Experimental report

16/02/2016

Proposal: 9-13-613 **Council:** 4/2015

Title: Lipid Exchange and Nanodisc Formation Experiments Using Stopped Flow

Research area: Soft condensed matter

This proposal is a new proposal

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Samples: DMPC, poly(styrene-alt-maleic acid) nanodiscs
DMPC/DMPG mixture, poly(styrene-alt-maleic acid) nanodiscs
DMPC, poly(styrene-alt-maleimide) nanodiscs
DMPC vesicles + poly(styrene-alt-maleic acid) solutions
phosphate buffer
acetate buffer

Instrument	Requested days	Allocated days	From	To
D33	2	2	27/10/2015	29/10/2015

Abstract:

Polymer stabilized lipid nanodiscs offer enormous potential as tools for enabling membrane protein structural studies & biophysics. Polymer stabilized lipid bilayer discs are easily made by adding polymer to a suspension of lipids or disrupted cell membranes, with discs forming within ~5 minutes. It is not clear how the polymer forms discs from these systems, since in vesicles or cell membranes the headgroups are exposed to solution, and the soluble polymer, but in the final discs, the polymer is wrapped around the tail regions of the lipid bilayer. We have also observed that nanodiscs are dynamic, and undergo exchange of lipids between discs or between discs and lipid bilayers on ~10 min timescales. Here we propose to use stopped-flow combined with SANS to study nanodisc formation from a solution of vesicles mixed with polymer, and also to probe the kinetics of lipid exchange between nanodiscs.

Introduction and background

Polymer stabilized lipid nanodiscs[1] offer enormous potential as tools for enabling membrane protein structural studies & biophysics.[2] Polymer stabilized lipid bilayer discs are easily made by adding polymer to a suspension of lipids or disrupted cell membranes, with discs forming within ~5 minutes.[3] It is not clear how the polymer forms discs from these systems, since in vesicles or cell membranes the headgroups are exposed to solution, and the soluble polymer, but in the final discs, the polymer is wrapped around the tail regions of the lipid bilayer. We have also observed that nanodiscs are dynamic, and undergo exchange of lipids between discs or between discs and lipid bilayers on ~10 min timescales (using hand-mixing, ILL experiment 9-13-468). Here we propose to use stopped-flow combined with SANS to study nanodisc formation from a solution of vesicles mixed with polymer, and also to probe the kinetics of lipid exchange between nanodiscs.

Experiments

The experiment was carried out on D33 and the beamline was used in two different modes: Stopped flow and sample changer. The stopped flow apparatus was used to investigate two different experiments:

- lipid exchange between nanodiscs and
- kinetic experiments on nanodisc formation looking at the modification of vesicles as polymer was added to generate nanodiscs.

For this experiment hydrogenated lipids, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC, purity ≥ 99%)and monoolein (purity ≥ 99%) were purchased from Sigma Aldrich. Deuterated DMPC was purchased from Avanti Polar Lipids (purity ≥ 99%). The commercial poly(styrene-alt-maleic acid) and poly(styrene-alt-maleimide) co-polymers used for the experiment were kindly provided from Cray Valley. Three different polymers were investigated, SMA2000P (Mw~6kDa, 2:1 styrene:maleic acid molar ratio), SMI1000 (Mw~6kDa, 1:1 styrene:maleimide) and SMI2000 (Mw~6kDa 2:1 styrene:maleimide).

Solutions of phospholipid vesicles were prepared in appropriate buffer solutions in D_2O (acetate buffer (ABS) pH5 and phosphate buffer (PBS) pH=8 for SMI and SMA polymer respectively, containing 0.2M NaCl) in order to give 10mg/mL concentration. This solution was sonicated for 30 min in an ice bath and then filtered using a 100μm filter to prepare uniform vesicles.

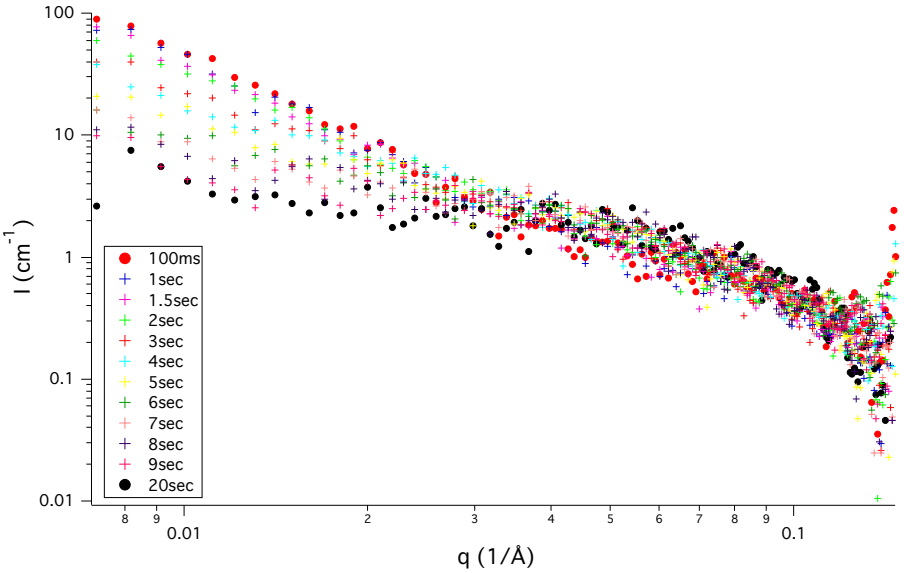

Figure 1: SANS patterns taken on D33, for a stopped flow experiment mixing SMI2000 (1.5wt%) and hDMPC (5mg/ml) in deuterated acetate buffer 50mM, 0.2M NaCl.

To study nanodisc formation using the stopped flow apparatus, the syringes in the stopped flow set up were charged with 10mg/mL of phospholipid in the same buffer (ABS or PBS) used to dissolve the polymer (polymer concentration 3mg/mL). The two solutions were mixed in a 1:1ratio with a final volume of 600μL. The final concentration in the neutron cell was the standard concentration used so far to make nanodiscs: 1.5%wt and 5mg/mL of polymer and phospholipid respectively.

Prior to mixing and after each set of kinetic runs, initial and final SANS patterns were collected for each of the separate components (ie polymer and vesicle solutions) and the final structures formed 30mins or so after the initial mixing. In order to get information on the early stages of nanodisc formation, after mixing in the

stopped flow apparatus, SANS patterns were acquired each 100ms (or faster) for the first 20 seconds and than each 200ms for 40 seconds or more. The injection was repeated 12-20 times (depending on signal level) and the time-slices for each run averaged to improve the statistics. A typical set of SANS patterns from one of the polymer/lipid vesicle mixing experiments is shown in Figure 1. This data demonstrates the initial high intensity scattering at low Q corresponding to the vesicle SANS pattern, which is eroded, with a small increase in intensity at high Q, as the nanodiscs form. The bilayer thickness is not greatly altered by the formation process, so the scattering at high Q remains similar for both structures.

Results

For the polymer/vesicle mixing experiments, SANS patterns showing the same kind of behaviour, as in Figure 1, were acquired with similar time slicing but using different combinations of polymers and phospholipid vesicles. In the table below the entire range of lipids and different combinations used to carry out the experiments are reported. In a first approximation the trend in the data shown in Figure 1 can be modelled as the sum of scattering from a vesicle component and a nanodisc component, with the first decreasing and the second increasing with time. We are now looking more closely at these fits to determine the detailed structures and to try to identify whether there are any transient intermediate structures formed during nanodisc formation.

Table 1: List of experiments carried out using the stopped flow apparatus on polymer-vesicle mixtures.

Phospholipid $(5mg/mL)_{fin}$	Polymer $(1.5\%wt)_{fin}$	Temperature (°C)	Frames
hDMPC +	SMA2000P	25	900s @ 15s, 60s @ 15ms
		10	60s @ 50ms
		5	40s @ 400ms, 40s @ 100ms
hDMPC +	SMI1000	5	20s @ 100ms 40s @ 400ms, 15s x 60
		25	10s @ 50ms, 20s @ 100ms
hDMPC +	SMI2000	25	12s @ 50ms, 20s @ 100ms
		5	40s @ 100ms 40s @ 400ms 40s @ 400ms, 10min, 5min x 3
hDMPC/monoolein+	SMI2000	5	40s @ 400ms, 10s @ 40ms 15s @ 100ms, 40s @ 400ms, 10s x 9
hDMPC/monoolein+	SMI1000	25	20s @ 100ms, 10s @ 100ms 10s @ 20ms, 1s x 30, 5min
		40	1min x 9 (while heating)
		5	10s @ 20ms 1s x 30, 5s x 12, 10s x 12

Experiments involving lipid exchange between nanodiscs were also attempted. In order to do this experiment, nanodisc samples made following the standard procedure (5mg/mL DMPC and 1.5%wt polymer), were purified by gel filtration in order to remove any free polymer left in solution. One disc sample was made using h-DMPC, and one using d-DMPC. Since the intensity depends on the number of object present in solution, a UV-Vis spectrum was acquired between 200 and 600nm to check that the concentration of the two different solutions was exactly the same comparing the signal from the styrene group in the polymers (absorption max at 254nm).

In Figure 2 below data is shown for the kinetic data for an experiment made by mixing h- and d-DMPC discs prepared SMI2000, in the stopped flow apparatus. The two nanodisc solutions were injected at 3mL/sec into the 1mm thick quartz neutron cell and scattering data collected as 200 ms frames for 120 sec. To obtain better statistics, 26 shots were repeated and averaged together. Both nanodisc solutions were in 65% D_2O acetate buffer (dABS), pH=5, 50mM, 0.2M NaCl, a solution contrast calculated to match that of discs containing a 1:1 proportion of h- and d-lipid so the signal should decrease with time as lipids move between discs and the match point is achieved. However the signal obtained did not significantly change with time, and is close to contrast matched, suggesting that the lipid exchange had occurred on a more rapid time-scale than the SANS measurements could be taken. The lipid exchange experiment was also run for nanodiscs made with h- and d-DMPC using SMA2000P, which is the most commonly used polymer for preparing nanodiscs used in

membrane protein purification. Previous experiments on D33 using mixing by hand, had suggested that lipid exchange should occur over about 5-10mins in these mixtures. Factors such as injection speed, and temperature were altered but no changes in the patterns were observed. Similar experiments were repeated with a new batch of SMA2000P nanodiscs, and also for SMI1000 nanodiscs, however we were unable to observe lipid exchange in these experiments.

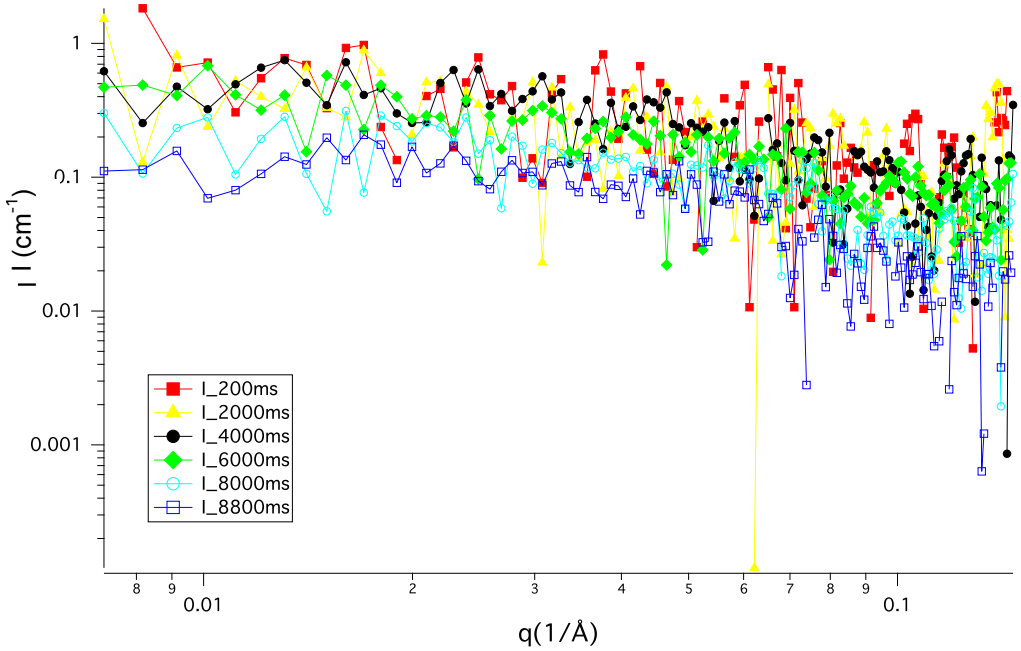

Figure 2 Lipid exchange experiment carried out with h-DMPC SMI2000 nanodiscs and d-DMPC SMI2000 nanodiscs in 65%dABS at 25°C

Unlike the results from the polymer/vesicle mixtures none of the lipid exchange experiments showed any significant change in signal and pattern with time. We assume that the turbulent mixing within the stopped flow apparatus is sufficient to cause lipid mixing within the flow lines before the solution reaches the SANS cell, and thus no change in intensity of the scattering is measured. Thus we concentrated on the use of the stopped flow apparatus to pursue the kinetics of disc formation, rather than the lipid exchange experiments. At the end of the experiment, we swapped to the sample changer to collect static SANS data, including for vesicles mixed with two RAFT synthesized polymers which form discs slowly, so that stopped flow is not required to follow the formation of discs.

Conclusion & Future Work

Successful results were obtained using the stopped flow apparatus to follow the interaction between lipid vesicles and the polymers used to form nanodiscs. While all of this SANS data followed a similar pattern (vesicle scattering decreasing as nanodiscs formed) the time for the equilibrium pattern to be achieved varied according to the polymer and lipid mixtures used. Data analysis on this aspect of the experiment is continuing. Unfortunately no useful data was obtained on the lipid mixing experiment from this set up. We are continuing to consider why this is the case, and to follow lipid mixing by fluorescence measurements (although the presence of the large fluorescent tag brings some uncertainty to such experiments).

We are continuing our studies on the lipid-polymer nanodisc systems by altering the polymer structure and molecular weight to determine the optimum polymer characteristics for disc formation and plan to continue structural studies on these systems, as well as on protein-containing nanodiscs to benchmark these systems for use in membrane protein purification and as supports for biophysical experiments.

References:

(1) Knowles, Finka, Smith, Lin, Dafforn, Overduin, *J. Am. Chem. Soc.* **2009**, *131*, 7484.

(2) Jamshad, Charlton, Lin, Routledge, Bawa, Knowles, Overduin, Dekker, Dafforn, Bill, Poyner, Wheatley, *Bioscience Reports* **2015**, *35*, e00188; Jamshad, Grimard, Idini, Knowles, Dowle, Schofield, Sridhar, Lin, Finka, Wheatley, Thomas, Palmer, Overduin, Govaerts, Ruysschaert, Edler, Dafforn, *Nano Research* **2015**, *8*, 774; Orwick, Judge, Procek, Lindholm, Graziadei, Engel, Gröbner, Watts, *Angew. Chem. Intl. Ed.* **2012**, *51*, 4653.

(3) Scheidelaar, Koorengevel, Pardo, Meeldijk, Breukink, Killian, *Biophysical Journal* **2015**, *108*, 279.