Structural Characterisation of Nanoparticle-Supported Lipid Bilayers

by Grazing Incidence X-ray and Neutron Scattering

Nicolò Paracini1*, Philipp Gutfreund2, Rebecca Welbourn3, Juan Francisco Gonzalez1, Kexin Zhu4, Yansong Miao4, Nageshwar Yepuri5, Tamim A Darwish5, Christopher Garvey6, Sarah Waldie1, Johan Larsson1, Max Wolff6, and Marité Cárdenas1,4*

1 Department for Biomedical Science and Biofilms – Research Center for Biointerfaces, Malmö University, Faculty of Health and Society, 205 06, Malmö, Sweden
2 Institut Laue-Langevin (ILL), 38000 Grenoble, France
3 ISIS Neutron & Muon Source, STFC, Rutherford Appleton Laboratory, Harwell, Oxfordshire, OX11 0QX, UK.
4 School of Biological Sciences, Nanyang Technological University, Singapore.
5 National Deuteration Facility, Australian Nuclear Science and Technology Organization (ANSTO), Australia
6 Heinz Maier-Leibnitz Zentrum (MLZ), Technische Universität München, Lichtenbergstraße 1, 85748 Garching, Germany
7 Department of Physics and Astronomy, Uppsala University, Box 516, 751 20, Uppsala, Sweden

*Email: nicolo.paracini@mau.se, marite.cardenas@mau.se

Abbreviations:

NP: nanoparticles, SLB: supported lipid bilayer, SLD: scattering length density, NR: neutron reflectometry, GISAXS: grazing incidence small angle X-ray scattering, GISANS: grazing incidence small angle neutron scattering; FRAP: fluorescence recovery after photobleaching

Abstract:

The structure of supported lipid bilayers formed on a monolayer of nanoparticles was determined using a combination of grazing incidence X-ray and neutron scattering techniques. Ordered nanoparticle arrays assembled on a silicon crystal using a Langmuir-Schaefer deposition were shown to be suitable and stable substrates for the formation of curved and fluid lipid bilayers that retained lateral mobility, as shown by fluorescence recovery after photobleaching. A comparison between the structure of the curved bilayer assembled around the nanoparticles with the planar lipid membrane formed on the flat underlying silicon oxide surface revealed a ~5 Å thinner bilayer on the curved interface, resolving the effects of curvature on the lipid packing and overall bilayer structure. The combination of neutron scattering techniques, which grant access to sub-nanometre scale structural information at buried interfaces, and nanoparticle-supported lipid bilayers, offers a novel approach to investigate the effects of membrane curvature on lipid bilayers.

Main text:
Supported lipid bilayers (SLBs) are robust and widespread models of biological membranes used in applications ranging from biophysical studies of membrane function (1) to biosensing (2). Planar SLBs typically consist of phospholipid bilayers formed via vesicle fusion (3), solvent-assisted bilayer formation (4) or Langmuir-Blodgett and Langmuir-Schaefer monolayer transfer techniques (5,6) onto flat hydrophilic interfaces of materials like quartz, mica and silicon oxide. There is growing interest towards the development of non-planar SLBs that deviate from canonical flat interfaces and instead display a degree of curvature of the lipid bilayer. These model systems find applications in the study of membrane curvature-mediated phenomena such as lipid and protein segregation and binding of curvature-sensitive proteins (7–13). For this purpose, nano- and micropatterned surfaces represent attractive substrates for the formation of SLBs with well-defined surface topologies imparted by the underlying interfacial nanostructure.

Both top-down and bottom-up approaches have been adopted for the modification of flat interfaces and the formation of surface patterns suitable to form curved SLBs. Top-down methods are primarily based on nanolithography techniques which afford a high degree of control and fine tuning over the resulting surface structure (8,9). The high precision of top-down methods however often comes at the cost of time and resources, which can become limiting factors when dealing with large surfaces and number of substrates to functionalise. Bottom-up approaches, on the other hand, typically rely on self-assembly processes driven by chemical and physical forces and can be exploited to fabricate patterned samples using nanoparticles (NP) to serve as substrates for SLB formation (7,10,14). Amongst bottom-up methods employed to form large arrays of NPs, Langmuir-Blodgett and Langmuir-Schaefer depositions offer an additional level of control on the self-assembly process by enabling the adjustment of the packing density of the Langmuir monolayer at the air/water interface prior to its transfer onto a solid substrate (15–17). Furthermore, Langmuir transfer techniques have the advantage of yielding large uniform monolayers which make well-suited samples for characterization by flux-limited grazing incidence scattering methods such as neutron reflectometry (NR) and grazing incidence neutron small angle scattering (GISANS) (18,19). Due to their unique ability to probe non-invasively buried interfaces and their differential sensitivity towards hydrogen and deuterium, neutrons are amongst the most powerful surface sensitive techniques for the structural characterization of complex biological thin films at solid/liquid interfaces, of which SLBs represent a primary example. Grazing incidence neutron scattering, particularly NR, has found wide application in the structural characterisation of planar SLBs, however the potential of techniques like GISANS, as well as NR, remains largely untapped when it comes to structural studies of model membranes with more complex topologies. In this context NR and GISANS, combined with selective lipid deuteration, can provide a novel approach to study the effect of curvature on the in-plane and out-of-plane structural features of SLBs.

In this article, we exploit a modified Langmuir-Schaefer deposition method to form large arrays of spherical silica NP on silicon oxide surfaces that are used as substrates to form nanoparticle-supported lipid bilayers (NP-SLB). First, we combine X-ray and neutron scattering to probe the structure of the NP arrays in both dry and aqueous environments (i.e. at the solid/air and solid/liquid interface). In-plane and out-of-plane structural properties of the NP-SLB system are resolved by NR and GISANS. Finally, using fluorescence recovery after photobleaching we show that lipids in the NP-SLB retain lateral mobility.
The formation of densely packed arrays of non-porous silica NPs onto a polished silicon substrate was achieved via a modified Langmuir-Schaefer transfer of NPs from the air/water interface onto a submerged substrate (20,21) as depicted in Figure 1A. To maximise the particle density in the monolayer, pressure-area isotherms were recorded to establish the maximum surface pressure applicable to the NP monolayer at the air-water interface before excessive compression resulted in a collapse, indicated by an abrupt change in surface pressure (Figure 1B). A value slightly below the collapse pressure was selected for NP depositions. Atomic force microscopy (AFM) confirmed the formation of a NP layer with high density of particles packed in a hexagonal lattice and interspersed with minor defects (Figure 1C).

![Figure 1 – Langmuir-Schaefer transfer of a NP monolayer onto a solid substrate](image)

**Figure 1** – Langmuir-Schaefer transfer of a NP monolayer onto a solid substrate (A) Schematic representation of the modified Langmuir-Schaefer transfer of the silica NP monolayer onto a submerged silicon crystal. Once the NP monolayer (red area) was compressed to the target surface pressure, measured by the Wilhelmy plate, the aqueous subphase was slowly removed from behind the barrier using a serological pipette tip connected to a pump. (B) Pressure-area isotherm of NP monolayer compressed above (black) and below (red) the collapse point at 15 mN/m and indicated by an abrupt change in the slope (dashed line), the arrow indicates the direction of compression. (C) NP monolayer (nominal diameter 2000 Å) after transfer onto a silicon wafer imaged by AFM. Black areas correspond to gaps between the particles whilst bright spots are particles absorbed on top of the NP monolayer. The scale bar is 2 µm. The inset shows a Fourier transform of the highlighted region in the AFM image and the corresponding hexagonal lattice.

Imaging techniques, such as AFM, only provide local information over small areas. Scattering methods on the other hand, probe the average structure over large areas in both dry and aqueous environments. Following the assembly method illustrated in Figure 1, monolayers of commercial NP with nominal diameters of 50, 100 and 200 nm were characterized by NR and GISAXS at the solid/liquid and air/solid interfaces. (Figure 2). The samples, assembled into custom built solid/liquid cells, were first characterised by NR in an aqueous environment. NR measurements enable the determination of the structure of buried thin films along the axis perpendicular to the substrate, yielding a profile describing the scattering length density (SLD) distribution, which determines the neutron refractive index along the normal to the surface. Reflectivity curves measured in the presence of H2O and D2O were fitted simultaneously to a model of the interface describing a monolayer of spheres (Figure S1).

The SLD profiles obtained from the constrained fits to the reflectivity curves collected in H2O and D2O (see methods and Figure S4 for details on the modelling), yielded a NP monolayer thickness of 2149 ± 12 Å, 1086 ± 8 Å and 604 ± 5 Å for the three samples, in good agreement with the size of the particles, confirming the formation of NP monolayers (Figure 2A). From the contrast provided by the hydrogenous and deuterated water the in-plane packing density of
the particles in the monolayers was extracted, ranging from 66 ± 1 % for the largest particles, 62 ± 5 % for the intermediate size to 40 ± 4 % for the smallest NPs (Table S1). A value of 100% corresponds to a defect-free layer of spheres tightly packed in a hexagonal lattice. The significantly worse packing of the smallest particles is indicative of larger areas occupied by defects in the NP monolayer, which might result from a size-dependent tendency to form inhomogeneous aggregates during the Langmuir-Schaefer procedure.

To complement the information obtained by NR, the samples were characterised by GISAXS which is sensitive to the in-plane arrangement and correlations between the NPs. The three particle sizes gave rise to a strong GISAXS signal displaying several orders of well-defined peaks (Figure 2C). The images were integrated across the $Q_x$ axis and over a $Q_z$ range corresponding to the angle of specular reflection and adjusted to include a single row of peaks in each integration box in order to extrapolate the in-plane correlation distances related to the patterns observed (Figure 2D, integrated regions shown by the yellow boxes in Figure 2C). The linear plot of the $Q_y$ position of the maxima against the order of the peaks indicates that the maxima are equidistant in $Q_y$, and the slope of the straight lines fitted through the data points corresponds to the average $\Delta Q_y$ (Figure 2E). According to the inverse relationship between the $Q_y$ spacing of the maxima and the corresponding real space distances, given by $d=2\pi/\Delta Q_y$, the signals from the different samples yielded correlation distance values of 2124 Å, 1081 Å and 609 Å. The parameters obtained from the model-free analysis of the GISAXS patterns were found to be in close agreement with the monolayer thickness values obtained from the NR fits.

Figure 2 – Structural characterization of NP monolayers by NR and GISAXS (A) Neutron reflectometry data (points) and best fit (lines) of three monolayers containing NP of different sizes, measured in D$_2$O and H$_2$O. Data and corresponding fits are offset vertically for clarity (B) corresponding SLD profile derived from the constrained fit of NR data from the two solution isotropic contrasts for each NP set. (C) GISAXS detector images of three monolayers measured at the air/solid interface (D) Plot of the integrated intensities within the yellow boxes shown in C. Absolute intensity is offset for clarity (E) Linear regressions of the maxima positions along $Q_y$ of the peaks shown in D.
The information on particles density and size obtained from NR were used as input parameters to generate simulated GISAXS signals using the distorted wave Born approximation (DWBA) implemented in the BornAgain software (22). The simulated scattering was in good agreement with the data, providing qualitative information on the average interparticle distances and on the degree of order in the monolayers (Figure S2). To obtain simulations that reproduced the intensity and $Q_y$ spacing of the data, an increasing level of disorder of the in-plane NP monolayer structure had to be assumed as particle sizes decreased, which is consistent with the lower packing densities extracted from the NR model.

The monolayers containing the largest NP displayed the highest level of order as well as the highest particle density and were therefore chosen as the substrate for the study of lipid bilayer deposition. Lipid deposition via vesicle fusion was monitored by quartz crystal microbalance with dissipation (QCMD) which was used to compare the process of bilayer formation on conventional “flat” silicon oxide sensors and on sensors coated with the NP monolayer (Figure 3A). The frequency shift ($\Delta f$) observed after injecting POPC vesicle and rinsing with water was $-24.2 \pm 0.7$ Hz and $-25.5 \pm 0.8$ Hz on the flat and NP-coated sensors respectively, in line with the values reported previously for the formation of lipid bilayers on flat silicon oxide sensors as well as on QCMD sensors coated with NP (7). The shift in dissipation ($\Delta D$) amounted to $+0.4 \pm 0.3$ ppm on the flat and $-5.8 \pm 1.2$ ppm on the NP-coated surfaces (Figure S3). The slightly higher $\Delta f$ value recorded on the NP sample shows a marginally higher amount of lipids adsorbed whilst the negative $\Delta D$ indicates that lipid addition affects the properties of the NP layer, suggesting that intercalation of lipids in between the NP increases the overall stiffness of the NP-SLB layer (23). The surfaces were then rinsed with ethanol to remove the POPC which in both cases caused $\Delta f$ and $\Delta D$ to return to the baseline measured at the beginning of the experiment, indicating complete removal of the lipids by the ethanol wash, with the NP-array remaining unperturbed. A second cycle of lipid deposition and removal was carried out yielding $\Delta f$ and $\Delta D$ values in line with the shifts observed in the first cycle, showing full reusability of the NP-array as a substrate for the formation of NP-SLBs (Figure 3A).

The NP-SLB was characterised by NR to access information on the structure of the lipid bilayer formed on the NP array. To fully exploit the ability of neutrons to differentiate between hydrogenous and deuterated molecules, the process investigated by QCMD was replicated on the neutron beam line using first hydrogenous hPOPC, followed by regeneration of the surface with ethanol and deposition of tail deuterated d$_6$POPC. After each lipid assembly, the sample was characterised both in H$_2$O and in D$_2$O. The reflectivity data sets collected in H$_2$O and D$_2$O on the bare NP array, the hydrogenous NP-SLB and the deuterated NP-SLB were then fitted to a common model of the interface where the structural parameters describing the NP monolayer were shared across the three conditions whilst the SLD of the lipids and their volume fractions were allowed to vary (see Figure 2D, E for the fits and SLD of the NP monolayer before lipids addition). The model that produced the most accurate fit to the reflectivity data from the NP-SLB samples contained a lipid bilayer coating the entire nanoparticle surface as well as part of the flat surface of the silicon substrate supporting the particles (See Figure S4 for a detailed description of the model used). Addition of hPOPC vesicles to the NP array caused a prominent shift of the reflectivity profile measured in D$_2$O whilst leaving the signal recorded in H$_2$O mostly unaffected, as expected from the deposition of hydrogenous material at the interface. Conversely, addition of d$_6$POPC resulted in a large shift in the reflectivity measured in H$_2$O.
but no significant changes in D₂O (Figure S5). Similarly to what was observed with QCMD, rinsing the NP-SLB with ethanol between bilayer depositions reverted the reflectivity back to the initial profile measured before lipid addition, confirming a nearly complete removal of the lipids whilst preserving the intact NP monolayer structure (Figure 3B).

Figure 3 – Formation of NP-SLB and vertical structure measured by NR (A) QCMD trace monitoring the deposition of POPC onto a silica sensor coated with a monolayer of NP. Labels indicate the injection of different solutions in the QCMD flow module. The light grey flat dotted line is a guide to the eye set at zero Hz frequency shift. (B) NR profiles of NP monolayer in D₂O before (black) and after (green) one cycle of lipid deposition and removal, showing recovery of the reflectometry signal upon EtOH rinsing (log-log scale) (C) NR data (points) and best fit (red lines) of the NP-SLB formed with hPOPC in H₂O and D₂O. (D) NR data (points) and best fit (blue lines) of the NP-SLB formed with d₆₄POPC in H₂O and D₂O (E) SLD profiles describing the NP monolayer in the absence of lipids (black dashed lines) in the presence of hPOPC (red lines) and in the presence of d₆₄POPC (blue lines). The expanded region shows a magnification of the flat SiO₂ interface highlighting the formation of a planar SLB on the flat silicon substrate.

The parameters obtained from the fits to the NR data described the formation of a lipid bilayer adhering to the NP, which coated the entire surface of the spheres with a high coverage as indicated by the low volume fraction of water detected in the tail region, below 4% in both the hPOPC and d₆₄POPC hydrophobic cores (Table S2, Figure S6). Additionally, a planar lipid bilayer formed on the silicon crystal surface underlying the NP monolayer, with an overall coverage of ~60%, indicating the formation of a less complete lipid layer in comparison with the bilayer coating the NP. The lower coverage of the underlying planar SLB is likely to result from the partial shadowing by the large NP which prevented the formation of a homogeneous continuous lipid layer (Figure S4A). Along with the differences in coverage, the structural data revealed that the NP-SLB displayed a ~27 Å thick hydrophobic core, ~5 Å thinner than the planar SLB (~32 Å), as well as a ~20% higher water content associated with the lipid head groups. Whilst the parameters obtained for the planar POPC SLB are in excellent agreement with previously reported values (24,25), the thinner structure of the NP-SLB pointed to a suboptimal packing of the lipids around the spheres resulting from the curved substrate. Mean
molecular area values, calculated assuming POPC tails molecular volume of 925 Å³ (24) yield values of ~58 Å² and ~69 Å² for the planar and curved SLB respectively, corroborating the looser packing of POPC in the latter.

The NR curves measured in D₂O showed a strong off-specular signal captured in the 2D detector images as horizontal stripes in the region of total reflection (Figure 5A) which caused the intensity dips visible in the specular reflectivity signal below the critical edge (Figure S7). This is similar to what is observed in the case of resonators, characterised by a potential well, formed of a region of low SLD in between two regions of high SLD (26). In the case of the spherical NP system under investigation here, the increasing volume fraction of silicon oxide (SLD of SiO₂ = 3.47x10⁻⁶ Å⁻²) towards the centre of the NP monolayer generates a region of low SLD in comparison to the D₂O rich regions above and below the monolayers centre (SLD of D₂O = 6.35x10⁻⁶ Å⁻²), which may explain the appearance of the resonances observed.

The addition of hPOPC caused both an increase in the intensity of the off-specular scattering and a shift in the Qᵥ position of the resonances (Figure 4A). Simulations of the off-specular data (27) reproduced qualitatively the signal change observed for the NP monolayer before and after addition of lipids (Figure 4B) confirming the validity of the specular reflectivity model used to fit the data. Moreover, the off-specular scattering simulations supported the GISAXS results indicating better ordering in the larger NP arrays. In order to reproduce the measured off-specular intensities of the 100 nm and 50 nm particles (Figure S8), micrometre sized D₂O clusters had to be included in between the NPs in the calculations, whilst these large D₂O clusters were not required to simulate the signal from the 200 nm particles.

Following up on the GISAXS data collected on the dry NP arrays, the process of NP-SLB formation was investigated by GISANS, which enables measurements of grazing incidence small angle scattering on buried (e.g. wet) biological thin films. The analysis of the integrated peak positions yielded a correlation distance of 2083 Å prior to the addition of lipids, in good agreement with the values obtained from the GISAXS and NR results (Figure 2). A comparison of the GISANS signals before and after addition of hPOPC to the NP array in D₂O revealed an increase in the intensity of the overall scattering in the 2D detector image, in line with the increased contrast in the sample caused by the addition of hydrogenous lipids in D₂O (Figure 4C, D). Notably, along with the change in intensity, the adsorbed lipids caused a shift of the maxima positions in Qᵥ, with a reduction of the average inter-peak separation, resulting in an increase in the correlation distance from 2083 Å to 2167 Å, corresponding to an overall increase of 84 Å in the particle diameter, as calculated form the linear regression of the peaks positions (Figure S9).
Figure 4 – NP-SLB formation monitored by GISANS and off-specular NR (A) Off-specular NR data in D$_2$O before (left) and after (right) the addition of hPOPC to the NP-array (dashed lines indicate the position of the critical edge) (B) Simulations of off-specular NR in the absence (left) and presence (right) of lipids coating the NP, $\theta_i$ and $\theta_f$ are the incident and reflected angles respectively (C) GISANS detector images of NP monolayer in D$_2$O before (left) and after (right) the addition of hPOPC (D) Integration across $Q_y$ of the GISANS images corresponding to the coloured boxes in C before (black) and after (red) addition of lipids (E) Magnified region of the peaks marked in D

According to the structural information obtained from the NR data the total bilayer thickness formed around the NP amounts to $\sim$39 Å, thus the expected increase in the apparent NP diameter upon bilayer formation would be $\sim$78 Å, in good agreement with the 84 Å obtained from the model-free GISANS analysis which corroborated the NR results.

Finally, diffusivity of the POPC molecules within the NP-SLB was investigated using FRAP. When compared to SLB formed onto flat glass surfaces, the measured lipid diffusion after bleaching was slower on the bilayers assembled on the NP coated substrate (Figure 5A). The final recovery was nonetheless comparable between the two substrates after 120s from the bleaching, indicating that lipid mobility in the NP-SLB, although more restricted, was still largely retained both in the planar bilayer and around the NP (Figure 5B). Further experiments are required to understand to what extent diffusion takes place directly between neighbouring NP compared to diffusion mediated by the planar underlying SLB. Imaging of the NP-SLB by super resolution (SR) and total internal reflection fluorescence (TIRF) microscopy provided a clear picture of the fluorescent bilayer and the NP array. SR and TIRF images were acquired on NP-SLB formed on particles with a larger nominal diameter (400 nm) that provide the optimal resolutions for the SLBs at different Z-positions, including the NP-SLB and the planar SLB on the underlying glass surface (Figure 5C).
The properties of NP-SLBs have been investigated both in bulk (28–32) and at interfaces (7,10,14,33) with a wide range of biophysical techniques. Here, for the first time, we provide a close up on the molecular structure of the lipid bilayer assembled on nanoparticles using a combination of surface sensitive techniques. Together the data demonstrate the possibility of using large NP arrays assembled via an accessible bottom-up method that does not involve complex nanofabrication, as substrates for the formation of high-coverage curved lipid bilayers. The combination of scattering and imaging methods provides access to accurate structural information on the vertical and in-plane structure of the NP-SLB. The NP-SLB platform described here provides a new tool that can be employed in the study of curvature dependent phenomena using both grazing incidence scattering and imaging techniques.
Acknowledgements

MC and NP thank the Swedish Research Council for financial support. NP acknowledges support from Nordforsk - Nordic Neutron Science Program (Grant 106881). MC thanks Wennergren foundation for financial support. The authors thank the ILL for beamtime (D22: doi:10.5291/ILL-DATA.8-02-912, FIGARO: doi:10.5291/ILL-DATA.8-02-889). The authors thank Prof. Jaume Torres for access to a Langmuir Trough at Nanyang Technological University. This study was supported by Singapore MOE Tier 3 (MOE2019-T3-1-012) to Y.M. The National Deuteration Facility in Australia is partly funded by The National Collaborative Research Infrastructure Strategy (NCRIS), an Australian Government initiative.


33. Brozell AM, Muha MA, Sanii B, Parikh AN. A Class of Supported Membranes: