Experimental report

15/02/2017

Proposal: 8-02-795 **Council:** 10/2016

Title: Neutron Reflectometry of clathrin-mediated endocytosis

Research area: Biology

This proposal is a continuation of 8-02-780

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Samples: synthetic lipids
Proteins - non-infectious, non-toxic, non-hazardous and non-pathogenic.

Instrument	Requested days	Allocated days	From	To
FIGARO Langmuir trough	2	2	30/01/2017	02/02/2017

Abstract:

Clathrin-mediated endocytosis is crucial for the internalisation of most eukaryotic cell surface proteins. Clathrin-coated vesicles (CCVs) assemble with their cargo at the plasma membrane then transport these to the early endosome inside the cell. A CCV consists of a clathrin scaffold coating a lipid vesicle, in which the cargo is bound, linked by adaptor proteins, like AP2, that are associated with effectors of CCV assembly, stability and disassembly.
In vivo, AP2 solely interacts with one leaflet of the cellular membrane. Therefore, we have been able to analyse the first stages of CCV assembly by using cargo embedded in a lipid monolayer and Langmuir trough methods. Importantly, the addition of AP2, then of clathrin, results in distinct physical changes. Available structural information about CCV formation has however been indirect. EM has only been on mature CCVs, either lacking lipids and AP2 or with a mixture of proteins. Crystal structures of AP2 (without lipids) have also suggested that large structural changes occur on binding the cellular membrane. To clarify between these different scenarios, neutron
reflectometry analysis of CCV formation will be very informative.

Neutron Reflectometry studies of clathrin-mediated endocytosis

Armando Maestro, David J Owen, Pietro Cicuta and Nathan Zaccai

***Abstract*—Clathrin-mediated endocytosis is crucial for the internalisation of most eukaryotic cell-surface proteins. Clathrin-coated vesicles (CCVs) assemble with their cargo at the plasma membrane then transport these to the early endosome inside the cell. A CCV consists of a clathrin scaffold coating a lipid vesicle, in which the cargo is bound, linked by adaptor proteins, like AP2. In vivo, AP2 solely interacts with one leaflet of the cellular membrane. Therefore, we performed neutron reflectometry analysis to address a quantitative characterisation of the interfacial dynamics and structure of AP2 forming the physical connection between clathrin and a lipid monolayer rich in PtdIns(4,5)P2 and cargo proteins.**

I. Introduction

Clathrin-mediated endocytosis CME is the main mechanism by which eukaryotic cells internalize and recycle most membrane proteins: Clathrin adaptor proteins bound to the plasma membrane, recruit and polymerize clathrin to form clathrin-coated pits into which transmembrane protein cargoes are packaged to build clathrin-coated vesicles CCVs inside the cytoplasm. We recently determined that a single adaptor protein AP2 is sufficient to initiate and drive clathrin-coated bud formation on appropriate membranes, enriched in PtdIns(4,5)P2 *(Kelly et al., Science, 2014)*. In vivo, AP2 solely interacts with one leaflet of the cellular membrane. An alternative in vitro approach, therefore, is the direct measurement of clathrin assembly on a flat lipid monolayer. We have been able to analyze the first stages of CCV assembly by using cargo embedded in a lipid monolayer. Importantly, addition of AP2, then of clathrin, results in distinct changes in lateral lipid pressure and compressibility. Besides, crystal structures of AP2 in complex with different cargos (but without lipids) have also suggested that large structural changes occur on binding the cellular membrane (see Fig. 1(a)). For these reasons, neutron reflectomery analysis of CCV formation are very useful offering structural information of AP2 binding to the lipid layer and clathrin polymerization.

II. Experimental details

Specular neutron reflectivity (SNR) experiments were performed to study the interaction of AP2 with a lipid monolayer that mimic a single membrane leaflet. A Langmuir lipid monolayer was prepared from a mixture of unsaturated lipids (DOPE, DOPS and DOPC), cholesterol and phosphatidyl inositol (PtdIns4,5P2). In addition, a TGN38 peptide, anchored to a maleimide chain, (also CD4 peptide) was also included in the mixture to evaluate the presence of a transmembrane cargo receptor. The SNR measurements were made on the time of flight (TOF) reflectometer on the FIGARO beam line at the ILL, Grenoble, France. Data were collected at two incident angles 0.62^o and 3.78^o giving a scattering vector Q from 0.011 to 0.047 $\mathring{A}^{-1}$ and from 0.034 to $0.26\mathring{A}^{-1}$ respectively. Calibration of FIGARO was performed using a pure D2O sub-phase. Backgrounds were subtracted from the data by the simultaneous acquisition of off-specular data for each measurement on the area detector. No off-specular scattering was observed for the monolayers under the conditions of the experiment. All SNR experiments were performed at a temperature of 22 ^{o}C.

Lipid monolayers were prepared on a Langmuir trough with a total area of 97 cm^2 and 60 cm^2 (Nima Technologies, Coventry, UK) by spreading from a chloroform solution (using a 50 μL Hamilton microsyringe) onto a pH 7.2 HKM-Tris buffer solution. Lipid monolayers at two surface pressures Π (namely 18 mN/m and 30 mN/m) were studied. Care was taking to ensure that a stable monolayer was created; *i.e.* Π is constant after lipids were spreading. (Note that the monolayer was not mechanically compressed by the barriers of the trough.) Then, SNR profiles of the lipid monolayers were obtained using two different H/D contrasts, namely D2O and air contrast matched water ACMW (a mixture of composition 92% H2O and 8% D2O by volume) as shown Fig. 1(b). For each experimental condition explored, once the lipid monolayer shows a stable value of Π, AP2 in buffer solution was injected (100 - 200 μL) into the aqueous sub-phase beneath the lipid monolayer. SNR profiles in ACMW at Q from 0.01 to 0.047 $\mathring{A}^{-1}$ were measured each five minutes to show the kinetics of AP2 binding to the lipid monolayer as shown Fig. 1(d). The kinetics of AP2 binding was also recorded by monitoring Π versus time (data not shown). Finally, when the interaction of AP2 with the lipid monolayer was almost complete (i.e. no further change in surface pressure was recorded after at least one hour), the SNR of the monolayer was measured as also shown Fig. 1(b).

The SNR profiles plotted in Fig. 1(b) were analysed by the Abeles matrix method using Motofit [A Nelson, J Appl. Crystallogr., 2006, 39, 273] assuming a stratified structure including the lipid monolayer and the proteins interacting. To find the parameters that show the most realistic model, the SNR data obtained for the two contrast at each experimental condition were simultaneously fitted with a genetic algorithm with least squares and refined with a Levenberg-Marquardt optimisation. The scattering length density σ of each layer, solvent and air were calculated as

$$\sigma = \frac{\sum b_i f_i}{\sum V_i f_i}, \tag{1}$$

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where b_i is the scattering length, V_i the molecular volume and f_i is the fraction of each component of a particular layer (see Fig. 1(c)). The lipid monolayer is divided in two sub-layers that corresponds to the the atoms that constitute the hydrophobic chains and the polar head. Furthermore, due to the fact that the monolayer is composed of mixed lipids, σ is calculated as the sum of the scattering lengths of the constituent atoms divided by their molecular volume. Concretely, the volume of the lipids head groups (PE, PC, PS and PI) was calculated as the sum of the corresponding C, O, H, N and P atomic radius. The volumes of the chains were calculated as the sum of CH2, C=C and CH3 groups. We assume the surface excess of the polar heads Γ_h is equal to the hydrophobic chains Γ_t so

$$\frac{\Gamma_h}{\Gamma_t} = \frac{\sigma_h d_h \beta}{\sigma_t d_t} \frac{\sum_i b_t}{\sum_i b_h} \quad (2)$$

where $\beta = 100 - SP/100$ and SP is the solvent penetration and d is the thickness of each layer. The best results of the fitting corresponds to the presence of the cholestanol molecules in the chain layer (with only the OH group in the polar head). The SLD density from TGN38 and AP2 were obtained from MULCH –ModULes For The Analysis Of Small-Angle Neutron Contrast Variation Data From Bio-Molecular Assemblies (http://smb-research.smb.usyd.edu.au/NCVWeb/input.jsp). TGN is anchored to the lipid monolayer thanks to a maleimide chain that is assembled in the monolayer. Fluorescence images confirm the existence of irregular lipid clusters densely packed. This mixed monolayer does not undergo a phase transition (constant Π with Area). The heterogeneous monolayer has different properties, such as charge density and lipid ordering, which obviously have implications for the binding of AP2 molecules (via electrostatic interactions to PIP2 clusters, and through a specific binding to TGN that should be placed close to the PIP2 clusters). Nevertheless, from the point of view of the SNR measurements, the sizes of the individual clusters ($< 15\,\mu m$) were smaller than the coherent length of the neutrons ($20-40\,\mu m$ for the slit geometries used in this study). In consequence, any diffuse scattering arising from these phase-separated domains would not be expected to contribute significantly to the measured speculate reflection. The results of the fittings, therefore, will represent an average of the monolayer; also, because drift makes the clusters to move. The existence of PtdIns(4,5)P2 clusters also justifies to average the SLD of all the lipid tails and heads for a final value to be used in the fittings. The values of σ for the different layers were fixed (data not shown). The hydrophobic regions were assumed to be solvent free, meanwhile the solvent penetration into the head group was a fitting parameter. The roughness between the layers was constrained to $4\,\AA$.

III. Results

The main goal of this experiment was to use SNR to probe the interaction of AP2 with the lipid monolayer by exploring the resultant interfacial structure before and after the injection of AP2 -the question we try to answer is whether the AP2 molecules pack tightly next to the membrane and even possibly embed themselves into the lipid outer leaflet. In detail, *FIGARO* was used to identify the structure of AP2 with respect to the lipid monolayer. In Fig. 1(b) we show the SNR profiles and associated fits for the lipid monolayer, containing TGN, respectively deposited on subphases of deuterated and ACMW at a surface pressure of 30 mN/m. At a first glance it can be seen in Fig. 1(b) that the presence of AP2 (after equilibration once injected beneath the lipid monolayer) results in a notable change of the SNR profile in both deuterated buffer and ACMW. The SNR data corresponding to the lipid monolayers in absence/presence of AP2 was fitted using a four-layers model assuming that TGN might be in between the lipid polar heads and the AP2c binding sites. The parameters used to obtain the best fits to the SNR data are given in Table I. It is worth mentioning that an AP2 containing layer was absent when the lipid monolayer was deposited on the subphases prior the injection of the protein.

TABLE I
Structural parameters obtained from fitting a four-layer model to SNR profiles of lipid monolayers dopped with TGN at a pressure of 30 mN/m in presence of AP2 (150μL) at 22 ^{o}C.

Layer	thickness ($\AA$)	σ (10^{-6} $\AA^{-2}$)	solvent (%)
Air	Inf	0	-
Tails	18.3	-0.17	0
Heads	10.5	1.375	0
TGN38 (D2O/ACMB)	18	3.5/1.92	95
AP2core (D2O/ACMB)	38	3.06/1.92	45
subphase (D20/ACMB)	Inf	6/0	-

From the fits, the remarkable difference in the SNR profiles before and after AP2 is injected beneath the lipid monolayer can be explained by the existence of an extra-layer that corresponds to AP2 molecules packed together binds directly to the

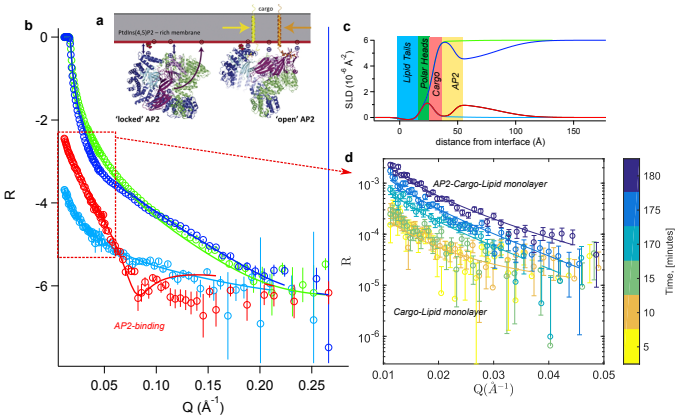

Fig. 1. **NR studies to address the mechanism of clathrin mediated endocytosis.** (a) Cartoon of the AP2 change of conformation when binding the cargo-lipid membrane (Kellie et al, Science 2014). (b) NR profiles for lipid monolayer doped with cargo and PtdIns(4,5)P2 in two contrasts (D20 buffer [green] and ACM buffer [light blue]). Injection of AP2 beneath the monolayer leads to a change in the NR profiles at both contrasts. A four layer model fit is shown as a solid line on each profile. (c) The scattering length density profiles calculated from the fit to the data in (b). (d) Kinetic of binding of AP2 to the cargo-lipid monolayer: Changes in the NR profile at low angle as a consequence of the AP2 binding. Lines are fits to the NR data that shows an increase in the coverage of the AP2 layer with time.

TGN molecules anchored to the lipid monolayer, and possible (via electrostatic interactions) also to the lipid membrane which is doped by PI(4,5)P2 lipids. Prior to get a plateau in the increase of Π once AP2 was injected, which is telling us that the lipid monolayer is in an steady-state, we performed a kinetic experiment that consists in collecting SNR data at low angle in both D2O and ACMW contrast every 5 minutes during 3 hours after the injection of AP2 (results shown in Fig. 1(d)). There is a clear change in the SNR profiles in ACMW –the profile changes at $t = 3\,\mathrm{h}$ with respect to the one at $t = 0\,\mathrm{h}$–. We analysed the ACMW profiles at different times by the 4-layers model fixing the parameters shown in Table I with the exception of the solvent penetration of the AP2 layer. The SNR profiles and the resultant fits are plotted in Fig. 1(d). The decrease of solvent penetration with time might be a clear indication of AP2 is binding to TGN and PI(4,5)P2.

We also measured SNR profiles in two contrasts for a cargo-lipid monolayer at lower pressure (around 20 mN/m) as shown Fig. 2. For the sake of comparison the profile at high pressure is also plotted Fig. 2. In summary, the 4-layers model is also valid for the SNR profiles at lower pressure, but the only difference is the larger solvent penetration ($\simeq 87\%$) meaning that less AP2 molecules are bound to TGN/Lipid monolayer.

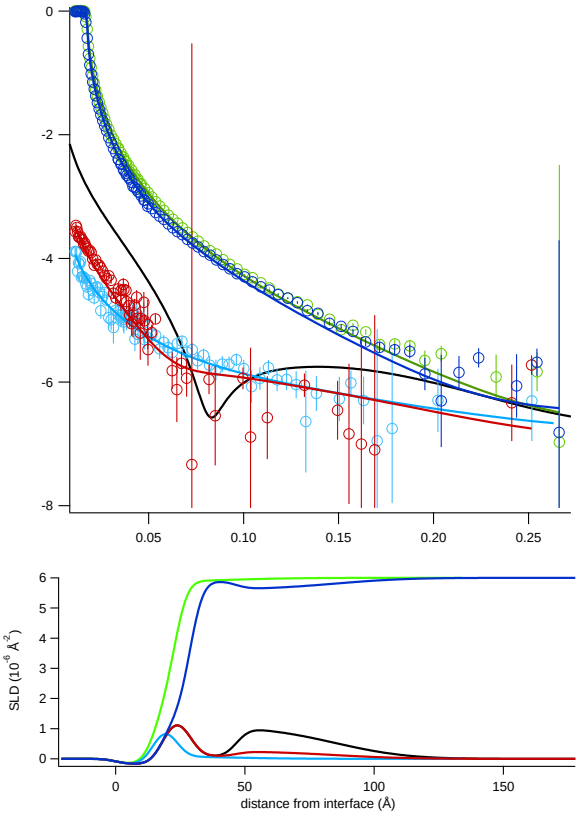

Fig. 2. (Top) SNR profiles for Lipid monolayer doped with TGN and PI(4,5)P2 at $\Pi = 20\,\mathrm{mN/m}$. The experiments shown are lipid on D2O buffer (green) and acmb (light blue) and lipid monolayer with AP2 in D2O (blue) and acmb (red). The four-layer model fit is shown as a solid line on each contrast–In black, the linear fit of the data at high pressure shown in Fig. 1. (Bottom) The scattering length density profiles calculated from the fit to the data as a function of the distance along the interface normal.

IV. Conclusions

We collected several complete NR datasets of AP2 interacting with different cargo-containing lipid monolayers at different lateral pressures (experiment 8-02-780 and 8-02-795). The analysis clearly demonstrated that structural changes could be directly observed in real time, with the AP2 ending positioned above the lipid head groups (see Fig.1(b-d)). We provided, therefore, a quantitative characterization of the interfacial dynamics and structure of AP2 forming the physical connection between clathrin and the PtdIns(4,5)P2- and cargo containing membrane that completely agrees with preliminary in-vitro experiments based on fluorescence microscopy and surface tensiometry of Langmuir lipid monolayers.