STUDY OF THE INTERACTIONS OF A MAJOR RUBBER PARTICLE PROTEIN (REF1) WITH SYNTHETIC PHOSPHOLIPIDS IN LANGMUIR MONOLAYERS

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Abstract

Rubber Elongation Factor (REF1) protein is located on the surface of rubber particles (RPs). As a RP-bound and hydrophobic protein, REF1 is suspected to be only partially eliminated by the water washing occurring during latex processing into raw natural rubber (NR) in the form of unsmoked rubber sheet (USS) and could thus affect raw NR properties. In this work, REF1 was identified as an abundant protein of raw NR and biophysical methods were used to describe the organization/structure of REF1 at the surface of RPs interacting with the lipid monolayer surrounding the poly-isoprene core. Therefore, an approach in Langmuir film was implemented to investigate the interactions between recombinant REF1 and synthetic phospholipids (POPC, POPG and POPA) by fluorescence (calcein-leakage measurements), ellipsometry, Brewster angle microscopy, and PM-IRRAS spectroscopy. REF1 was shown to interact differently depending on lipid headgroup type: no interaction with POPC, intermediate interactions with POPG and strong interactions associated to a conformational switch from α-helices to β-sheets with POPA.

Keywords: Hevea brasiliensis rubber particle; Langmuir monolayer; Rubber elongation factor; Synthetic phospholipids.

INTRODUCTION

Latex from *H. brasiliensis* is a suspension of rubber particles (RPs) and lutoids in cytoplasmic serum. The micrometric and spherical RPs are made of a poly-isoprene core surrounded by a lipid/protein biomembrane [1,2]. When latex is processed into natural rubber (NR), it remains obscure whether RPs keep their structure and/or composition in dry NR. This question is crucial as non-isoprene molecules including proteins, lipids, carbohydrates and minerals are known to impact the final properties of NR. Recently, Wu *el al.* characterized latex and NR samples with a super-resolution fluorescence imaging technique and suggested that there is some interactions between proteins and lipids in dry NR [3]. Therefore, a precise description of the RP membrane at latex stage is required to better apprehend the interactions between poly-isoprene chains, lipids and proteins occurring in NR.

Among non-isoprene molecules, proteins and lipids are known to be involved in NR structure [4-7]. Qualitative and quantitative analysis of lipid contents in both latex and NR have been reported [8,9], but proteins of raw NR have received little attention in comparison to latex proteins. Moreover, while protein content in NR is documented [10,11], much less is known about protein composition of NR. Some authors extracted proteins from end-products such as chirurgical gloves and performed SDS-PAGE electrophoresis gels of protein extracts [12] but little is known regrading SDS-PAGE profiles of extractible protein from raw NR grades.

Rubber Elongation Factor (REF1[1], Accession Number P15252) is an abundant protein in latex [14]. As a strong allergen, REF1 (also named Hevb1), was intensively studied to solve its allergenicity issue in healthcare facilities [15,16]. At first, REF1 was detected on large RPs (diameter > 0.4 μm), but it is now established that it can be found on small RPs (diameter < 0.4 μm) as well [16-19]. As a RP-bound and hydrophobic protein [20], REF1 is suspected to be only partially eliminated by the water washing occurring during latex processing into raw NR and might be present in NR in significant amount, thus affecting raw NR properties.

Extensive works have been carried out to elucidate the organization/structure of REF1 protein at the surface of RPs at latex stage [21,22]. An original approach consisted in mimicking the lipid/protein membrane surrounding the poly(cis-1,4-isoprene) core of the rubber particle by lipid/protein Langmuir monolayers [21,22]. This strategy was recently applied to investigate the interactions of recombinant REF1 protein with native lipids extracted from latex [23].

In this work, REF1 was firmly identified among proteins extracted from raw NR (in the form of an unsmoked rubber sheet). Using gel-enhanced liquid chromatography coupled with tandem mass spectrometry (Ge-LC-MS/MS) and database search program, REF1 was found to be the major protein of the most intense band of the SDS-PAGE gel performed on the NR protein extract. This finding confirmed the importance of characterizing the interactions of REF1 protein with Langmuir monolayers made of various lipids.

Three synthetic phospholipids were then selected (*i.e.* POPC, POPG and POPA) and their interactions with recombinant REF1 protein were characterized by calcein-leakage measurements, polarization modulated- infrared reflection adsorption spectroscopy (PM-IRRAS), ellipsometry, and Brewster angle microscopy (BAM). Surprisingly, no interaction was detected with POPC, although phosphatidylcholine specie (PC) amounts to ~80% of total phospholipids of latex [9]. Calcein-leakage experiments applied on liposomes confirmed the absence of interaction of REF1 with POPC and the strong interaction with POPA. While PM-IRRAS spectra indicated no conformation change of REF1 with POPG (α-helices), a strong variation of amide I band was observed with POPA attributed to modification of secondary structure of REF1 from α-helices to β-sheets. Other indicators including surface pressure, monolayer thickness variation.

MATERIALS AND METHODS

1. Latex collection

Hevea brasiliensis rubber trees of RRIM600 clone planted in 2002 were selected from a plantation belonging to Union Rubber Co. Ltd., Chanthaburi, Thailand. Since opening of trees, the tapping system applied was S/2, 2d3. Trees were tapped in early morning (5 AM) and latex was let flow for 1 to 2 hours until the required volume of latex was collected, *i.e.* 3 L to prepare one single USS (unsmoked rubber sheet). Lattices from 10 trees were pooled together and filtered through a stainless steel sieve of 2 mm mesh size.

2. Unsmoked rubber sheet (USS) manufacturing

Unsmoked sheet (USS) sample was prepared on plantation from 3 L fresh latex according to the RRIT technical recommendation for rubber sheet making process [24].

[1] The notation of Tang *et al.* [13], *i.e.* REF1, was used to name the major isoforms of REF family.

3. Protein extraction from USS, protein separation by SDS-PAGE and protein identification by LC-MS/MS

3.1. Extraction of proteins from USS

100 mg USS in the form of small rubber pieces were solubilized in 40 mL of a solution made of cyclohexane/ethanol mixture (39:1, v/v). Sample was let solubilized for 3 days in a water bath at 35°C and then centrifuged at 10000 x g for 30 min at 20°C. The supernatant was poured in a glass bottle while the pellet was collected with a spatula and let dry for 1-2 days under hood until complete evaporation of solvent. The dried protein extract was then collected and stored in an Eppendorf at -20°C.

3.2. Separation of proteins by 1D electrophoresis sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The proteins contained in the rubber protein extract were separated through one dimension (*i.e.* molecular weight) by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as previously described [25] and the gel was analyzed by CLIQS image analysis software (http://totallab.com/cliqs/).

3.3. Protein identification by gel-enhanced liquid chromatography coupled with tandem mass spectrometry (Ge-LC-MS/MS)

The most intense protein band from SDS–PAGE gel was manually cut from the gel and excised to smaller pieces of approximately 1 mm-cubes. Proteins were subjected to in-gel tryptic digestion and analysis of peptides was performed by nanocapillary liquid chromatography and tandem mass spectroscopy (LC-MS/MS) as previously described [26,27]. Peptide mass data were analyzed by Mascot (Matrix Science) database search program.

4. Expression and purification of recombinant REF1 protein

Recombinant *Hevea brasiliensis* REF1 protein was produced in *Escherichia coli* and purified as previously described [22]. Protein aliquots were kept at -80 °C. Protein sample was defrozen just before experiments and kept on ice before injection in the Langmuir trough to prevent its aggregation.

5. Synthetic phospholipids

Synthetic lipids (Avanti, USA) used for Langmuir film studies were 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphate (sodium salt) (POPA), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), and 1-palmitoyl -2-oleoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) (sodium salt) (POPG).

6. Characterization of the interactions between lipids and REF1 protein

6.1. Calcein-leakage measurements on large unilamellar vesicles (LUVs)

Calcein-leakage experiments were performed by monitoring the fluorescence signal after adding REF1 protein to large unilamellar vesicles (LUVs) made of POPA, POPC and POPG lipids. The ability of REF1 to interact with LUVs results in a destabilization of the membrane of LUVs and thus in a calcein release in the medium which is recorded by fluorescence. LUVs preparation and calcein-leakage experiments were performed as previously described [21]. Fluorescence measurements were made with a microplate reader (TECAN infinite M1000PRO). After the addition of the reactants, the 384-well microplate (Grenier Flat bottom, black polystyrene) was shaken just before measurement. Data were collected every 1 h at 25 °C, λ excitation at 485 nm and λ emission at 515 nm. Lipid concentration was set at 100 µM and concentration of REF1 varied from 0.01 µM to 50 µM (total volume 30 µL). After 24 h, 1 µL of 10%Triton X-100 (Sigma) solution was added to achieve complete liposome leakage. The percentage of calcein release was calculated according to the following equation: $L(t) = [(Ft - F0) / (Fmax - F0)] * 100$ where L(t) is the percentage of the calcein released (%), Ft is the measured fluorescence intensity at time t, F0 is the fluorescence intensity at time t = 0 and Fmax is the fluorescence intensity after addition of Triton X-100.

6.2. Surface tension

Adsorption of REF1 into lipid monolayers was followed by surface pressure (Π) measurement. Experiments were performed at 25 ± 1 °C on a circular Teflon trough of 20.4 cm^2 filled with 8 mL of subphase (TBS 1× buffer pH 7.5). The surface pressure (Π) was measured with a plate of Whatman filter paper held by a Nima Wilhelmy balance. The interaction of proteins with lipid films was performed in two steps. First, the lipids were spread at the air-buffer interface from chloroform/methanol (4:1 v/v) solution at 1 $mg.mL^{-1}$ to reach the desired surface pressure (around 28 $mN.m^{-1}$). Second, the protein REF1 was injected at a final concentration of 2 µM into the subphase using a microsyringe. The surface pressure was measured continuously during protein adsorption in the lipid monolayer until an equilibrium pressure was reached (plateau).

6.3. Ellipsometry and Brewster angle microscopy (BAM)

The thickness of the films formed at the air-buffer interface was determined on a 6 mL Teflon trough using a NFT IElli2000 ellipsometer (Göttingen, Germany) equipped with a doubled frequency Nd-Yag laser (532 nm, 50 mW), a polarizer, an analyzer, and a CCD camera. The imaging ellipsometer works at an incidence angle close to the Brewster angle (54.58°) and it operates on the principle of classical null ellipsometry. The morphology of films at the air-buffer interface was observed by the CCD camera. The spatial resolution was about 1 µm and the size of BAM images was 450×600 µm with the ×10 magnification objective used. The angles of the polarizer, compensator, and analyzer that obtained the null condition allow one to get the (Δ, Ψ) ellipsometric angles which are related to the optical properties of the sample [28,29]. For ultrathin films, Δ is proportional to the film thickness. The value of the film thickness mainly depends on the refractive index used. Since it is difficult to determine an accurate experimental refractive index value, we used the same average value of the refractive index 1.45 for both lipid layers and proteins to perform thickness estimations. The BAM pictures presented in the various figures were corrected from the tilt angle observation. Note that a shutter was used with various timing to avoid camera saturation.

6.4. Polarization modulated-infrared reflection adsorption spectroscopy (PM-IRRAS)

Polarization modulated-infrared reflection-adsorption spectra were recorded on a Nicolet (Madison, WI) Nemus 870 spectrometer equipped with a HgCdTe (MCT) detector (SAT, France) and cooled at 77 K by liquid nitrogen at a resolution of 8 cm^{-1} by adding 600 scans. Details of PM-IRRAS experiments were previously described [21,30]. PM-IRRAS spectra were normalized by the TBS 1× buffer spectrum or lipid spectrum. The room temperature was regulated at 25 ± 1 °C. Each experiment was repeated at least 2 times.

RESULTS AND DISCUSSION

REF1 is found in large amount in extractible proteins of NR

In this work, proteins were extracted from rubber in the form of USS (unsmoked rubber sheet) by solubilizing rubber pieces in cyclohexane containing 2.5% ethanol. Indeed, ethanol was shown to increase the solubility of rubber pieces in toluene [31] which could help to release proteins from rubber matrix. Protein standard solution and NR protein extract were subjected to SDS-PAGE electrophoresis and the obtained gels are presented in Lane 1 and Lane 2 of **Figure 1A**, respectively. The standard containing six proteins of known molecular weights (MW) provided the calibration curve allowing to assign MWs to migration distances (Rf) of protein bands.

Four bands were detected on the gel of NR proteins (**Fig. 1A,** Lane 2) by image analysis software. They were assigned at 34.2 kDa, 30.3 kDa, 27.7 kDa and 14.7 kDa (**Fig. 1B**). The intensity of the band assigned at 14.7 kDa (circled in red on **Fig. 1A**) appeared to be much more intense than others. The assigned MW of this band suggests the presence of REF1 (Rubber Elongation Factor, MW: 14.7 kDa), the major isoform of REF protein found in *Hevea* latex [13]. To verify this assumption, the band was manually cut from the SDS-PAGE gel and proteins contained in this band were subjected to trypsin-digestion. Peptides were analyzed by LC/MS-MS and mass data search by Mascot is summarized in **Table 1**.

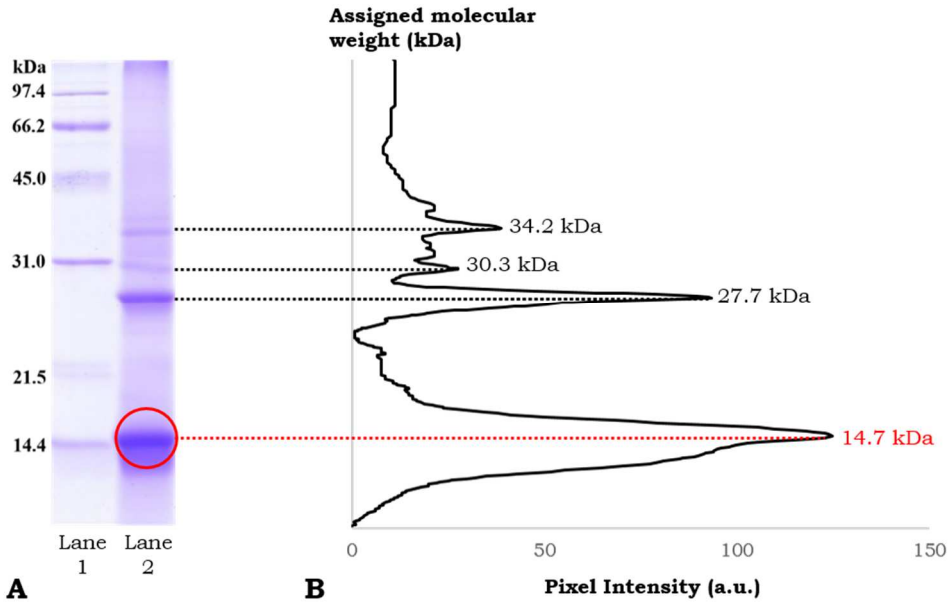

Figure 1. A) SDS-PAGE gels of protein standard (Lane 1) and raw NR protein extract (Lane 2). Known molecular weights of the six proteins contained in standard appear on left side (kDa). B) Intensity profile of lane 2 of the gel shown on Fig.1A with assigned molecular weights indicated for the four detected bands.

Table 1. Proteins identified by LC-MS/MS in the most intense band of SDS-PAGE gel obtained from proteins extracted from USS rubber (red-circled band on lane 2 of gel shown in Fig. 1A). Mascot search parameters: Database = SwissProt, Taxonomy = Viridiplantae (Green Plants).

Accession No.	Identification	MW (kDa)	Isoelectric point (pI)	Score	Exponentially modified protein abundance index (emPAI)	Other names
P15252	Rubber Elongation Factor (REF)	14.713	5.04	3981	2.58	REF1, Hev b 1
O82823	Small Rubber Particle Protein (SRPP)	22.331	4.80	98	0.15	SRPP1, Hev b 3

Two proteins were identified in the most intense band of SDS-PAGE including Rubber Elongation Factor (REF1) and Small Rubber Particle Protein (SRRP1). The values of score and exponentially modified protein abundance index (emPAI) [32] indicated that REF1 was very abundant in the band while SRPP1 was only present as trace. Both SDS-PAGE gel and LC-MS/MS data indicated that REF1 protein is present in large amount in NR sample confirming the importance to better describe the organization/structure of this protein at the surface of RPs where it interacts with the lipid monolayer surrounding the poly-isoprene core. An approach in Langmuir film was applied in the second part of this study to investigate the interactions between recombinant REF1 protein and synthetic phospholipids.

REF1 interacts strongly with POPA while no interaction is detected with POPC

First, calcein-leakage experiments were led to evaluate the ability of REF1 to interact with lipids in the form of large unilamellar vesicles (LUVs). A strong interaction between protein and lipid corresponds to high calcein leakage due to the destabilization (*i.e.* bursting) of LUVs. Then, REF1 protein was injected in the subphase at 2 µM below lipid monolayers

stabilized at an initial surface pressure of about 28 $mN.m^{-1}$. When REF1 adsorbs into lipid monolayer, the surface pressure (Π) increases regularly until a plateau is reached. The difference between the initial surface pressure of the lipid (Π_i) and the surface pressure reached at the plateau (Π_p) provided the surface pressure increase ($\Delta\Pi = \Pi_p - \Pi_i$). In addition, ellipsometry coupled to Brewster angle microscopy (BAM) allowed the determination of the film thickness and the visualization of the film at micrometer scale (450 x 600 μm^2). Finally, PM-RRAS spectra provided information on the secondary structure and orientation of REF1 protein when it interacts with lipids.

The above-described methods were applied to REF1 interacting with three synthetic phospholipids including POPC (zwitterionic), POPA (negatively charged) and POPG (negatively charged). Calcein-leakage experiments were performed with solution of LUVs at 100 µM and REF1 protein with concentrations varying from 0.01 to 50 µM. The level of calcein in the medium was measured by fluorescence at time T_0 (*i.e.* time when the experiment was started) and at time T_0+24h (**Table 2**). For REF1 with POPC, a very slight interaction was detected only for a REF1 concentration of 50 µM while intermediate and strong interactions were highlighted for POPG and POPA, respectively.

Table 2. Calcein release from LUVs made of POPG, POPC and POPA lipids at 100 µM in the presence of REF1 protein injected at concentrations ranging from 0.01 to 50 µM. Dark and bright colors correspond to calcein release recorded at T_0 and T_0+24h, respectively.

	% of calcein release in REF1 protein at several concentrations							
	REF1 0.01 µM	**REF1 0.05 µM**	**REF1 0.1 µM**	**REF1 0.5 µM**	**REF1 2 µM**	**REF1 10 µM**	**REF1 20 µM**	**REF1 50 µM**
POPG leakage at T_0	2.0	4.7	6.5	9.7	11.7	27.3	31.3	47.7
POPG leakage at T_0+24 h	1.9	5.2	13.8	28.7	32.6	56.7	63.6	78.0
POPC leakage at T_0	0	0	0	0	0	0	0	3.0
POPC leakage at T_0+24 h	0	0	0	0	0	0	0	5.0
POPA leakage at T_0	1.2	2.4	3.6	4.5	5.6	8.8	16.3	30.8
POPA leakage at T_0+24 h	5.1	4.5	8.9	24.4	43.8	48.0	59.9	75.5

Before REF1 injection in the subphase, thickness of lipid monolayers compressed at 28 $mN.m^{-1}$ were determined and reported in **Table 3**. All lipids displayed thickness in the range of 14-16 Å (14.4 ± 0.8 Å, 14.3 ± 1.4 Å and 15.8 ± 1.6 Å for POPA, POPC and POPG, respectively) which is in agreement with formation of lipid monolayer [21,23].

The surface pressure increases induced by REF1 adsorption in lipid films are listed in **Table 3**. The strongest increase in surface pressure was recorded with POPA: 2.5 ± 1.3 $mN.m^{-1}$, a value in the same range as the one measured for REF1 interacting with native phospholipids (PL) from latex [23]. The values obtained for POPG (0.8 ± 0.3 $mN.m^{-1}$) and POPC (1.4 ± 1.0 $mN.m^{-1}$) suggest that there is poor insertion of REF1 in the lipid monolayer.

Thicknesses of REF1/lipid monolayers are listed in **Table 3** and showed a great disparity depending on the lipid nature. While the thickness of REF1/POPA monolayers was 77.7 ± 0.8 Å, the ones of POPG and POPC were 62.1 ± 1.0 Å and 14.4 ± 3.0 Å, respectively. In the case of POPC, the thicknesses of lipid monolayer and REF1/lipid monolayer were similar confirming the absence of interaction between REF1 and POPC. For POPG, although the surface pressure increase was low (0.8 ± 0.3 $mN.m^{-1}$), a high increase in film thickness was noticed (+ 46.3 Å) suggesting that REF1 interacts slightly with lipid headgroups without insertion (no increase in surface pressure) and accumulates beneath the lipid monolayer (increase in film thickness). The strong interaction between POPA and REF1 was confirmed by the strong increase in film thickness (+ 63.3 Å).

Table 3. Thicknesses of lipid and mixed REF1/lipid monolayers and increases in surface pressure (ΔΠ) due to REF1 adsorption (2 µM) under lipid monolayers. The star symbol (*) indicates data previously obtained with native phospholipids of latex [23].

	Layer thickness and increase in surface pressure (ΔΠ)		
	Lipid monolayer	Lipid monolayer in interaction with REF1 protein	
	Thickness (Å)	Thickness (Å)	ΔΠ (mN.m^{-1})
POPA	14.4 ± 0.8	77.7 ± 0.8	2.5 ± 1.3
POPC	14.3 ± 1.4	14.4 ± 3.0	1.4 ± 1.0
POPG	15.8 ± 1.6	62.1 ± 1.0	0.8 ± 0.3
Native PL*	14.5 ± 1.5	51.7 ± 10.9	2.2 ± 0.1

PM-IRRAS spectra of REF1 (2 µM) adsorbed at air/buffer interface and at lipid/buffer interface are presented in **Figure 2A**. On the PM-IRRAS spectrum of REF1, the amide I band is a broad band centered at 1650 cm^{-1} with shoulders at 1630 cm^{-1} and 1690 cm^{-1} indicating that several secondary structures coexist in REF1 at the air/buffer interface, mainly α-helices (1650 cm^{-1}) as well as β-sheets (1630 cm^{-1}, 1690 cm^{-1}) and turns (1670 cm^{-1}). No amide I band was detected for REF1/POPC indicating the absence of interactions between those components in this range of concentration. The shape of REF1/POPG spectrum (*i.e.* position of amide I and II bands, ratio between intensities of amide I over amide II) indicated that REF1 kept its secondary structure in α-helices without change in the helices orientation. In contrast, a strong change was observed for REF1 interacting with POPA monolayer. The amide I band position was clearly shifted toward lower wavenumbers indicating that REF1 switched from an α-helice to a β-sheet conformation in the presence of POPA. The BAM pictures presented in **Figure 2B** show that REF1 adsorbs in a homogeneous manner with all studied phospholipids. No aggregation or segregation was noticed.

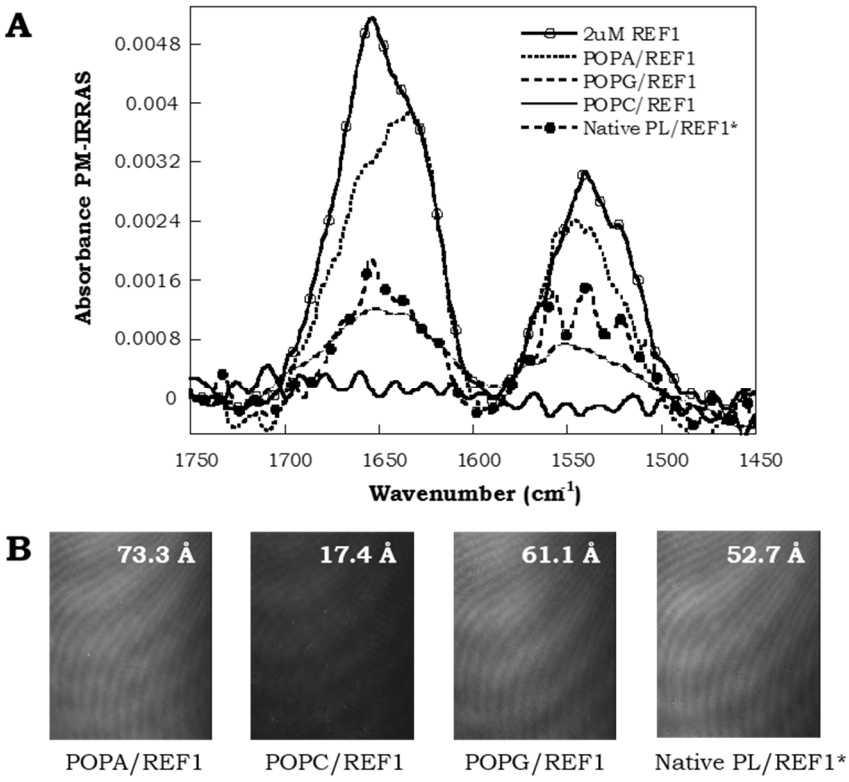

Figure 2. A) PM-IRRAS spectra of REF1 protein film and mixed REF1/phospholipid monolayers recorded with POPA, POPG and POPC lipids. B) BAM pictures (450 × 600 µm²) of REF1/phospholipid films captured when the surface pressure reaches a plateau with a shutter timing adjusted to 250, 50, and 120, respectively. Film thicknesses (Å) are indicated in white color on each picture. The star symbol (*) indicates data previously obtained with native phospholipids of latex (shutter timing at 120) [23].

Surprisingly, all experiments conducted in this study indicated that there is poor interaction between REF1 and POPC (lipid with zwitterionic headgroup) in the form of monolayer or vesicle (LUV). This is remarkable especially as REF1 was already shown to interact with POPC on dot blots [21]. Phosphatidylcholine specie (PC) amounts to ~80% of total phospholipids of RRIM600 latex followed by 11% phosphatidic acid (PA), 5% phosphatidylinositol (PI) and 4% phosphatidylethanolamine (PE) [9]. The weak interaction between REF1 and PC specie could explain why the interaction of REF1 with native phospholipids [23] is rather weak as compared to synthetic POPA.

Interactions of REF1 with POPG and POPA (lipids with negatively charged headgroups) were highlighted suggesting a strong contribution of electrostatic force. In particular, drastic effects were observed for REF1/POPA including: high surface pressure increase, high thickness increase of monolayer, high calcein leakage of LUVs and strong PM-IRRAS signal. The lower steric hindrance of PA headgroup as compared to larger PG headgroup could induce stronger REF1/lipid interactions. Moreover, a switch of REF1 secondary structure from α-helices to β-sheets was observed with POPA. This phenomenon was already observed with neutral lipids from latex but in this case, the conformational switch was accompanied by strong aggregation [23] which was not observed in the present work with POPA.

CONCLUSION

In this work, we clearly identified REF1 protein in large amount in dry NR (USS). REF1 was shown to interact differently with synthetic and native phospholipids depending on lipid headgroup type: poor interaction with synthetic POPC, intermediate interactions with synthetic POPG and native PLs [23] without conformational change of the protein, strong interactions associated to a conformational switch from α-helices to β-sheets without and with aggregation for synthetic POPA and native NLs [23], respectively. The ability of REF1 to interact differently depending on the lipid headgroup might result in different structuration of REF1 protein at the surface of RPs and in dry NR.

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