Experimental Report

Proposal: **9-13-485** **Council:** 10/2012

Title: Understanding the interfacial structure in lipid/dendrimer mixtures

This proposal is continuation of: 9-13-448

Researh Area: Other...

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Samples: PAMAM Dendrimers
POPC & POPG Lipids

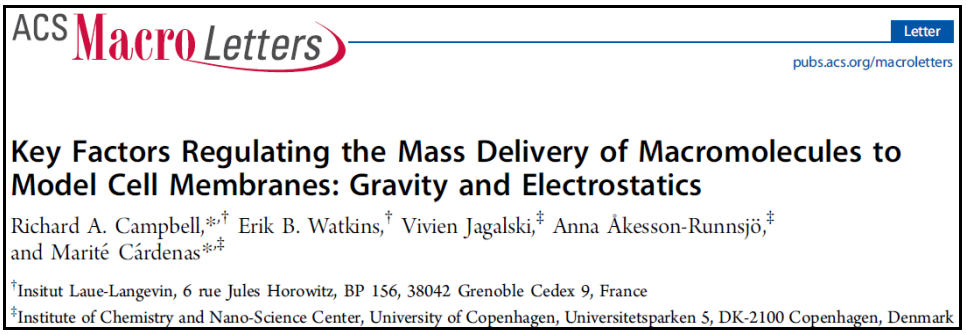
Instrument	Req. Days	All. Days	From	To
FIGARO User-supplied	2	2	04/03/2013	06/03/2013

Abstract:

Supported Lipid Bilayers (SLBs) are important substrates for the study of drug/polymer/nanoparticle interactions and they are commonly used in neutron reflection and other surface sensitive techniques. We have widely investigated the interaction of PAMAM dendrimers with lipid vesicles and SLBs using neutron reflection and found contradictory results depending on the protocol for vesicle fusion (pH, ionic strength, hydrogenated vs deuterated lipids in H2O or D2O) or the orientation of the interface (vertical vs horizontal up and down geometries). We have carried out an initial experiment on FIGARO which demonstrated that phase separation and gravity massively influence the interfacial properties. Despite this important finding, three issues concerning (1) the role of bound vesicles, (2) the underlying mechanism of multilayer formation and (3) the presence of a lamellar structure only on silica and not the lipid bilayers remain unanswered. We request 2 days of beam time to resolve these issues and write up the work for publication. This clarification is of outmost importance if reliable data on complex mixed systems can be reliably extracted from neutron reflection experiments.

Final Report for Figaro Experiment #9-13-485

Publication. As a result of this FIGARO experiment we published the work in *ACS Macro Letters*:[1]



Scientific background. An immense effort has been invested to understand the mechanisms of interactions between macromolecules and cell membranes.[2] Positively charged macromolecules can be recruited into the cell by the endocytosis pathway,[3] and then trafficked by different organelles according to their surface charge.[4] A key challenge is to develop drug delivery systems involving the efficient transport of therapeutic agents to lipid membranes.[5] It can be advantageous to position reservoirs of the drug in contact with the membrane for continuous delivery by slow diffusion.[6] In this case the drug may be encapsulated into aggregates of liquid crystalline phase.[7]

The present work concerns interactions with model membranes of lamellar aggregates comprising phospholipids and macromolecules with strong biomedical potential. The aim of the work was to highlight key factors that regulate the efficient delivery to supported lipid bilayers (SLBs) of large reservoirs of macromolecules. Factors under consideration are (1) the location of the interface with respect to the gravitational field and (2) the electrostatic interactions between the aggregates and the SLB. The system chosen for study is lipid vesicles comprising a mixture of 90 mol% POPC (zwitterionic) and 10 mol% POPG (anionic) combined with PAMAM dendrimers of generation 6 (cationic). A lipid-to-dendrimer molar ratio of 130 was used and the resulting aggregates have slight positively charge. The experimental technique used was neutron reflectometry, which is sensitive to the adsorbed layer structure (isotopic substitution), presence of interfacial multilayers (Bragg diffraction peaks), and in-plane surface arrangements (off-specular scattering). We used an approach involving the reflection up vs. down modes of FIGARO with surfaces located above and below the liquid. The reason for this approach is that different properties were previously revealed on surfaces located above and below a synthetic mixture due to the transport under gravity of bulk aggregates,[8] and we sought to probe the significance of such effects in the present system.

Experimental results. A fresh POPC/POPG/PAMAM sample is unstable with time: spatial separation of a condensed phase above a dilute phase occurs due to gravity (fig. 1A). The dilute phase interacts with the lower silica surface. After 2 h the interfacial structure was approximately 90 Å thick, but the dendrimers and the lipids could not be discerned into defined layers, probably due to the coexistence of domains of dendrimer-below-bilayer and bilayer-below-dendrimer. With time, dendrimer molecules bound to the outer leaflet of the SLB slowly translocated across the membrane. After 30 h, a dendrimer layer (thickness 54 Å; coverage 29 %) was in direct contact with the silica and a lipid bilayer (thickness 35 Å; coverage 65 %) was floating on top (figs 1B/1C). The lack of Bragg peaks in the specular reflectivity data (fig. 1C) and the absence of off-specular scattering, otherwise exhibited as horizontal lines in a $Q_z(Q_x)$ projection of the neutron data (fig. 1D), suggests that (1) lamellar aggregates did not adsorb to the surface, and (2) the surface itself did not template a multilayer assembly. A schematic of the interfacial structure is shown (fig. 1E).

The interaction of the condensed phase with the upper surface is dramatically different. Four Bragg peaks in the specular data reveal that a lamellar stack of lipid/dendrimer repeating layers is in contact with the surface (figs 1F/1G). Further, a close inspection of the data at low vertical

momentum transfer values (Q_z) shows that there are two contributions to the total reflection resulting in a double critical edge (fig. 1H). Fitting the two critical edges in the data required a model involving the incoherent addition of contributions to the reflectivity from two macroscopic domains of different surface structures. The length scale for the domains is above the neutron coherence length of the measurement (~ 10 micrometers). After 30 h, the structure of the first domain, with 55 % area coverage, is similar to that on the lower surface with a dendrimer layer (thickness 55 Å; coverage 40 %) and a floating lipid bilayer (thickness 35 Å; coverage 80 %). The second domain, with 45 % area coverage, involves macroscopic stacks of dendrimers (thickness 84 Å; coverage 35 %) with lipid bilayers (thickness 35 Å; coverage 90 %). This latter structure matches the lamellar spacing for the bulk liquid crystalline aggregates measured by small-angle X-ray scattering.[9] A schematic of the interfacial structure at the upper surface is shown (fig. 1J).

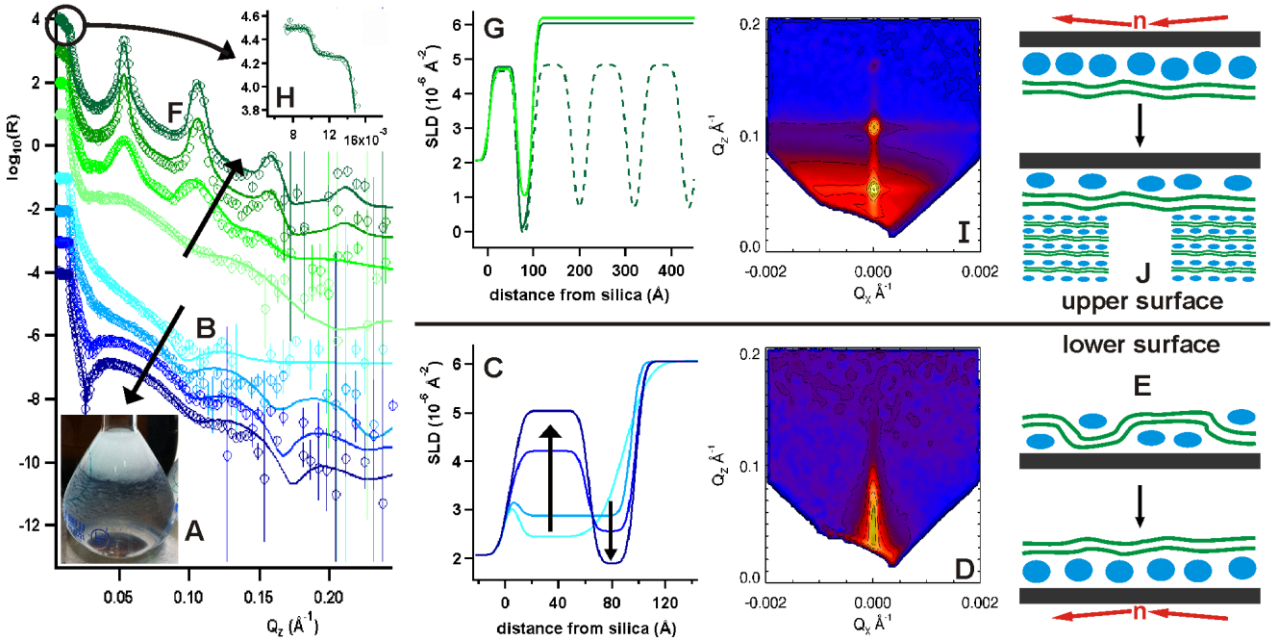


Figure 1. (A) POPC/POPG/PAMAM sample after 9 h showing phase separation; NR data and fits of hydrophilic silica surfaces (B) below and (F) above the POPC/POPG/PAMAM sample with respect to the sample age (2, 5, 16 and 30 h with increasing darkness), where data sets and fits are offset in reflectivity for clarity, and their respective scattering length density profiles for silica surfaces (C) below the sample after 2, 5, 16 and 30 h and (G) above the sample after 2 and 30 h where the full and dashed dark lines are contributions to the reflectivity from the two domains described in the text; (H) magnification of the data and fit involving an incoherent addition model for the double critical edge of the 30-h data in F; off-specular scattering plots of $Q_z(Q_x)$ with a logarithmic intensity scale for surfaces (D) below and (I) above the sample after 30 h; schematic indicating the change in interfacial structure from 2 to 30 h for surfaces (E) below and (J) above the sample where black slabs are silicon crystals, blue ovals are dendrimers, green double lines are bilayers, the edge of each crystal facing the adsorbed molecules marks 'zero distance' in C and G, and red arrows depict the reflection of neutrons. The gap between the two bound lamellar aggregates in J is solvent. Repeating structure in G continues for 100 repeating units. Straight black arrows mark the passing of time.

We turn our attention now to the interactions of the bulk lipid/dendrimer aggregates with pre-formed SLBs made from the fusion of vesicles comprising 10, 17.5 and 25 mol% POPG in POPC (fig. 2). As the bulk aggregates had been shown to float, these measurements were carried out only on SLBs located above the bulk liquid. The pre-formed SLBs all had excellent coverage of > 95 %.

Bragg peaks are present in the specular data for SLBs made from 25 and 17.5 mol% charged lipids (figs 2A and 2B, respectively) and the data are consistent with the coexistence of two different surface structures on the micrometer scale. The structure involved domains of 98 % area coverage with a translocated dendrimer layer (thickness 45 Å; coverage 70 %) and a floating lipid bilayer (thickness 35 Å; coverage 90 %), and domains of 2 % area coverage with adsorbed lamellar aggregates (structure as above). The final interfacial structure determined is shown schematically in fig. 2D. In contrast, for the SLB made from 10 mol% POPG (fig. 2C), the lamellar stacks are practically absent, and only a small first-order Bragg peak at $Q_z = 0.055$ Å^{-1} is observed after 30 h. The surface structure fits reasonably well to a translocated dendrimer layer with a floating lipid

bilayer (filled line), but the fit improved slightly by including a sparse additional dendrimer layer on top (dashed line). Even after 30 h the surface excess of dendrimer in contact with the silica is only about half that for the higher charged SLBs. The translocation process is clearly suppressed by the low content of negatively charged lipid. The layer structure is shown schematically in fig. 2E.

In our published Letter we discussed the implications of our findings in terms of drug delivery investigations and applications.[1] First, while there is routine research in the investigation of potential new drugs to interact with cell membranes, and formulations often involve complex mixtures, effects of gravity on membrane interactions are probably neglected in the vast majority of cases. The present study, where two interfaces of the same sample exhibit different behavior, emphasizes the importance of understanding such effects, especially when the bulk phase separation may be less apparent with small sample volumes. Second, as negative surface charge on membranes has been shown to direct positively charged macromolecules into the endocytosis pathway,[3] there is scope to trigger this mechanism in drug delivery systems via attachment of lamellar aggregates to the cell. Given the different charge densities of healthy and cancerous cells in the body,[10] we touched on the potential for tuning the electrostatic nature of therapeutic agents to gain selectivity in targeted deliveries to specific cell types.

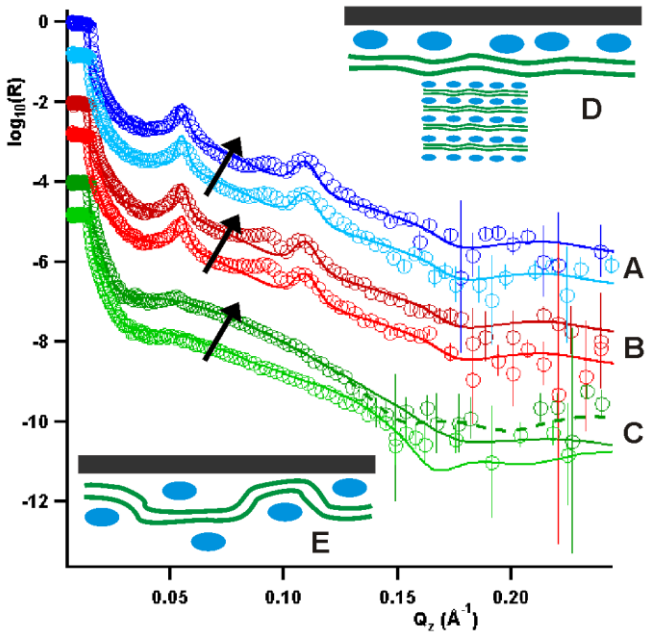


Figure 2. Specular NR data and fits of the interaction of POPC/POPG/PAMAM mixtures with pre-formed SLBs from vesicles with (A) 25, (B) 17.5 and (C) 10 mol% POPG in POPC after (light color) 12 h and (dark color) 30 h. Data sets and fits are offset in reflectivity for clarity. The small Bragg peak at 0.090 $Å^{-1}$ result from residual multilamellar vesicles in the sample used for experiments A and B. For the fit to (C) the filled line is a 2-layer dendrimer/lipid model and the dashed line is a 3-layer dendrimer/lipid/dendrimer model. Schematics of the layer structure for the (D) 17.5/25 mol% experiments and (E) 10 mol% experiment are shown.

Future work. This work has raised some important questions we seek to answer in 2014. Is the slow translocation and aggregate attachment with lower charged SLBs a result of kinetic or thermodynamic barriers? Can we also trigger the aggregate attachment mechanism by adjusting the charge of the aggregates? Is the translocation process facilitated initially by negatively charged lipid in the outer leaflet followed by aggregate attachment due to its movement into the inner leaflet thus extinguishing the exposed surface charge? Can we also induce surface self-assembly of multilayers through the interaction of dendrimers with surface-bound vesicles on the SLB? These questions will prompt an additional beam time application for the January 2014 deadline with the intention of following up this work with one further scientific paper as well as one methodological paper.

References. [1] Campbell, R. A.; Watkins, E. B.; Jagalski, V.; Åkesson-Runnsjö, A.; Cárdenas, M. *ACS Macro Lett.* **2014**, *3*, asap; [2] Leroueil, P. R.; Hong, S.; Mecke, A.; Baker, J. R.; Orr, B. G.; Banaszak Holl, M. M. *Accounts Chem. Res.* **2007**, *40*, 335; [3] McLaughlin, S.; Murray, D. *Nature*, **2005**, *438*, 605; [4] Yeung, T.; Gilbert, G. E.; Shi, J.; Silvius, J.; Kapus, A.; Grinstein, S. *Science* **2008**, *319*, 210; [5] Faraji, A. H.; Wipf, P. *Bioorg. Med. Chem.* **2009**, *17*, 2950; [6] Esposito, E.; Cortesi, R.; Drechsler, M.; Paccamiccio, L.; Mariani, P.; Contado, C.; Stellin, E.; Menegatti, E.; Bonina, F.; Puglia, C. *Pharm. Res.* **2005**, *22*, 2163; [7] Bitan-Cherbakovski, L.; Libster, D.; Aserin, A.; Garti, N. *J. Phys. Chem. B* **2011**, *115*, 11984; [8] Campbell, R. A.; Yanez Arteta, M.; Angus-Smyth, A.; Nylander, T.; Varga, I. *J. Phys. Chem. B* **2012**, *116*, 7981; [9] Åkesson, A.; Bendtsen, K. M.; Beherens, M. A.; Pedersen, J. S.; Alfredsson, V.; Cárdenas Gómez, M. *Phys. Chem. Chem. Phys.* **2010**, *12*, 12267; [10] Szachowicz-Petelska, B.; Dobrzynska, I.; Skrodzka, M.; Darewicz, B.; Figaszewski, Z. A.; Kudelski, J. *J. Membrane Biol.* **2013**, *246*, 421.