Experimental report

17/09/2018

Proposal: 8-02-804 **Council:** 4/2017

Title: Neutron Reflectometry of clathrin-mediated endocytosis

Research area: Biology

This proposal is a continuation of 8-02-780

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Experimental team: Nathan ZACCAI

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Samples: synthetic lipids
Proteins - hydrogenated
Proteins - deuterated

Instrument	Requested days	Allocated days	From	To
FIGARO Langmuir trough	4	4	20/04/2018	24/04/2018

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

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***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 binding dynamics and structure of AP2 forming the physical connection between clathrin and a lipid monolayer rich in PtdIns(4,5)P2 and cargo proteins such as TGN and CD4.**

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. 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. Langmuir lipid monolayers with different cargo content were prepared from a mixture of unsaturated lipids (DOPE, DOPS and DOPC), cholesterol and phosphatidyl inositol (PtdIns4,5P2). We included in the lipid monolayer different polypeptides as transmembrane cargoes receptor: Cd4 and TGN38 peptide, both anchored to a maleimide chain. The SNR measurements were made on the horizontal time of flight (TOF) reflectometer FIGARO at the ILL, Grenoble, France. We used a Langmuir trough as a sample environment to monitories the surface pressure during the experiment. 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 (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 with different composition at one surface pressure Π (namely 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. 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 study the kinetics of AP2 binding to the lipid monolayer as shown Fig. 2(a). 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.

The SNR profiles plotted in Fig. 1 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

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$$\sigma = \frac{\sum b_i f_i}{\sum V_i f_i}, \quad (1)$$

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). Both CD4 and TGN are anchored to the lipid monolayers 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 $3\,\mathring{A}$.

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 the SNR profiles and associated fits for the lipid monolayer, containing TGN, CD4, TGN:CD4 and a control sample, respectively deposited on sub-phases of ACM buffer at a surface pressure of 30 mN/m. At a first glance it can be seen in Fig. 1 that lipids monolayers have different lipid packing density even when the protocol of preparation is the same. This is due to the fact that the cargoes made an impact in the lipid distribution across the Langmuir monolayer.

We performed a kinetic experiment that consists in collecting SNR data at low Q in ACM buffer with a time resolution of 5 minutes during 3 hours after the injection of AP2 (results shown in Fig. 1(a)). There is a clear change in the SNR profiles in ACMW for all the samples studied that corresponds to TGN, CD4, CD4:TGN samples and in the absence of cargo. We analysed the ACMW profiles at different times to get an estimation of the binding of AP2 to the different monolayers. The resultant fits to a single exponential first order reaction are plotted in Fig. 1(b) and the resulting binding times in Fig. 1(c). It is remarkable the increase of binding time when cargo is present. We are now trying to rationalise the different founds

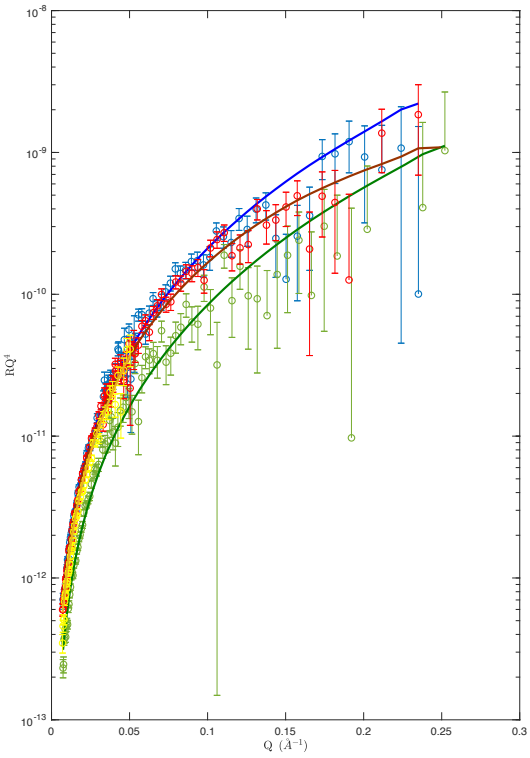

Fig. 1. SNR profiles for different lipid monolayers doped with cargo (CD4, TGN and CD4:TGN 1:1) and PtdIns(4,5)P2 in ACM buffer. Lines correspond to fits to the data using a two layers model in Motofit & Aurore software.

between TGN and CD4 samples. Further analysis of these data is ongoing at this moment.

Finally, 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 (data not shown) and ACMW (see Fig. 3). 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 AP2 binding sites.

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 both the CD4 and 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.

IV. Conclusions

We collected several complete NR datasets of AP2 interacting with different cargo-containing lipid monolayers. The ongoing 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. 2). We estimate to 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 SNR experiments as well as in-vitro experiments based on fluorescence microscopy and surface tensiometry of Langmuir lipid monolayers.

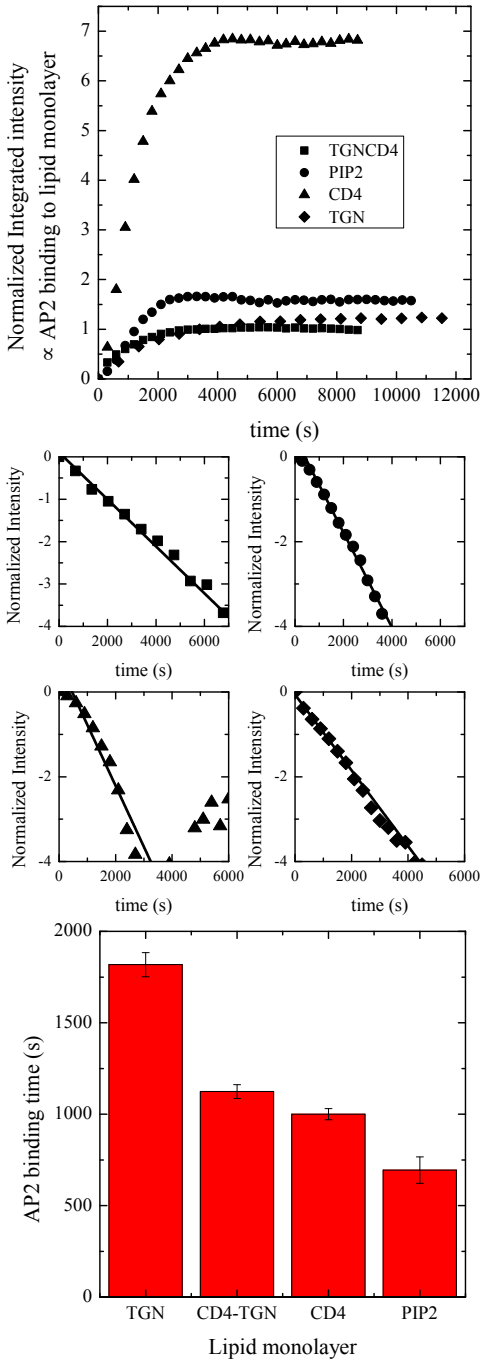

Fig. 2. (top) Low-Q kinetic analysis of AP2 binding to different lipid monolayers doped with PtdIns(4,5)P2 and (no cargo, CD4, TGN and CD4:TGN 1:1) in ACM buffer. (middle) Normalized binding kinetic assays. Lines correspond to fits to the data using a model based on an exponential first order reaction. (bottom) Binding times observed for the different samples measured.