distance of 0.3 nm above the phosphate groups, searching simulations were conducted with a 1:1 MUS:OT NP to acquire configurations with limited contact between the NP and protrusion, then unbiased probing simulations were launched, but none ended in insertion. To test an even more extreme deformation, three lipid protrusions were induced adjacent to each other in the bilayer and the NP was even pulled into simultaneous contact with all three using an applied force. The resulting configuration had a much larger value of $H_C$ due to significant NP-protrusion contact as shown in Fig. S1a. Note that the configurations used for probing simulations in the main text all only involved contact between the NP and a single protrusion. Despite the large amount of hydrophobic contact, after 10 ns the protrusions rapidly relaxed and no insertion was observed. Similar behavior was identified in three consecutive simulations. Given the failure of any simulation with a box-spanning bilayer to show insertion despite significant bias, we believe that the free boundary of the ribbon is critical.

As discussed in the Methods section of the main text, free boundaries are necessary because box-spanning bilayers with periodic boundary conditions (PBCs) inhibit the generation of membrane curvature. The imposition of PBCs enforce the constraint that the total area of the top and bottom leaflet remain equivalent and restricts lipids from transferring between the two leaflets without overcoming a large free energy penalty. During NP insertion, the asymmetric incorporation of the NP will displace lipids and try to expand the top leaflet while lipids in the bottom monolayer will prefer to maintain their equilibrium area per lipid. This differential expansion leads to bilayer curvature as has been previously shown during peripheral protein insertion. Importantly, the largest curvature is generated when a protein is shallowly inserted into the bilayer akin to the initial insertion of the NP. In physical systems, such as cell membranes or lipid vesicles, membrane bending is possible and is a mechanism for the relaxation of this perturbation and resulting stress. PBCs, however, do not permit the asymmetric expansion of one bilayer leaflet, thus unphysically inhibiting bending and effectively creating an additional stress that opposes insertion. These considerations explain why we do not observe insertion in conventional box-spanning membranes, despite large starting values of $H_C$, and why box-spanning bilayers are unsuitable for studying NP-bilayer fusion. Previous simulations of membrane curvature induced by proteins have overcome this restriction using lipid systems with free boundaries, such as the lipid ribbons used here or free lipid patches. Alternatively, some simulation protocols use unconventional boundary conditions, explicitly predetermine an asymmetric lipid distribution between the top/bottom leaflets, or use much larger membranes to allow curvature to relax within the periodic box. We chose to use the ribbon system to minimize the computational expense associated with a very large membrane system and avoid making any a priori assumptions regarding the distribution of lipids in the bilayer.

To further demonstrate the need for a free boundary, Fig. S1b shows the length of the ribbon along the x-axis as a function of time during the insertion of a MUS:OT NP (trajectory 1 in Fig. 4 of the main text). The length is determined as the difference between the maximum and minimum x-coordinates of the ribbon during insertion. The plot shows that the ribbon length fluctuations significantly, experiencing deviations on the order of 1-1.5 nm during approximately 10 ns of simulation time. The increase from 0-15 ns occurs as the NP first excludes volume and expands the top leaflet of the bilayer, the decrease from 15-50 ns is due to the significant curvature, and the plateau over the final 50-150 ns is due to the relaxation of curvature during continued NP insertion. The snapshots in Fig. S1 illustrate these different effects. The change in ribbon size also emphasizes the need for a sufficiently large box size; the ribbon length never approaches the x-dimensions of the box, showing that the initial system set up (21.26 nm in the x-direction) was large enough to avoid edge-edge interactions. The free boundary is thus necessary due to the need to accommodate large ribbon length deformations, induced curvature, and permit the transfer of lipids between monolayers to maintain a preferred area per lipid. Moreover, we expect that in general systems with free boundary conditions should be employed to study insertion processes for the same reasons described above; we again note that a recent study of a related NP system also pointed to constraints imposed by the geometry of PBCs.

Details on extended simulations

In the main text, the solvent-accessible surface area (SASA) is shown as a function of time for several trajectories over 150 ns. This parameter was chosen as it correlates with the insertion of the NPs and reflects the underlying hydrophobic driving force. For comparison, the distance between the NP center of mass and the center of mass of lipids within a cylinder passing through the NP center of mass along the z-axis was calculated. The cylinder had a radius of 2.0 nm, but the center of mass of the lipids was only determined for lipids at least
1.0 nm away from the center of the cylinder. This definition ensured that neither curvature effects nor monolayer excluded volume affected the distance measurement. The distance between the NP and the selected lipids was then projected along the z-axis. Fig. S5 shows both the change in the SASA and change in the NP-bilayer distance, ∆z, as a function of time for the full 400 ns trajectories of all six insertion trajectories. The baseline values are calculated as the average of the values for the control simulations, which are also shown in the plot. As expected, both the SASA and distance between NP and bilayer show similar trends, confirming that the SASA decreases while the NP inserts. Furthermore, the values plateau within approximately 200 ns with no significant change over the remainder of the trajectory, and moreover plateau at nearly the same values independent of the starting configuration. The similarity in final SASA value indicates that the change in SASA, and thus the hydrophobic driving force, depends on the NP composition but not the initial NP configuration. The values of both ∆SASA and ∆z are also slightly larger in magnitude for the MUS:HDT NPs than the MUS:OT NPs.

Fig. S3 shows several snapshots from a MUS:HDT insertion trajectory to complement the snapshots in Fig. 4 and Fig. 5 of the main text. Fig. S3a shows snapshots during initial “stalk” formation for the first MUS:HDT trajectory plotted in Fig. 5. As in the MUS:OT trajectory from Fig. 4, initial contact with a lipid protrusion leads to the lipid tail preferentially contacting exposed hydrophobic surface on the NP, triggering hydrophobic ligands to align with the lipid tail and eventually access the hydrophobic core. Fig. S3b shows a longer-timescale insertion with the same general features as the MUS:OT insertion shown in Fig. 5. Over 25 ns, significant curvature is generated that begins to relax as ligands extend farther into the bilayer. As with the MUS:OT NP, the MUS ligands consistently snorkel charges to aqueous solution. The snapshots thus show similar pathways for insertion for both the MUS:OT and MUS:HDT NPs. The snapshots in Fig. S3c and Fig. S3d again emphasize the curvature generated during insertion.

Control simulations without NP-protrusion contact do not lead to insertion

During the control simulations, the NPs diffused along the bilayer surface and insertion was not observed within 400 ns as shown in Fig. S5. However, monitoring the value of $H_C$ did show occasional transient spikes consistent with some hydrophobic contact between the NP and the bilayer even without a protrusion being induced. Fig. S4 shows $H_C$ as a function of time for both control NPs as well as snapshots of configurations with large $H_C$ values. The snapshots show lipid behavior consistent with the configurations induced from the workflow used to generate NP-protrusion contact. In the first snapshot (labeled with a black dot), a lipid tail is in contact with the NP, indicating that a protrusion occurred but did not induce insertion. Given that even with induced protrusions NP-protrusion contact only leads to insertion for a fraction of total simulations (c.f. Fig. 3 in the main text), this observation is consistent with the induced protrusion results. Moreover, the finding that lipid protrusions can occur, albeit rarely, indicates that the biases used to induce protrusions are realistic and mimic actual lipid fluctuations. The snapshot labeled with the red dot resembles an attempt at MUS:OT anchoring, and as a result leads to the rapid reduction of contact consistent with the anchoring results discussed in the main text (Fig. 8).

Finally, the last snapshot shows an attempt at MUS:HDT anchoring with a ligand inserting partially into the hydrophobic core before the system again relaxes although it persists for longer. The finding that occasional spikes in $H_C$ occur during completely unbiased simulations, with configurations resembling those induced by the biased workflow, supports the idea that spontaneous insertion would be observed over sufficiently long periods of time, agreeing with experimental results in vesicle systems. However, we again emphasize that these are clearly rare events as none of the transient increases in $H_C$ led to insertion, agreeing with the relatively low proportion of trajectories observed to insert during the probing simulations and the finding of a large energy barrier for protrusion generation (Fig. 7).

Calculation of lipid tail protrusion PMF

The main text presents analysis of the potential of mean force (PMF) for inducing a protrusion in a bilayer. The PMF measures the free energy change associated with the formation of a protrusion and was calculated from umbrella sampling simulations using the weighted-histogram analysis method. A smaller model bilayer system was first created to calculate the PMF efficiently given the significant computational expense of US simulations. A 64 lipid DOPC system was created from the reference 128 lipid system provided by Poger et al., then additional sodium and chloride ions were added to form a 150 mM solution consistent with the conditions used in the main simulations. The total system contained 64 lipids, 3,937 water molecules, 11 Na$^+$ ions and 11 Cl$^-$ ions. The system was equilibrated for 100 ns at 310 K using a velocity-rescale thermostat with a time constant of 0.1 ps and at 1 bar using a semi-isotropic Parrinello-Rahman barostat with a time constant of 1.0 ps and a compressibility of $4.5 \times 10^{-5}$ bar$^{-1}$. Electrostatic interactions, cutoffs, the timestep, and the use of constraints followed the same parameters as in all other simulations.

The reaction coordinate for umbrella sampling was defined as the distance of the protruding tail atom from the center of mass (COM) of the bilayer projected onto the mem-

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brane normal (i.e. the z-axis). Umbrella sampling windows were spaced by 0.1 nm along the z-axis using a total of 26 windows. For each window, the last united atom bead in the chosen lipid’s sn-1 tail was first pulled for 2 ns by applying a harmonic potential between the bead’s initial position and the desired position with a spring constant of 500 kJ/mol/nm². The spring constant was then increased to 3000 kJ/mol/nm² for another 2 ns of equilibration (with a Berendsen barostat) before 70 ns of umbrella sampling was performed with the same spring constant and a Parrinello-Rahman barostat. All atoms of the lipid being pulled were excluded from the bilayer COM calculation. To increase sampling without additional computational expense, two lipid protrusions were induced simultaneously by pulling on atoms in both monolayers. A similar method has been used previously in the study of lipid flip-flop and lipid desorption. The two lipids were chosen to be spaced apart by at least 3 nm in-plane to minimize interactions between the two perturbed lipids. Finally, the two different atoms were pulled to different positions such that the sum of the distances to which both beads were pulled was always 2.6 nm to minimize the overall bilayer disruption. After the completion of umbrella sampling, the PMF was calculated using the program *g_wham* with 201 bins. The use of the two-lipid umbrella sampling methodology allowed two uncorrelated PMFs to be generated simultaneously so the results are plotted as an average of both PMFs with corresponding error bars.

**Workflow for calculating anchoring / protrusion timescale**

To test whether ligand anchoring can persist long enough for protrusions to occur, we developed a workflow to estimate the timescales for both processes. First, ligand anchoring was induced by pulling the end of a HDT ligand into the bilayer from an initial starting configuration extracted from the control simulations. The ligand was pulled at a rate of 1 nm/ns to the same depth measured for the anchoring ligand in the unbiased simulations. The ligand was pulled until it had similar initial values of $H_C$ as the HDT simulations and could be used as a suitable comparison to calculate the detachment time. Separately, the ten post-equilibration configurations for the MUS:HDT NPs were continued with the bias maintained to retain anchoring and $H_C$ was monitored. These simulations were used to determine the approximate time anchoring had to be maintained before a spontaneous protrusion occurred as judged by a rapid rise in $H_C$ and visual inspection. For both sets of simulations, runs were ended after 50 ns if neither NP detachment nor protrusion behavior was observed. No significant difference in detachment timescale was observed for MUS:HDT NP configurations originated from either the control simulations or extracted directly from the trajectory in Fig. 6. Representative snapshots of the starting anchored configurations and outcomes are shown in Fig. S6.

**References**

Fig. S1 Necessity of free boundary condition for modeling NP-bilayer insertion. a Snapshots illustrating that insertion is not observed in box-spanning bilayers even with significant initial hydrophobic contact and multiple induced protrusions. b Ribbon length fluctuations during spontaneous insertion over 150 ns, leading to changes on the order of 1-1.5 nm. c Snapshots illustrating extreme curvature and length deformations of ribbon which would be inhibited in a bilayer constrained by periodic boundary conditions.

Fig. S2 Configurations extracted from searching simulations where NPs first contact protrusions, used to launch probing simulations. Four configurations for both MUS:OT (a) and MUS:HDT (b) NPs were used with varying, although similar, numbers of hydrophobic contacts as labeled.
Fig. S3 Additional snapshots of 1:1 MUS:HDT NP insertion with images taken from the MUS:HDT 1 trajectory shown in Fig. 6 of main text. 

a Initial lipid/ligand mixing during stalk formation after protrusion contact in analogy to the snapshots in Fig. 4 of the main text. 
b Long timescale insertion snapshots in analogy to Fig. 5 of the main text. 
c Additional zoomed-out snapshots to illustrate extent of induced curvature and relaxation after 400 ns during MUS:OT insertion. 
d Additional zoomed-out snapshots to illustrate extent of induced curvature and relaxation after 400 ns during MUS:HDT insertion.
Fig. S4 Control simulations show sparse hydrophobic contact with bilayer. $H_C$ is shown as a function of time for MUS:OT (a) and MUS:HDT (b) NPs. c Snapshots of high $H_C$ configurations identified during control simulations.
Fig. S5 Measurements of insertion over full 400 ns trajectory. The change in the SASA and change in the NP-bilayer distance are shown as a function of time for the MUS:OT (a) and MUS:HDT (b) NPs. The control simulations are included for reference.

Fig. S6 Representative snapshots from simulations used to assess anchoring/protrusion timescales. a Starting configuration with anchored OT ligand that detaches within 5 ns. b Starting configuration with anchored HDT ligand with protrusion occurrence within 25 ns. c Starting configuration with anchored HDT ligand that remains anchored in excess of 50 ns.