Direct observation of alpha-lactalbumin, adsorption and incorporation into lipid membrane and formation of lipid/protein hybrid structures

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\textbf{A B S T R A C T}

The interaction between proteins and membranes is of great interest in biomedical and biotechnological research for its implication in many functional and dysfunctional processes. We present an experimental study on the interaction between model membranes and alpha-lactalbumin (\textalpha-La). \textalpha-La is widely studied for both its biological function and its anti-tumoral properties. We use advanced fluorescence microscopy and spectroscopy techniques to characterize \textalpha-La-membrane mechanisms of interaction and \textalpha-La-induced modifications of membranes when insertion of partially disordered regions of protein chains in the lipid bilayer is favored. Moreover, using fluorescence lifetime imaging, we are able to distinguish between protein adsorption and insertion in the membranes. Our results indicate that, upon addition of \textalpha-La to giant vesicles samples, protein is inserted into the lipid bilayer with rates that are concentration-dependent. The formation of heterogeneous hybrid protein-lipid co-aggregates, paralleled with protein conformational and structural changes, alters the membrane structure and morphology, leading to an increase in membrane fluidity.

1. Introduction

The interaction of proteins with cellular membranes controls fundamental cellular processes such as membrane trafficking, cytokinesis, and intracellular signaling [1–7]. Beside cytoplasmic, peripheral or integral membrane proteins, there is a class of proteins known as amphitropic that interacts with lipids and membranes during their biological activity [8,9]. These soluble proteins or enzymes can reversibly and transiently interact with membranes, having affinities for both aqueous and hydrophobic environments [9–11]. Moreover, they are implicated in key biological functions such as enzyme regulation [12], cell signaling [9], apoptosis [13], transmembrane signaling [8] and also regulate the interaction between membrane and cytoskeleton [8].

Proteins may also affect membrane integrity leading to cellular dysfunction or death [2,14–16]. This is the case for the interaction between partially unfolded proteins/protein aggregates and lipid bilayers [17–21] possibly generating cytotoxicity and, in vivo, the onset and progression of several human diseases [2,4,15,16,19]. Membrane-protein interactions are of the same origin [19,22,23] and are regulated by electrostatics, hydrophobic forces [24,25] and protein flexibility [26], being this true for both pathological and non-pathological proteins [15,27–29]. However, the mechanisms ruling protein-induced membrane destabilization are still out of reach. In the context of protein-related diseases, unraveling them is fundamental in order to better understand both the amyloid formation in vivo and the related toxicity [30]. To this aim a great attention has been recently addressed to the interaction between intrinsically disordered regions of proteins and membrane, which may be involved both in protein function and dysfunction in cellular environment [31–33].

Finally, understanding membrane-protein interactions is also crucial for pharmaceutical applications. Indeed, a complete understanding on how therapeutic or carrier proteins adsorb on, or penetrate the membrane is fundamental for the optimization and efficiency of pharmacological treatments [34]. Moreover, novel protein-membrane hybrid structures may also be developed as novel drug delivery systems [35].

Bovine \textalpha-La is a small (14.2 kDa) acidic (pI 4.8) Ca\textsuperscript{2+} binding milk protein, composed of 123 aminoacids [36]. It has been widely used as a model for protein molten globule state in folding studies [37–40] and largely studied for its capacity to interact with lipids [11,41]. The interaction of \textalpha-La with lipids plays an essential role in many biological processes such as the biosynthesis of lactose and the secretion of the protein in the milk [41]. Due to these peculiar properties, \textalpha-La has been classified as an amphitrophic protein and it is considered a good model to clarify the mechanisms at the basis of the amphitropic behavior, in particular for proteins with an intrinsic ability to reversibly bind to a lipid bilayer [11,13,24]. At acidic pH, \textalpha-La is in its molten globule state...
This is a compact denatured state partially retaining the native secondary structure but lacking of a well-defined tertiary structure [39,42] that is known to rapidly insert into the lipid membrane [29,43,44]. Moreover, this state is characterized by a high conformational flexibility [42,45] and thus represents a good model for monitoring protein-membrane interactions in conditions where a stable global protein fold is missing [11,32,46].

A great number of experiments were performed on α-La interaction with lipid bilayers, using both small unilamellar vesicles (SUVs) and large unilamellar vesicles (LUVs) as model membranes [13,24,26,41,43–45,47–49]. Besides being an excellent model system for studying protein-membrane interactions and describing specific mechanisms for amphitrophic protein behavior [11], the specific interest in the analysis of α-La-membrane interaction is mainly based on the fact that the bovine and human variant of this protein can alter its biological function and gain a tumoricidal property if partially unfolded and bound to oleic acid [32,50–52]. These complexes are named HAMLET (human α-La made lethal to tumor cells) and BAMLET (bovine α-La made lethal to tumor cells) [50,51] and their interaction with membrane has been shown to be crucial to trigger tumor cell death [32,53,54]. Nevertheless, results remain controversial and a clear unifying picture of membrane and protein structural changes related to the formation of membrane-protein complexes is not clear. This is mainly due to the high sensitivity of such systems toward the experimental conditions [24,41]. Subtle changes in the physico-chemical conditions such as proton concentration, temperature as well as curvature and charge of the lipid membrane can indeed significantly alter the experimental outputs [24,41]. In particular, the combination of hydrophobic and electrostatic interactions determine both membrane structural modifications and the properties of the membrane-bound protein states, which were found to range from native-like to molten globule conformation [24,26,45,49].

Using a combination of fluorescence microscopy and spectroscopy techniques, here we present an experimental study on the interaction between molten globule α-La and Phospholipid Giant Vesicles (GVs). We show changes at mesoscopic scale in the morphology of GVs upon accumulation of α-La at the membrane surface, leading to novel hybrid protein-lipid structures. For these structures, we were able to distinguish and localize different membrane-bound states of the protein, with structural features of protein and lipid packing depending on the protein concentration. Moreover, our results highlight the need of control of a heterogeneous distribution of multilamellar giant vesicles with diameter of several micrometers. After liposomes formation, sample was diluted to a final lipid concentration of 200 μM and used within 24 h for spectroscopy and microscopy measurements. A stock solution of Laurdan (500 μM) was prepared in DMSO and stored protected from light exposure. Laurdan was added to the GVs in a probe-lipid molar ratio of 1:500 and left to equilibrate for 3 h before measurements.

2.1. Giant Vesicles preparation and staining

Phospholipid giant vesicles (GVs) were prepared mixing POPC and POPG in chloroform:methanol 3:2. Using a rotary evaporator (Buchi, Rotavapor R-215, equipped with the Buchi Vacuum Controller V-855), the lipids were dried to form lipid films. After drying overnight, the lipids were hydrated with NaCl 0.1 M, pH 2. Resulting sample is made of a heterogeneous distribution of multilamellar giant vesicles with diameter of several micrometers.

After liposomes formation, sample was diluted to a final lipid concentration of 200 μM and used within 24 h for spectroscopy and microscopy measurements. A stock solution of Laurdan (500 μM) was prepared in DMSO and stored protected from light exposure. Laurdan was added to the GVs in a probe-lipid molar ratio of 1:500 and left to equilibrate for 3 h before measurements.

2.1. Protein solution preparation and staining

Bovine α-La was dissolved in NaCl 0.1 M, pH 2. Samples were freshly prepared and filtered through 0.10 μm filters (16,553, Sartorius). Protein concentration was determined by means of a Jasco V-760 UV–Vis spectrophotometer, using an extinction coefficient ε1%λ = 20 at λ = 280 nm

Staining procedure: Bovine α-La in 0.1 M potassium phosphate buffer (pH 8.3) was mixed with Atto 647 NHS ester at approximatively 1:3 protein:dye concentration and incubated for about 2 h at room temperature in the dark. The labeled protein was separated from unreacted dye using a Sephadex G-25 column equilibrated with 0.1 M phosphate buffer at pH 7.4. Labeled-α-La was added to α-La sample at molar ratio 1:100; measured pH at the end of this procedure is pH 7.2 ± 0.2. α-La was added to GVs at Lipid:Protein molar ratios (L/P) 250:1 and 20:1. Final α-La concentration was 10 μM (L/P 20:1) and 0.8 μM (L/P 250:1), for fluorescence spectroscopy and microscopy measurements. A final protein concentration of 1.6 μM was used in circular dichroism measurements, at L/P 250:1.

2.2. Fluorescence measurements

Fluorescence measurements were carried out at 25 °C in 1 cm path quartz cuvettes using a Jasco FP-8500 spectrophotometer equipped with a Jasco ETC-815 peltier as temperature controller. At L/P 20:1 protein and lipid concentration were 10 μM and 200 μM, respectively. At L/P 250:1 protein and lipid concentration were 0.8 μM and 200 μM, respectively.

2.2.1. Intrinsic fluorescence emission

Fluorescence emission spectra were acquired using an excitation wavelength λexc = 290 nm and detected in the range 285–550 nm, with 0.5 nm wavelength interval, emission and excitation bandwidth of 2.5 nm and 5 nm respectively.

2.2.2. Laurdan fluorescence emission

Fluorescence emission spectra were acquired in the range 360–600 nm, using an excitation wavelength λexc = 350 nm. Spectra were recorded with 0.5 nm wavelength interval, emission and excitation bandwidth of 5 nm and 10 nm, respectively. According to Parasassi et al. [55], the Laurdan Generalized Polarization (GP) function is defined as:

20 μM (L/P 250:1) and 0.8 μM (L/P 250:1), for fluorescence spectroscopy and microscopy measurements. A final protein concentration of 1.6 μM was used in circular dichroism measurements, at L/P 250:1.

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GP = \left(\frac{I_{440} - I_{900}}{I_{440} + I_{900}}\right)

where I_{440} and I_{900} are the emission intensities at 440 and 490 nm respectively. Laurdan emission spectrum is centered on about 440 nm in the membrane gel phase and on about 490 nm in the membrane liquid crystalline phase [67,68]. Due to Laurdan properties, GP value analysis allows determining the phospholipidic phase state as well as the variations in membrane fluidity due to changes in membrane water content. Laurdan GP variation as a function of time was calculated after α-La addition to POPC:POPG vesicles at L/P 20:1 and 250:1. As control measurement, we show that Laurdan GP value doesn’t change after adding 0.1 M NaCl-HCl solution at pH 2 ± 0.2.

2.3. CD measurements

CD spectra of α-La and samples at the two different L/P were recorded on a Jasco J-715 spectropolarimeter in the far-UV region (194–270 nm), using quartz cuvettes. For each spectrum 20 accumulation have been acquired, with data interval 0.1 nm, bandwidth 1.0 nm, scan speed 50 nm/min. All spectra have been acquired at room temperature. At L/P 20:1 spectra were acquired using a path length of 1 mm, with protein and lipid concentration of 10 μM and 200 μM, respectively. At L/P 250:1 spectra were acquired using a 5 mm path cuvette, with protein and lipid concentration of 1.6 μM and 400 μM, respectively. In the latter case, in order to improve S/N ratio, path length was changed to 5 mm and sample concentration was doubled, maintaining L/P ration constant (250:1). As control, we also verified that at L/P 20:1 the observed effects were the same also after doubling sample concentration.

2.4. 2-photon and confocal fluorescence microscopy

GVs were imaged before and after α-La addition. 500 μL sample aliquots were deposited in Chambered Coverglass (Nunc Lab-Tek II) and imaged using a Leica TSC SP5 confocal laser scanning microscope, with a 63× objective, NA = 1.4. Scanning frequency was 200 Hz. For all measurements, at L/P 20:1 protein and lipid concentration were 10 μM and 200 μM, respectively; at L/P 250:1 protein and lipid concentration were 0.8 μM and 200 μM, respectively. Control measurements have shown that results are the same independently of the sample holder. The same changes in vesicles morphology were observed in samples incubated in quartz cuvette (3 ml volume) or in chambered coverglass (500 μL volume).

2.4.1. Colocalization experiments

1024 × 1024 pixel images have been acquired with a sequential acquisition of two channels: Atto 647 emission was acquired in the range 653–750 nm, using λ_{exc} = 633 nm; Laurdan emission was acquired in the range 430–505 nm (λ_{exc} = 380 nm) using 2-photon excitation (Spectra-Physics Mai-Tai Ti:Sa ultra-fast laser), at 780 nm.

Several measurements as a function of time were performed using α-La samples containing 1:100 Atto 647-labeled α-La and multiple conditions were tested. Presented measurements are obtained at L/P 20:1 or at L/P 250:1. To monitor labeled protein signal, 512 × 512 fluorescence confocal images have been acquired as a function of time in the range 653–750 nm, with λ_{exc} = 633 nm, and phase contrast channel was also acquired when needed.

2.4.2. Laurdan GP imaging

256 × 256 pixels images were acquired using 2-photon excitation at 780 nm and collecting the emission in the range 430–450 nm and 485–505 nm, simultaneously. In order to obtain 256 × 256 GP maps, providing GP value in each pixel of the image, data were analyzed using SimFCS software (Laboratory for Fluorescence Dynamics, University of California, Irvine, CA, available at www.lfd.uci.edu). Calibration of the GP scale has been obtained using a Laurdan GP = 0.2 for a standard Laurdan solution in DMSO at 22 °C [61,69].

Control measurements were performed assessing that possible unsought interactions occurring between the fluorescent dye used to label α-La sample and model lipid membranes, in the present experimental conditions are negligible. The effects of protein addition to liposomes are the same regardless of the use of labeled or unlabeled protein (data not shown).

2.5. Fluorescence lifetime imaging microscopy

256 × 256 pixels fluorescence Lifetime Imaging Microscopy (FLIM) images were acquired in the time domain using a Leica TSC SP5 inverted microscope with a 63× /1.4 oil objective (Leica Microsystems), coupled with a PicoHarp 300 TCSPC Module (PicoQuant). The excitation wavelength for Atto 647 NHS ester was set to 633 nm, using the pulsed White Light Laser (Leica Microsystems) with 80 MHz repetition rate. In the presented experiments protein and lipid concentration were, respectively, 10 μM and 200 μM (at L/P 20:1), and 0.8 μM and 200 μM (at L/P 250:1).

FLIM measurements have been processed by the SimFCS software (Laboratory for Fluorescence Dynamics, University of California, Irvine, CA, available at www.lfd.uci.edu). Calibration of the phasor plot for FLIM data was obtained measuring the lifetime of Atto 647 NHS ester in DMSO, which has a known single exponential decay with 2.4 ns lifetime (www.sigmaaldrich.com).

We report for FLIM the Phasor analysis, introduced by Digman et al. [70]. The signal from each pixel is mapped to a single point called “phasor” in the phasor plot, details of this analysis are described in Digman et al. [70]; briefly the cosine (g-coordinate) and sine (s-coordinate) Fourier transforms of the emission decay collected in each pixel of the image, are represented in a polar plot (Phasor plot) according to the following definitions [70,71]:

\[ g_{ij}(\omega) = \frac{\int_{0}^{\infty} I_i(t) \cos(\omega t) \, dt}{\int_{0}^{\infty} I_i(t) \, dt} \]

\[ s_{ij}(\omega) = \frac{\int_{0}^{\infty} I_i(t) \sin(\omega t) \, dt}{\int_{0}^{\infty} I_i(t) \, dt} \]

where I_i(t), is the fluorescence decay measured at each pixel of an image, i and j identifies the single pixel and ω is the angular modulation frequency, equal to 2πf with f (laser repetition frequency) is 80 MHz. A direct relationship between a phasor location and decay lifetime exists and lifetimes can be found with a fit-free procedure after suitable system calibration. In this representation single exponential decays lie on the “universal circle”, defined as the semicircle of radius \(1/2\) going from the point (0,0), corresponding to a lifetime \(\tau = \infty\), to the point (1,0), corresponding to a lifetime \(\tau = 0\). Phasors follow vectors algebra so that the addition rules of vectors are valid [70,71].

3. Results and discussion

3.1. Interaction of α-La with GVs

Using giant vesicles (GV) as a model in a protein-membrane system allows visualising membrane deformation at microscopic scale as well as the localization of the protein in/onto the membranes at the same scale. We then prepare POPC:POPG liposomes at pH 2 and add to them α-La in a molten globule state [72] as a protein system, labeled with Atto 647 (Atto 647-labeled α-La, at pH 2 is 1:100, see Materials and Methods).

In absence of protein, GVs morphology is stable for 24 h (see SI, Fig. S1), with the vesicles being highly heterogeneous both in shape and size. Upon addition of the protein, the progressive growth of fluorescence signal co-localized with liposomes indicates that α-La readily
Fig. 1. POPC:POPG morphology changes as a function of time after the addition of 10 μM α-La containing 1:100 Atto 647-labeled α-La (L:P 20:1). (A–C) Representative images of liposomes morphology (A) before protein addition and (B) 5 min and (C) 30 min of incubation with protein at RT. Arrows indicate a liposome whose change in morphology and position is evident. (D) Representative image of final structures after incubation with protein for 24 h. The overlap of confocal fluorescence (Atto 647 fluorescence emission) and phase contrast (liposomes) channel is reported. Fluorescence confocal images have been acquired in the range 653–750 nm, with λ_{exc} = 633 nm. Scale bar is (A–C) 10 μm and (D) 7.5 μm.

accumulates on GVs membrane over time (Fig. 1A–C). Within few seconds after protein addition, α-La locates on accessible liposome surfaces and modifies vesicles morphology and position, possibly inserting into the lipid bilayer (Fig. 1A–C). Time-lapse analysis as shown in the video in SI further confirm these early changes. Interestingly, after 24 h of incubation of liposomes with α-La, we detect the formation of regular, roundish and full structures (Fig. 1D) with an average size smaller than the one of vesicles in absence of proteins, suggesting the formation of micrometric protein-lipid structures.

Membrane modification, such as disruption [73,74] and collapse [52], are dependent on protein structure, folding and aggregation state [29,73] as well as protein concentration, lipid composition [13,72] and membrane curvature [24]. α-La is reported to induce the loss of bilayer integrity in unilamellar vesicles, depending on membrane composition and charge, but also on the pH controlling protein conformation and charge [13,29,45,72]. Similar changes in giant unilamellar vesicles morphology are observed upon interaction with bovine α-La in complex with oleic acid (BLAOA) [52]. These changes are mainly attributed to the presence of oleic acid [52]; however, the effect of specific regions of BLAOA cannot be neglected, with α-La component being crucial for the specific antitumor effect [32]. This is possibly related to α-La tendency of assuming partially disordered structure and preferentially interacting with negatively charged lipids [24,45,49]. Indeed, fluctuating tertiary structure in α-La molten globule state contains a partially unstructured region possibly exposing hydrophobic patches to the solvent [39,75,76]. Being in this conformational state is a necessary condition for α-La partition into GUVs [29] and explains the data of Fig. 1 in terms of both adsorption and insertion of the protein on/into the lipid bilayer. In this scenario, the combination of electrostatic interactions with the polar heads of lipids and hydrophobic interactions with the lipid chains may result in a fast α-La adsorption to the membrane followed by the partial or total insertion into the lipid bilayer.

3.2. Effect of lipid/α-La ratio on hybrid structures

To further investigate the membrane-protein co-aggregates, we form the hybrid structures at different lipid:protein ratios (Fig. 2). From now on, we will refer to the samples obtained by adding α-La to POPC:POPG GVs at Lipid:Protein (L:P) 20:1 and at L:P 250:1 as “20:1 sample” and “250:1 sample”, respectively (see Materials and Methods). Representative images of the final hybrid protein-lipid structures formed after 24 h of incubation are shown in Fig. 2, with the GVs stained by Laurdan (green channel, 2-photon microscopy) and α-La stained by Atto 647 (red, confocal microscopy). Control measurements on protein-free GVs are reported in the supplementary materials (see SI, Fig. S1).

Our data indicate a high level of co-localization of the fluorescence signal for the two channels in both samples, confirming that α-La is partitioned onto the membrane. Micrometric protein-lipid structures have different shapes and organization in both samples; they contain both proteins and lipids, and are heterogeneous in morphology and distinct from the initial lipid structure (Fig. S1A). A high heterogeneity in morphology is also evident in the 250:1 sample (Fig. 2A, B, E, F). In this sample, POPC:POPG vesicles and protein are indeed wrapped into large structures and budding between different structures is enhanced when compared to the control sample (see SI, Fig. S1). However, even if in minor extent, we also detect GVs retaining their initial morphology. These liposomes show a reduced fluorescence intensity in the green channel if compared with others structures in the same image as well as a negligible co-localization with protein signal (red channel, see arrows, Fig. 2E and F). Quite in contrast with this observation, for the 20:1 sample, we detect a significantly different morphology. In this case, fluorescent structures are clearly homogenous in size and shape (Fig. 2C and D), with a diameter of approximately 2 μm (smaller than the bare vesicles) and with a tendency to cluster. In line with the data reported in Fig. 1D, the structures appear roundish with fluorescence signals of the Laurdan and Atto 647 almost fully co-localized. This suggests that the structures consist of both lipids and proteins.

Our data unequivocally points toward a key role of the lipid:protein ratio in regulating the morphology of the final hybrid structures. Measurements in Fig. 2C–2D clearly show that in 20:1 sample, α-La is homogeneously distributed on the newly formed structures (Fig. 2C–2D) while in 250:1 sample α-La does not allocate on each vesicle and appears to be unevenly distributed on the liposomes surface (Fig. 2A–B). This suggests that the protein concentration in 250:1 sample is low enough to not saturate all the available interaction sites on the membrane. Moreover, the inhomogeneity of the Atto fluorescence signal reflects another important aspect, which is the inhomogeneity in protein distribution on protein surface. The reason of this is likely to be related to the charge on both proteins and vesicles. α-La has positive charge at pH 2, while the charge distribution on the lipid surface can be highly heterogeneous. This is not surprising if one things that at pH 2 small fraction of POPC can be positively charged and a small fraction of POPG can be negatively charged. This would indeed lead to an uneven surface charge distribution of the POPC:POPG liposomes (See Materials and Methods).

3.3. FLIM reveals differences in the interaction between α-La and GVs

Based on our results, we then speculate that different molecular interactions are at the basis of differences in the μm-morphology of lipid-protein structures. If this is true, the analysis of Atto 647 fluorescence signal in terms of lifetimes may give insights in the interactions as well as on the different environment experienced by the fluorophore (covalently bound to α-La). We performed FLIM analysis in terms of the so called “phasor approach” [70] for image analysis. This allows
overcoming fit procedures and provides a global view of the fluorescence decay occurring at each pixel in the images [63,70,71,77–81]. When using this method each pixel of the image is mapped to a point in the phasor plot in relation to the fluorescence lifetime measured. All single exponential lifetimes lie on the so-called “universal circle” (see Materials and Methods). Long lifetimes are located near the origin (0 on the x axis) while short lifetimes are shifted on the circular line toward the bottom right intersection with the x axis (1 on the x axis). This analysis allows distinguishing (without imposing a model) distributions of multi-lifetime species or a mixture of different single lifetime species which result in the appearance of clouds inside the universal circle [71].

We compare Atto 647-labeled α-La in aqueous solution (Fig. 3A, no co-aggregates), 250:1 sample (Fig. 3B) and in 20:1 sample (Fig. 3C) and perform the phasor analysis on those images (see Materials and Methods). We identify three different lifetimes distributions as well-distinct clouds of points in the phasor plot and (cyan, green and red circles, Fig. 3G) allowing the selection of corresponding pixels in the image. Based on this color code, we can identify for each sample the areas in the micrographs corresponding to the specific lifetime distributions (phasor color maps, Fig. 3D, E and F). As expected, for protein in solution the color map shows a uniform cyan signal over the entire image (i.e. same lifetime, Fig. 3D) that is related to the longest (in average) lifetime observed.

Fig. 2. Morphology of Laurdan stained giant vesicle after addition of α-La containing 1:100 Atto 647-labeled α-La. (A–D) Representative images of POPC:POPG GV after α-La addition at (A, B) L/P 250:1 and (C, D) L/P 20:1. Images are the overlap of two-photon microscopy measurements (Laurdan fluorescence emission-green channel) and confocal microscopy images (Atto 647 fluorescence emission- red channel). (E–F) Detail of 250:1 sample after 24 h of incubation with α-La, showing (E) Laurdan fluorescence emission channel and (F) Atto 647 fluorescence emission. Arrows indicate a group of vesicles where protein is absent or barely visible. Two-photon and confocal microscopy images have been acquired in the range 430–505 nm (λexc = 780 nm) and 653–750 nm (λexc = 633 nm), respectively. Scale bar is (A, B, C, D) 2.5 μm and (E, F) 5 μm.

Fig. 3. Phasor analysis of FLIM measurements on Atto 647-signal. GV were incubated for 24 h with α-La containing 1:100 Atto 647-labeled α-La, at (B, E) L/P 250:1 (protein concentration 10 μM) and (C, F) L/P 20:1 (protein concentration 0.8 μM). (A, D) FLIM measurement on 10 μM α-La containing 1:100 Atto 647-labeled α-La in solution (NaCl 0.1 M, pH 2). (A-B-C) Intensity images, showing the different morphology of final structures. Images have a 256 × 256 pixel resolution and image size is (A, B) 30.75 μm × 30.75 μm and (C) 15.08 μm × 15.08 μm. (D–F) Phasor color maps, in which each pixel is colored depending on the lifetime of the dye. (G) Phasor plot of FLIM measurements on Atto 647-signal. Separate Clusters of pixel have been identified in the phasor plot and are highlighted by colored circles; these pixels are mapped in panel D E and F with corresponding colors.
The color map for 250:1 sample shows the majority of pixels highlighted in green (Fig. 3E), while for the 20:1 sample we detect a dominance of red pixels (shortest lifetimes detected). This indicates that the two protein-vesicle samples are characterized by different lifetime distributions of Atto dye. However, it is important to note that in panel E, even if they are minor components, cyan and red pixels are also observed as well as green pixels in panel F. Interestingly, the difference in the lifetime distribution can be related to the differences in morphology within the samples. Pronounced changes in morphology of the protein-vesicle system are likely related to a significant change of the environment experienced by the labeled protein and will then result into a significant modification in the lifetime value when compared to the sole protein in solution. Indeed, when we observe a substantial morphological change of the protein-vesicle system (20:1 sample), the Atto fluorescence lifetime distribution is significantly shifted toward shorter value (red) and the protein is located to the regular, roundish, clustered structures (Fig. 3C and F). The lifetime shift is less pronounced when one looks at 250:1 sample, and the dominant lifetime distribution is represented in green and detected on the structures with modified morphology and resembling the ones reported in Fig. 2A and B. In the same sample and for liposomes maintaining their original morphology, the lifetime corresponds to the one measured for Atto 647-labeled α-La sample in aqueous solution (cyan) (Fig. 3B and E).

The differences in the lifetime distribution can be attributed to an enhanced rigidity or different polarity of the environment in proximity of the dye upon interaction of the protein with the model membrane. Atto fluorescence lifetime is known to weakly depend on the environmental viscosity and to be largely influenced by environment polarity [82]. Namely, differences in fluorescence properties result from differences in polarity between aqueous and lipid phases [83]. We can then speculate that the three distinguishable lifetimes (Fig. 3E-F) correspond to different levels of insertion of the Atto labeled protein in the lipid membrane (green and red) and to a simple adsorption onto the membrane with a protein mostly interacting with solvent (cyan pixels in Fig. 3E). This allows highlighting a marked distinction between regions in which: 1) proteins mainly interact with aqueous phases (cyan, Fig. 3D), 2) proteins mainly interact with lipids (green, Fig. 3E and F) and 3) proteins are confined in a rigid environment and mainly interact with other proteins or with a more compact lipid environment (red, Fig. 3F).

Taking the data in Figs. 1–3 together, it is possible to say that α-La insertion into membrane leads to changes in liposome morphology, being this dependent on the L/P ratio and in line with the literature [13,15,28,45,52,73,74,84,85]. Importantly, structural perturbations of the membrane and lipid reorganization are also dependent on the concentration of the protein partitioned into or adsorbed to the membrane surface [85,86]. Indeed, for globular proteins and below a certain concentration, adsorption to lipid bilayer is suggested to happen along with small membrane reorganization [85]. This is in line with our results in Fig. 3E in which α-La adsorption to POPC:POPG vesicles occurs at low concentrations (α-La 0.8 μM, L/P 250:1), with a limited destabilization of the lipid bilayer as well as a negligible effect on its morphology. The interaction process is likely ruled by electrostatic and hydrophobic interaction [24,45]. Insertion will probably occur into the membrane surfaces only if the protein concentration exceeds the concentration threshold, otherwise adsorption will be the dominant interaction with a consequent low destabilization of the membrane morphology and structure.

3.4. Effect of α-La-GVs interaction on GVs properties

While we can detect the differences in the interaction mode between α-La and GVs (Figs. 1–3), the mechanisms at the basis of the observed phenomenon and how changes in GVs morphology are linked to membrane structural reorganization at the molecular level need to be unraveled. To this aim, we use a combination of both spectroscopy and microscopy methods. We use Laurdan dye to monitor variations in membrane phase properties [67,68] occurring upon α-La addition to the GVs sample (see Materials and Methods). Changes in Laurdan optical properties provided by spectroscopy measurements can be coupled with spatial information by means of two photon microscopy as we previously reported [20,73]. Analysis of Laurdan fluorescence signal allows characterizing membrane structure and investigating the environment-induced changes of the lipid bilayer [67,68]. Indeed Laurdan fluorescence emission spectrum is sensitive to lipid packing and membrane fluidity [67,68]. Changes in the spectrum (i.e. in lipid bilayer fluidity) are estimated in terms of Generalized Polarization (GP, see Materials and Methods) [28,55–65] allowing one to distinguish between liquid (−0.3 < GP < 0.3) and gel phase (GP > 0.5) [68] of the membranes.

Laurdan has been used to investigate the effects on LUV membranes caused by the addition of bovine α-La complexed with oleic acid (BLAOA) and at different pHs [52]. A shift of the fluorescence emission spectrum toward lower wavelengths was observed after the addition of BLAOA to the vesicles, indicating that the lipid bilayer becomes more densely packed with a decreased solvent accessibility to Laurdan probe. Interestingly, it was highlighted that, at acidic pH, both BLAOA and bovine α-La have the same effect on membranes [52]. We previously used Laurdan GP to investigate interaction between proteins and live cells [28] and the effect of α-synuclein addition to GVs. In the latter case we highlighted an increase in membrane rigidity for anionic membranes leading to liposomes burst [73]. Moreover, a decrease in membrane rigidity together with the formation of stable micro-domains with different fluidity for zwitterionic membranes containing cholesterol was also observed [20].

Fig. 4A shows Laurdan GP variations as a function of time in bulk experiments performed in cuvette. We also report GP variations measured for Laurdan dye initially dispersed in a 0.1 M NaCl solution at pH 2, when 10 μM α-La is added using the same experimental protocol (pink circles, Fig. 4B). This measurement is an important control indicating that Laurdan dye also interacts with protein in the same time scale inducing GP growth.

Using steady state fluorescence spectroscopy, we observe that the GVs are initially in the gel phase (Laurdan GP ~0.55, not shown). After adding α-La to GVs, we detect a sudden GP reduction respect to the control value (Fig. 4A, gray) for both L/P 250:1 (Fig. 4A, red) and 20:1 (Fig. 4A, blue). The GP value reaches a steady value after approximately 10 min and for both samples, the signal is reduced if compared to the value for bare GVs, with a larger variation at lower L/P.
Furthermore, the Laurdan fluorescence intensity increases for the protein-membrane systems compared to the control sample, being larger in the sample 20:1 (data not shown). Interestingly, when we add α-La solution to Laurdan the dye fluorescence intensity increases (a 3-fold increase compared to the initial value), showing also a blue shift likely due to changes in the environment of the dye (See SI Fig. S2). Moreover, GP index grows also for the α-La-Laurdan system, resembling the development of the signal in the presence of lipids (Fig. 4B) and reaching a final value lower than the one for bare GVs (0.55). The variation in GP index due to α-La-Laurdan interaction is about four times larger than the one observed when protein is added to stained liposomes. This is not surprising if one considers that this hydrophobic molecule is initially in unfavorable water environment.

The GP reduction observed for Laurdan in lipid membranes and reported in Fig. 4A is likely to reflect a change in membrane fluidity. This indicates that, upon protein addition, an initial change in lipid organization takes place leading to a more disordered phase accessible to solvent. The subsequent growth may be ascribed to further changes in the membrane structure toward a more ordered phase similar to the initial one. However, data in Fig. 4B suggest that a direct interaction between Laurdan and α-La also occurs, leading to the observed GP growth in the first 10 min. Importantly, we note that similar changes were not observed in analogous experiments [20,73] where no interaction between Laurdan and α-synuclein was found. We can then conclude that GP changes in Fig. 4A are potentially due to a redistribution of the dye on both protein and vesicles. In this specific case, the final spectrum of Laurdan (and then GP) may result from the superimposition of at least two main effects arising from both protein and lipid environment around the fluorescent molecule. After the sudden reduction, the increase of GP signal, could be due to the spectral component shifted toward lower wavelengths due to direct interaction between the fluorescent dye and α-lactalbumin. This reflecting a reduced content of water molecules around it. As Laurdan is initially staining lipid membrane, these data together to the ones in Fig. 1 certainly indicate that a large effect occurs within few minutes after α-La addition to GV samples. α-La immediately accumulates at the membrane surface inducing modification in lipid organization, possibly penetrating the bilayer and inducing significant modification on lipid some structures. This resulting in the global reduction of measured GP.

In order to obtain detailed spatial information on the observed phenomenon, we use 2-photon microscopy using Laurdan as extrinsic probe. In Fig. 5, the comparison between representative measurements on Laurdan stained 20:1 and 250:1 samples is shown. Again, in order to show stationary conditions of final state, we show the results on the lipid-protein structure recorded 24 h after protein addition. Control measurements on POPC:POPG giant vesicles in absence of protein are reported in SI (see SI, Fig. S3).

Intensity images for 20:1 and 250:1 samples (Fig. 5A, B and C) show the vesicle morphology, in line with previous data (Figs. 2 and 3). GP maps at pixel resolution were obtained by analysis of the intensity images and GP value for each pixel is reported in a color scale (from blue to red, −1 < GP < +1, Fig. 5D, E and F) together with the related histograms (Fig. 5G).

Despite of the differences in shape, size or fluorescence intensity, GP distribution for all the samples is quite uniform (Fig. 5D–F), showing a slight spatial inhomogeneity in 20:1 samples (Fig. 5E), where some micrometric regions with lower GP are detectable. In particular, the mean value of GP for each map is around 0.55 for initial liposomes, 0.50 for 20:1 sample and 0.53 for 250:1 sample, all of these values being compatible with the lipid bilayers in the gel phase in the whole liposomes. As expected, the decrease in the average GP value is more evident at ratio 20:1 where, according to FLIM measurements, protein insertion is more effective. However, we have to note that, although these data stem for a reduction in the membrane organization and an increased water permeability of the membrane, Laurdan interaction with protein, which leads to an increased GP signal, has to be taken into account.

The width of the histogram remains similar even after α-La addition, with a little shift of the average GP toward small values, more evident at L/P 20:1 (Fig. 5G). These results are in line with what observed in bulk measurement. The presence of protein adsorbed onto the membrane surface could modify water accessibility to Laurdan and the direct interaction between the dye and the protein inserted/adsorbed may also modify the measured GP.

At high protein concentration, the inhomogeneity in the GP spatial distribution is evident, which likely reflects an inhomogeneity of the protein distribution in the final structures. Due to the interaction between Laurdan and α-La (Fig. 4B), the areas with a lower GP value (yellow pixels) reflect an increased fluidity in the membrane. These experimental results are in line with recent results indicating the softening of membrane bilayer upon interaction with α-La at pH 2 [29]. Internalization of the protein and the formation of such hybrid structures may result in an enhanced water permeability of the membrane as suggested by Laurdan measurements, leading to a change of the membrane mechanical properties.

This should result in a structural modification of the protein, which is dependent on the L/P ratio. Changes in Laurdan fluorescence intensity and GP index reported above have to be considered as the superimposition of the effect of 1) Laurdan-protein interaction and 2) changes in lipid packing in the surrounding of the dye. This is in line with fast internalization of protein in the liposome structure leading to hybrid microstructures, whose size and morphology depends on L/P ratio and on the amount of protein accumulated on and into the membrane.

3.5. Effect of α-La-GVs interaction on α-La structure

To verify if the observed changes also affect protein structure, we investigated α-La secondary structure upon interaction with GVs using far-UV circular dichroism. At pH 2, α-La is in the molten globule state, which has native-like secondary structure but a not well-defined tertiary structure [39]. The CD spectrum on α-La sample in solution (Fig. 6, blue dashed line) reveals the presence of both α-helices and β-sheet structures [87], in line with several studies on both Human and Bovine α-La in acidic conditions [24,75,88–92].

In presence of GVs at L/P 20:1 (Fig. 6A), only minor changes in the spectrum can be observed, with an increase of the signal at both 208 nm and 222 nm. On the contrary, for the sample at L/P 250:1 (Fig. 6B) we detect a reduction of the CD signal as well as a dramatic modification of the shape with a pronounced peak at 208 nm. This indicates the loss of the α-helical structure in favor of β-sheets. The marked difference between the two samples is further clear if one looks at the normalized CD spectra (insets in Fig. 6A and B). This is in line with the result obtained by means of FLIM measurements, according to which in the 250:1 sample the population of protein inserted into the membrane is the dominant one (see green pixel, Fig. 3). Importantly, control measurements show that the observed changes in CD spectrum only depend on the L/P, and not on protein concentration (not shown).

We note that the observed modifications in spectroscopy measurements may arise from changes occurring in different sample sub-populations. CD signals in Fig. 6 are the result of the superimposition of two main components: one due to the modified structure of protein interacting with lipid membrane and one ascribable to α-La still free in solution or interacting with other protein molecules. The weights of these two contributions are likely to depend on L/P. At lower L/P, i.e. at the higher protein concentration used in our experiments (10 μM), it is likely that final sample includes both membrane-bound and unbound proteins: the two states would lead to different CD signals, having different secondary structures. At the equilibrium, the signal coming from the unbound proteins could prevail on the other, resulting in a small difference between the initial and the final CD spectrum. At high L/P, i.e. at the lower protein concentration, the clear modification of
the CD spectrum possibly arises by a reduced concentration of “free” α-La not interacting with lipids.

α-La structure in its native or molten-globule state is known to contain two alpha-helix rich hydrophobic cores [93]. In particular, the molten globule is characterized by a core with a native-like packing, well defined secondary structure surrounded by highly mobile peripheral regions [76] and with hydrophobic regions more exposed and flexible than in the native state [39,75,76]. These partially disordered regions are likely the key players in the membrane-protein interaction. Indeed, they may quickly interact with giant vesicles, being easily inserted into the lipid bilayer. Since these regions are mainly located in the alpha-domain, their insertion in the phospholipidic bilayer could be responsible of the reduction of the protein CD signal corresponding to α-helices.

Another possible explanation of the modification in CD spectrum may also arise from changes in aromatic and sulfur side chains contribution in the far-UV region [94]. In particular, a study carried out on helical peptides shows that the presence of aromatic residues results in a positive/negative contribution to CD signal at about 222 nm [95]. The contribution of aromatic residues to far-UV spectrum have been highlighted also in studies about thermal denaturation of equine lysozyme, in which changes at about 222 nm have been ascribed to a local rearrangement of the tertiary and secondary structures and to the transfer of some tryptophan residues to a more hydrophobic environment [96,97]. This can also be the case for the change in Fig. 6, potentially determined by a modified environment for the aromatic residues also affecting their packing. A similar change in the far-UV spectrum was not observed in previous studies focused on α-La interaction with SUV or LUV [24,49,72]. Far-UV spectra from α-La conformers and PC:PG vesicles were found to be similar in shape but higher in signal with respect to those for α-La measured in solution, indicating an increase in α-helical content of α-La upon interaction with vesicles [49]. Changes in the CD signal for protein:lipid systems are also temperature dependent [72].

Either modification in secondary structure or in aromatic packing discussed above is consistent with the idea of a protein insertion driven by hydrophobic interaction. In native α-La, aromatic residues form the two aromatic clusters located in the hydrophobic core of the protein [93]. Although some of them are located in the molten globule core with a native-like packing [76], the insertion of α-La in the lipid bilayer likely results in a change of environment for the aromatic residues located in the flexible regions and in the variation of their structural
packing.

To directly probe changes in aromatic environment experienced during α-La-GVs interaction we monitor α-La intrinsic fluorescence emission. The major contribution to α-La intrinsic fluorescence is due to the four tryptophans (TRP) residues located in different sites of the protein [36]. Changes in intrinsic fluorescence emission spectra reflect variations in the TRP local environment. Moreover, TRP emission is strongly dependent on the polarity of the surrounding environment [98,99]. Intrinsic fluorescence of low pH α-La molten globule state has been previously characterized and it resulted red-shifted with respect to the one typical of the native state [24,75,88], indicating the exposure of TRP residues. In particular, according to Lala et al. [75], two of the four TRPs are exposed to the solvent.

We observe an increasing in intensity and a blue-shift of the spectrum both for 20:1 and 250:1 samples (Fig. 7A and B, straight lines), being these changes more pronounced for the 250:1 sample. A blue-shift of TRP emission spectrum indicates a less polar environment and both a decrease or increase of TRP emission quantum yield may occur upon conformational changes [100–104]. These data are consistent with the idea that partially disordered hydrophobic regions insert into the phospholipidic bilayer. The blue-shift reveals that tryptophan residues in the new formed structures are buried compared to their solvent exposure typical in the molten globule.

An increase in intrinsic fluorescence intensity is also reported for α-La upon protein interaction with LUVs or SUVs at pH 4.5 occurred [13,26,49,72], indicating the disappearance of protein tertiary interactions that quench the fluorescence in the native state [49]. The extent of fluorescence intensity increase was shown to be dependent on vesicle composition [13]. The intrinsic fluorescence maximum was found both red-shifted and blue-shifted depending on the considered α-La conformer [26,49], as well as on vesicle composition and temperature [72]. At pH 2, the fluorescence spectrum of DMPC-bound α-La was blue-shifted compared to the one of free protein, indicating that TRP residues were less accessible in the membrane-bound case [44]. Our results (Fig. 7A and B) obtained at pH 2, where α-La is in the highly plastic molten globule state, are in agreement with those obtained by Cawthorn et al. [44]. This proves the high reactivity of α-La to subtle changes in the physico-chemical conditions of the surroundings, probably affecting its function. The difference in the extent of spectral variation, in both maximum position and fluorescence intensity is likely due to the presence of two dominant components of the spectrum ascribable to two distinct protein conditions. In the 250:1 sample the major component of the spectrum is probably represented by protein molecules interacting with membrane, whilst in the 20:1 sample a large contribution may also be due to proteins in solution or interacting with neighbors proteins. Intrinsic fluorescence measurements clearly suggest the insertion of α-La into the lipid bilayer and the formation of α-La–GV structures with TRPs being less exposed to the solvent if compared to the molten globule state. Moreover, in the 250:1 sample almost all protein is inserted in the bilayer.

4. Conclusions

The ability of proteins of interacting with membranes is at the basis of cellular function and dysfunction. Analyzing and quantitatively monitoring this phenomenon is extremely challenging. This is mainly due to the fact that, in complex systems as the biological ones, molecules of different nature interact and form new hybrid structures with different properties. Our work shows the formation of new hybrid lipid-protein structures, from α-La interacting with model liposomes. Hydrophobic forces appear to be the driving forces of adsorption/insertion of the protein on/into the membrane, being the latter dependent on lipid/protein ratio.

After addition of α-La molten globule to POPC:POPG giant vesicles, protein molecules accumulate on accessible liposome surfaces and cause an immediate destabilization of the lipid bilayer, modifying vesicles morphology and inducing vesicles budding. The change in morphology is likely due to a fast initial adsorption followed by protein insertion into the membrane. The combination of electrostatic interactions of the protein with the polar heads of lipids and hydrophobic interactions with the lipid chains results in a quick adsorption of α-La to lipid bilayer. At high concentration, protein molecules are located at the membrane surface and cover all accessible regions: above a critical protein surface density, the insertion into the lipid bilayer takes place, mainly driven by hydrophobic interactions, squeezing liposomes and inducing their compaction and eventually a collapse into full, regular and roundish structure. A homogeneous distribution of roundish structures is observed at high protein concentration, whereas structures with a more heterogeneous morphology are found at low protein concentration. Simultaneously, changes in the membrane fluidity occur. Laurdan GP analysis reveals that the interaction with protein leads to a decrease of the final GP value, attributable to changes in the membrane fluidity toward a more disordered lipid phase. This can be related to a membrane softening caused by the reorganization of the lipids around proteins leading to water penetration. Importantly, α-La interacts with Laurdan dye also in the absence of lipid membranes, with the effect of inducing an increase in the GP index. As a consequence, the overall GP variations are ascribable to a redistribution of the dye on both protein and vesicles. To the best of our knowledge, this effect was not reported before and has to be taken into account when quantitative information of membrane physical stability in presence of protein needs to be obtained. Indeed, GP calculations are done at fixed wavelengths (or...
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