

Article

A QCM-D Study of the Interaction of Early Endosomal Antigen 1 (EEA1) Protein with Supported Lipid Bilayers Mimicking the Early Endosomal Lipid Composition

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Abstract

The combination of supported lipid bilayers (SLBs) with the Quartz Crystal Microbalance with Dissipation monitoring (QCM-D) has been proven to be a powerful tool to simultaneously monitor mass and viscoelastic changes related to membrane binding-events. In this work, the above methodology is employed for the study of the interaction of the Early Endosomal Antigen 1 (EEA1) to a model lipid bilayer that mimics the early endosome (EE) membrane, focusing on the membrane composition. Starting with the formation of a lipid bilayer through the vesicles fusion technique, we investigated the formation of SLBs that incorporate phosphatidylinositol 3-phosphate (PI(3)P), a key component for EEA1 binding, in combination with other lipids, e.g., (1,2-dioleoyl-sn-glycero-3)-phosphocholine (DOPC), -phosphoserine (DOPS), -phosphoethanolamine (DOPE), and cholesterol (Chol). The interaction of the full-length coiled-coil EEA1 to the formed SLBs was further studied in real time with the QCM-D and characterized with respect to the lipid composition and pH. Our findings confirm that PI(3)P is essential for the EEA1–membrane interaction, while it was shown that Chol and phosphatidylserine greatly influence the binding event. In fact, including 30% Chol in a PI(3)P (3%):PS (6%) SLB resulted in almost double EEA1 binding than in the absence of Chol. Moreover, we employed the QCM-viscoelastic model available to analyze the QCM-D data with emphasis on the study of the protein conformation. Our results showed that, in our in vitro system, EEA1 is not fully extended and/or highly packed, but is mainly in a bent, distorted conformation with an average size close to 100 nm. This study complements previous works employing in vitro assays, also demonstrating the ability to reconstitute more complex biomimetic EE membranes containing inositol phospholipids on a QCM surface for the study of EEA1 binding.

Keywords: Early Endosomal Antigen 1; Quartz Crystal Microbalance with Dissipation; early endosomal lipid membranes; viscoelastic modeling; supported lipid bilayer; phosphatidylinositol 3-phosphate; protein–membrane interactions



Academic Editor: Maria João Moreno

Received: 18 December 2025

Revised: 9 January 2026

Accepted: 14 January 2026

Published: 26 January 2026

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1. Introduction

The reconstitution of a biologically relevant membrane in a biophysical model system is of major importance for the study of lipid–protein interactions and membrane-binding events, providing both qualitative and quantitative information. A significant tool for the above studies is the successful formation of a supported lipid bilayer (SLB) on a solid surface, normally combined with a surface-sensitive technique, such as microscopy (such as atomic force or fluorescence) [1–3], neutron reflectometry [4], optical (surface plasmon resonance or dual polarization interferometry) [5,6], acoustic (quartz crystal microbalance or surface acoustic wave) biosensors [7,8], or a combination of the above [3,4]. Examples of such studies include the interaction of SLBs with nanoparticles [9], anesthetic compounds [10], antimicrobial peptides [11], or cytokines [2].

Early Endosomal Antigen 1 (EEA1) is an effector of the small GTPase Rab5 that is localized to early endosomal (EE) membranes via binding to the phosphatidylinositol 3-phosphate (PI(3)P) lipid. It functions as a tether of endocytic vesicles that fuse with EEs. EEA1 is a long coiled-coil protein (1411aa) with a characteristic domain organization. It contains two zinc finger domains localized at the *N*- and *C*-termini separated by heptad repeats that are predicted to form a homodimeric parallel α -helical coiled-coil [12]. The *N*-terminal N_2C_2 zinc finger domain has been shown to bind Rab5 while the *C*-terminal FYVE zinc finger domain has been shown to bind PI(3)P lipids with high specificity [13]. Structural studies have revealed that each 325kDa EEA1 homodimer binds two distinct PI(3)P head groups in its association with the EE membranes. The interaction of the FYVE domain with PI(3)P-membranes is found to be multivalent, involving dimerization, activation of the histidine switch, non-specific electrostatic interactions with acidic lipids, and hydrophobic insertion into the membrane [6,14]. In addition to PI(3)P, the EE membrane is composed of phosphatidylcholine (PC), negatively charged lipids such as phosphatidylserine (PS), phosphatidylethanolamine (PE), and sphingolipids. Cholesterol (Chol) is another critical component in regulating membrane structure and lipid organization, and is important for the formation of liquid-ordered membrane microdomains [15–17]. The work of McBride et al. [18] proposes that EEA1 assemblies in oligomers on the EE membrane and further interactions with SNARE proteins lead to membrane fusion. Furthermore, ultrastructural analysis of EEA1 *in vivo* suggests a dense filamentous network on EEs in HeLa cells [19,20]. Moreover, the interaction of EEA1 with PI(3)P-containing membranes was recently described during the “trigger-and-convert” mechanism where endosomal interaction is underpinned by EEA1 binding to EEs through the *C*-termini [21].

The Quartz Crystal Microbalance with Dissipation monitoring (QCM-D) is a useful tool for studying membranes and protein–membrane interactions. It is based on the real-time monitoring of the frequency (*f*) and dissipation (*D*) of an acoustic wave propagating within a thin piezoelectric crystal. As the QCM crystal is driven to oscillation at the fundamental frequency (here 5 MHz), the Sauerbrey equation [22] can be used to calculate the adsorbed mass (Δm) of the elastic thin film from the relevant frequency change (Δf), as a result of the proportionality between Δm and Δf [23]. However, if the adsorbed mass layer is not elastic but deforms during oscillation, dissipating acoustic energy, the above equation does not hold true and the viscoelastic film approach is adopted. In this case, the frequency and dissipation monitored in the fundamental and various overtone frequencies ($n = 1$ –13) can be used in combination with a viscoelastic model to calculate the mass and thickness of the biofilm, as well as the elastic shear modulus and film viscosity [24–27]. There are several models that interpret the generated data of the formation of a viscoelastic film depending on the type of the adsorbed mass, i.e., whether it is considered a homogeneous or heterogeneous film, including the Voigt, FEM, and Voinova models [27,28]. In an alternative approach, bound biomolecules are treated as independent,

discrete entities attached through a single point, rather than a film. In this model, the acoustic ratio of $\Delta D/\Delta f$ (the change in measured dissipation per surface-coupled unit mass) has been correlated to hydrodynamic parameters such as the intrinsic viscosity ($[\eta]$) of the attached biomolecule, where $[\eta]$ is the direct measure of molecular conformation (i.e., shape and size) [29,30]. This methodology has been used with QCM-D to quantify the length, curvature, and helical structure of DNA molecules [31], as well as the conformational transitions of proteins [32] and characterization of liposomes bound to DNA [33].

The QCM-D technique has been used extensively for the study of SLBs for subsequent membrane-binding events. The mechanism of the formation of an SLB on a hydrophilic surface via the vesicle fusion technique was first proposed in 1998 [34]. Moreover, several studies reported the use of vesicles of various lipid compositions to form an SLB [1,8,35,36], including phosphatidylinositol (PIP) lipids [2,37], e.g., phosphatidylinositol-4,5-bisphosphate (PI(4,5)P₂) [4,38] or phosphatidylinositol-3',4',5'-triphosphate (PIP₃) [37]. Some of the above PIP-containing SLBs have served as a biomimetic membrane to study the specific binding of protein molecules on PIP-containing SLBs [2].

In this study, we created an SLB model system mimicking the EE membrane through vesicle fusion on a hydrophilic SiO₂-coated acoustic device, while monitoring the interaction in real time using the QCM-D technique. To mimic the EE membrane, vesicles of various lipid compositions were employed, while the resulting SLBs were tested for EEA1 protein binding. The lipid composition of the vesicles was chosen based on the lipid composition of an enriched early endosomal fraction determined in a previous study [39]. The specificity of the PI(3)P-FYVE domain system and the requirement for PI(3)P and DOPS in the membrane were validated. Our findings show that Chol affects the binding of EEA1 with membranes, presumably by promoting the hydrophobic insertion of the FYVE domain into the lipid membranes. On the other hand, SM or PE lipids did not affect EEA1 association with SLBs. Finally, by using the viscoelastic acoustic model, we derived information on the thickness of the EEA1 layer on the SLB and showed that this was independent of the lipid composition, except for in membranes containing Chol or high amounts of PS lipid.

2. Materials and Methods

2.1. Chemicals

Analytical-grade chemicals were purchased from Merck or Sigma (Merck KGaA, Darmstadt, Germany), Tris-Sodium citrate was obtained from ITW Reagents (Milano, Italy), and NaCl was obtained from Riedel-de Haën-Honeywell Research Chemicals (Solstice Advanced Materials Inc., Morris Plains, NJ, USA). All buffers were prepared in ultrapure water (water conductivity 0.055 μ S/cm). Lipids were purchased from Avanti Polar Lipids (Alabaster, AL, USA) in powder form: 1,2-dioleoyl-sn-glycero-3-phosphocholine 18:1 (Δ 9-Cis) PC (DOPC), 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (sodium salt) 18:1 PS (DOPS), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine 18:1 (Δ 9-Cis) PE (DOPE), cholesterol (Chol) (ovine wool, >98%), and Chicken Egg Sphingomyelin. PI(3)P (Phosphatidylinositol 3-phosphate diC16) (sodium salt) was purchased from Echelon biosciences (Salt Lake City, UT, USA). All lipids were stored as received at -20°C until used.

2.2. Protein Expression and Purification

Three protein constructs were used, EEA1_(1–1411), GFP-EEA1_(1289–1411), and GFP-2xFYVE. Human EEA1 (EEA1_(1–1411)) was purified with 6xHis-MBP fusion from pOEM vector. SF9 cells were grown in ESF921 media (Expression Systems) and co-transfected with linearized viral genome and an expression plasmid. P1 and P2 viruses were generated per the manufacturer's protocol, and yield was optimized by expression screens and infection

time course experiments. The P2 virus was used to infect SF9 cells (grown to a density of 1 million cells/mL) at 1% (*v/v*). Cells were harvested after 30–40 h spinning in a tabletop centrifuge at $500\times g$ for 10 min. Cell pellets were resuspended in 20 mM Tris (pH 7.5, 150 mM NaCl, 5 mM MgCl₂, 0.5 mM TCEP; STD) supplemented with DNase one and a protease inhibitor cocktail (chymostatin 6 µg/mL, leupeptin 0.5 µg/mL, antipain-HCl 10 µg/mL, aprotinin 2 µg/mL, pepstatin 0.7 µg/mL, and APMSF 10 µg/mL). Pellets were flash frozen and stored at -80°C . All subsequent steps were performed at 4°C or on ice. Cells were thawed on ice and lysed by sonication. Similar procedures were followed for the purification of GFP-EEA1_(1289–1411). The GFP-2xFYVE construct was expressed in BL21 cells. EEA1_(1–1411) was purified on a Superose6 Increase column in 20 mM Bis-Tris, 150 mM NaCl, 2 mM MgCl₂, and 0.5 mM TCEP pH 6 buffer. The GFP-EEA1_(1289–1411) and GFP-2xFYVE constructs were purified on a superdex200 column. Concentrations for EEA1_(1–1411) were determined by a bicinchoninic acid protein assay (Pierce BCA Protein Assay Kit, ThermoFisher, Waltham, MA, USA). Concentrations for the GFP-EEA1_(1289–1411) and GFP-2xFYVE constructs were determined with a NanoDrop Spectrophotometer (ThermoFischer) using the extinction coefficient of the GFP tag. Purity for all protein constructs was assessed by SDS-PAGE followed by colloidal Coomassie staining. Proteins were aliquoted, flash frozen in liquid nitrogen, and stored at -80°C .

2.3. Preparation of Unilamellar Vesicles (ULVs)

Various lipids were dissolved in 1:1 chloroform: methanol to a concentration of 10 mg/mL; and in the case of PI(3)P, in 1:2:0.8 chloroform–methanol–water to 0.5 mg/mL as instructed by the manufacturer. For liposome formation, lipids were mixed in the desired ratios, and the solvent was evaporated under nitrogen (30 min), followed by hydration with 20 mM Bis-Tris, 150 mM NaCl, 2 mM MgCl₂, and 0.5 mM TCEP (pH 6.0) to a final concentration of 1 mg/mL for all lipid compositions. In the case of Chol-containing liposomes, hydration occurred in 10 mM trisodium citrate, 150 mM NaCl, pH 4.6. Multilamellar vesicles were extruded 25 times through 50 nm pore polycarbonate membranes (LiposoFast extruder, Avestin, Ottawa, ON, Canada). Unilamellar vesicles (ULVs) were stored at 4°C and used the same day. Hydration and extrusion were performed at room temperature or at 50°C for Chol- and sphingomyelin-containing lipids to ensure processing above the lipid phase transition temperature:

1. DOPC:PI(3)P, 97:3 (%mol);
2. DOPC:DOPS:PI(3)P, 91:6:3;
3. DOPC:DOPS:PI(3)P, 80:15:5;
4. DOPC:DOPS:PI(3)P, 82:15:3;
5. DOPC:DOPS:PI(3)P:DOPE, 85:6:3:6;
6. DOPC:DOPS:PI(3)P:DOPE:SM, 73:6:3:6:12;
7. DOPC:DOPS:PI(3)P:Chol, 61:6:3:30.

2.4. Quartz Crystal Microbalance with Dissipation

A Q-Sense E4 (Biolin Scientific, Gothenburg, Sweden) was used to measure changes in the resonance frequency (Δf) and dissipation (ΔD) of 5 MHz SiO₂-coated (50 nm) sensors (QSX 303 SiO₂, Biolin Scientific) as a function of time. The SiO₂ sensors were cleaned by immersion in 2% SDS (30 min), followed by a rinse with MilliQ water, drying under nitrogen, and oxidative cleaning (30 min) in a UV–Ozone cleaner (E511, Ossila, Sheffield, UK). The flow cells and tubing were cleaned in a 2% Hellmanex, rinsed with MilliQ water, and dried under nitrogen. Buffer was flown over the sensors at a flow rate of 50 µL/min with a peristaltic pump (Gilson MiniPlus Evolution, Gilson, Knox County, IL, USA). Frequency f and dissipation D were collected on multiple overtones, $n = 3$

(15 MHz) to $n = 13$ (65 MHz). All overtones were used in our subsequent analysis apart from the fundamental f ($n = 1, 5$ MHz). Changes in frequency are plotted scaled by the overtone order, $\frac{\Delta f}{n}$, because this quantity is proportional to the mass of the adsorbed layer [22].

2.5. Acoustic Monitoring of SLB Formation Followed by EEA1 Binding

Supported lipid bilayers (SLBs) were formed on SiO₂-coated quartz crystal sensors to mimic the early endosomal membrane. Prior to SLB formation, unilamellar vesicles (ULVs) were prepared, as described in Section 2.3. SLB formation was initiated by introducing the ULV solution onto the sensor surface at a flow rate of 50 µL/min. The concentration of the ULV solution was optimized to 0.05 mg/mL for the formation of SLBs without events. In this work, the above methodology is employed for the study of the interaction. The total volume of ULV solution used was 700 µL and a buffer composition of 20 mM Bis-Tris, 150 mM NaCl, 2 mM MgCl₂, and 0.5 mM TCEP at pH 6.0 was used. The formation of the SLB was monitored in real-time using QCM-D, assessing changes in frequency and dissipation, to confirm successful bilayer formation. For SLBs containing Chol, a distinct protocol was employed. ULV solution was prepared at a concentration of 0.1 mg/mL and a total volume of 1.5 mL. The ULV solution was suspended in the same buffer composition used for ULV production, 10 mM trisodium citrate and 150 mM NaCl, at pH 4.6 (also described in Section 2.3). The lower pH was necessary to facilitate the fusion of the Chol-containing vesicles, which are otherwise resistant to rupture at pH 6 [38]. The flow rate was increased to 80 µL/min to facilitate faster liposome fusion. Following injection, the flow was halted for 30 min to allow for optimal SLB formation. After stabilization of the acoustic signals, the surface was rinsed with buffer, and then the buffer was exchanged to match the protein solution buffer, concluding with a final buffer rinse. Buffer conditions for EEA1 binding assays were initially set to pH 7.5 based on established protocols. Following preliminary results and an investigation into the stability and activity of EEA1, a strong pH-dependence of EEA1 binding was determined (see Section 3). To ensure optimal and consistent EEA1 binding for the remaining studies, all subsequent experiments were performed at pH 6.0 (20 mM Bis-Tris, 150 mM NaCl, 2 mM MgCl₂, and 0.5 mM TCEP). The protein concentrations used were 0.0315–0.5 µM for EEA1_(1–1411); 0.25–1.5 µM for GFP-EEA1_(1289–1411); and 0.25–2.5 µM for GFP-2xFYVE. Protein solutions were prepared and applied to all SLBs in the same buffer used for purification (Bis-Tris, 150 mM NaCl, 2 mM MgCl₂, and 0.5 mM TCEP at pH 6). All measurements were performed at 25 °C.

2.6. Data Analysis and Viscoelastic Modeling

Acoustic data were collected by QSoft 401 (Biolin Scientific, Gothenburg, Sweden) software and exported by Q-tools (version 3, Biolin Scientific, Gothenburg, Sweden) software. Thickness and viscoelastic properties of EEA1 films were estimated by fitting the QCM-D data to a continuum viscoelastic model [27,28,40]. The model relates the measured QCM-D responses, Δf and ΔD as functions of n , to the viscoelastic properties of the adsorbed layer and the surrounding solution. Averaged data were fitted with the software QTM (version: PyQTM_2022, D. Johannsmann, Technical University of Clausthal, Clausthal-Zellerfeld, Germany). The EEA1 film was modeled as a homogeneous viscoelastic layer with thickness h_f , density ρ_f , and elastic and viscous compliances at the reference frequency, $J'_{f_{ref}}$ and $J''_{f_{ref}}$, respectively. The shifts in the resonance frequency, Δf , and bandwidth, $\Delta \Gamma = \frac{\Delta D f}{2}$, due to a film in liquid are given by

$$\frac{\Delta f + i\Delta \Gamma}{n} \approx -\frac{2f_0^2}{Z_q} \rho_f h_f \left[1 - i \frac{\widetilde{J}(f) \omega \rho_{liq} \eta_{liq}}{\rho_f} \right] \approx -\frac{2f_0^2}{Z_q} \rho_f h_f [1 - (J'(f) - iJ''(f)) i \omega \eta_{liq}], \quad (1)$$

where $\widetilde{J}(f) = J'(f) - iJ''(f)$ is the complex shear compliance of the film, ρ_f and h_f are its density and thickness; their product, $\rho_f h_f$, is mass per unit area of the film, $\omega = 2\pi f$ is the radial frequency, f_0 is the fundamental resonance frequency, ρ_{liq} and η_{liq} are, respectively, liquid density and viscosity, and $Z_q = 8.8 \times 10^6 \left[\frac{\text{kg}}{\text{m}^2\text{s}} \right]$ is the acoustic impedance of quartz.

The frequency dependence of the compliance is approximated through power laws:

$$\widetilde{J}(f) = J'_{f_{ref}} \left(\frac{f}{f_{ref}} \right)^{\beta'} - iJ''_{f_{ref}} \left(\frac{f}{f_{ref}} \right)^{\beta''},$$

where f is the frequency, f_{ref} is a reference frequency, and β' and β'' are the respective power law exponents describing the frequency dependence of elastic and viscous compliances.

The objective of the fitting is to find a set of parameters ($\rho_f h_f$, $J'_{f_{ref}}$, $J''_{f_{ref}}$, β' , and β'') that reproduce the frequency and dissipation shifts observed experimentally. Limits on the values of the exponents are known: $-2 < \beta' < 0$ and $-1 < \beta'' < 1$. In our case, the density and viscosity of the liquid are set to those of water: $\rho_{liq} = 1.000 \text{ g/cm}^3$, $\eta_{liq} = \eta'_{liq} = 0.89 \text{ mPas}$; β' , β'' , and η'' for water are zero and $\rho_f = \rho_{liq}$ is assumed. In our experiments, the frequency and dissipation changes arise from the assembly of macromolecules—lipids and lipid-bound proteins—and their re-arrangements at the resonator surface in the solution. An example of dataset fitting to obtain thickness is included in the Supplementary Materials.

3. Results and Discussion

3.1. Methodology of the Work

To study the interaction of EEA1 to the membrane, we used SLBs mimicking the EE membrane's lipid composition. In addition to the full-length EEA1 (MW 325 kDa), we used two truncated constructs that bind PI(3)P, the C-terminus of EEA1 (C-term EEA1, MW 28.2 kDa) tagged with GFP (MW, 27 kDa) and a synthetic construct including two tandem FYVE domains linked and tagged with GFP (2xFYVE, MW 45.2 kDa). The two truncated constructs were used as an additional verification that our PI(3)P model membranes have functional protein-binding capabilities for further comparison studies. The geometries of the three molecules are depicted in Figure 1A, while specific parameters related to their size and molecular weight, etc., are provided in Table S1. Since both of the truncated constructs were tagged with GFP molecules, we also performed control experiments verifying that GFP does not produce a non-specific acoustic signal response (Figure S1).

A typical experimental set of our methodology, depicted in Figure 1B, includes the SLB formation through vesicle fusion followed by protein addition. For the composition of the SLBs, two parameters were of importance: the physiological relevance of the SLB to the EE membrane and the ability of vesicles to fuse on the hydrophilic surface. Typical % of lipids found in an EE membrane (Table S2) were used to prepare the vesicles applied to the surface. We used DOPC as the main lipid combined with various molar ratios of PI(3)P, DOPS, DOPE, SM, and Chol. In mimicking EE membranes, PI(3)P was particularly important, since it plays a key role in the recruitment of EEA1 protein [21]. For this reason, all vesicles contained at least DOPC and PI(3)P. Moreover, DOPS, DOPC, DOPE, SM, and Chol were added at various mol % to reach a physiologically relevant composition [39]. Most lipid mixtures resulted in the formation of an SLB upon the addition of vesicles on the clean SiO₂-QCM-coated device surface. Vesicles containing 20 and 30 mol% of Chol also resulted in the formation of SLBs, unless DOPE or SM were included in the lipid mixture where no vesicle fusion was observed. Lastly, vesicles composed of the exact mol % of EE lipid composition (Table S2), although prepared successfully, did not fuse to produce SLBs.

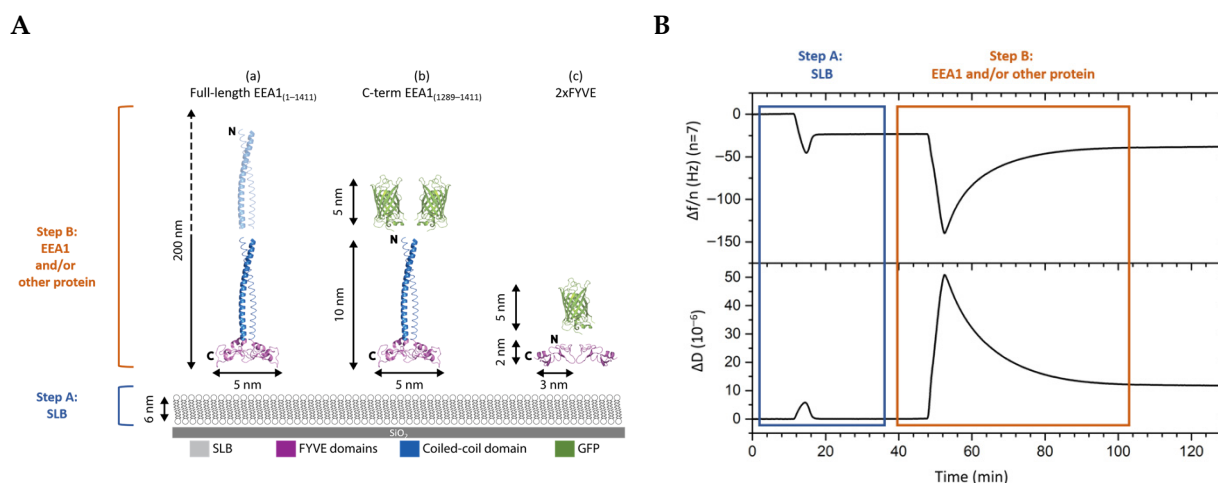


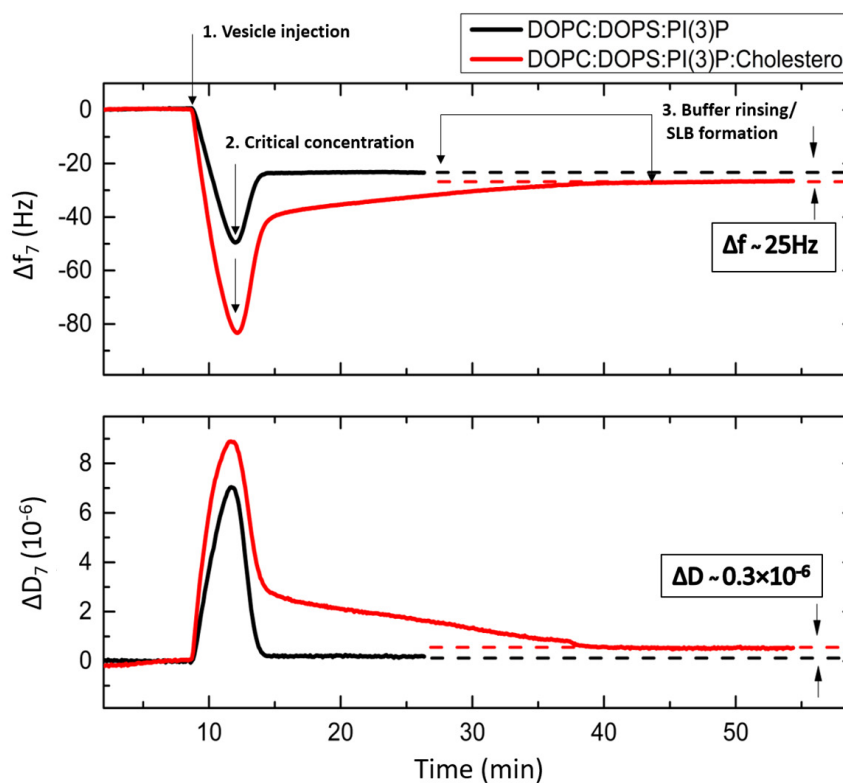
Figure 1. (A). (a) Full-length EEA1 protein ($200 \times 5 \times 2 \text{ nm}^3$), (b) C-term EEA1 structure ($10 \times 5 \times 2 \text{ nm}^3$), and (c) 2xFYVE construct ($7 \times 6 \times 2 \text{ nm}^3$). The C-term EEA1 and 2xFYVE are labeled with the GFP tags. (B). Real-time curves showing Δf and ΔD upon formation of SLB and EEA1 binding to SLB. SLB composition is DOPC:DOPS:PI(3)P, 91:6:3 (mol %); EEA1 concentration and volume is 250 nM and 200 μL , respectively.

3.2. Real-Time Formation of Supported Lipid Bilayers

To form SLBs on the QCM-D surface with a composition as close as possible to the EE membrane, we investigated several experimental parameters. The optimal conditions for SLB formation were determined through systematic variation in the buffer pH, liposome concentration, sample volume, and flow rate. Initially, motivated by published protocols [41,42], we explored the use of EDTA and Triton X-100, since they have been reported to facilitate liposome fusion, particularly for lipid mixtures containing sphingomyelin (SM), dioleoylphosphatidyl-ethanolamine (DOPE), and Chol. However, in our case, these additives were not required for successful SLB formation (Figure S2). The final, optimized conditions for SLB formation occurred at pH = 6 for all vesicle lipid compositions apart from the Chol-containing ones, which fused at a pH = 4.6, following the experimental protocols described in the Section 2.

Figure 2A shows the real-time monitoring of Δf and ΔD during the formation of DOPC:DOPS:PI(3)P and DOPC:DOPS:PI(3)P:Chol-containing SLBs after the addition of the corresponding vesicles on the QCM surface. Both responses follow the well-described two-step fusion response in the presence of salts [1,34], including an initial change in both the f (decrease) and D (increase) signals, followed by a sharp transition to the opposite direction until equilibrium is reached. These steps are attributed to the adsorption of intact vesicles up to a critical surface density after which vesicles rupture and fuse to form an SLB rigid film. Comparing the two responses, the adsorption of the Chol-containing vesicles was slower than the non-Chol ones and could be observed only under static conditions. This is attributed to the more rigid structure of Chol membranes, which are more resistant to fusion. Based on the two-step formation kinetics during the vesicles addition (Figure 2A), we concluded that the presence of PI(3)P and Chol in combination with unsaturated lipid mixtures (DOPC, DOPS, and DOPE) did not prevent the formation of a relevant SLB.

A



B

Lipids Composition	Mol %	$\Delta f/n$, $n = 7$ (Hz)	ΔD (10^{-6})	# Exp	$\Delta D/(\Delta f/n)$ ($10^{-9}/\text{Hz}$)	$\Delta D/\Delta f$ ($10^{-6}/\text{Hz}$)
1. DOPC:PI(3)P	97:3	24.20 ± 0.25	0.24 ± 0.05	4	9.91	0.001
2. DOPC:DOPS:PI(3)P	91:6:3	24.06 ± 0.53	0.13 ± 0.11	12	5.40	<0.001
3. DOPC:DOPS:PI(3)P	80:15:5	23.89 ± 0.22	0.10 ± 0.05	4	4.18	
4. DOPC:DOPS:PI(3)P	82:15:3	23.89 ± 0.22	0.10 ± 0.05	3	4.18	
5. DOPC:DOPS:PI(3)P:DOPE	85:6:3:6	24.30 ± 0.78	0.16 ± 0.11	9	6.58	
6. DOPC:DOPS:PI(3)P:DOPE:SM	73:6:3:6:12	24.52 ± 0.36	0.16 ± 0.15	8	6.52	
7. DOPC:DOPS:PI(3)P:Chol	61:6:3:30	27.54 ± 0.84	0.93 ± 0.29	4	33.77	0.005

Figure 2. (A). Comparison of the formation of two SLBs by using vesicle fusion for two different lipid compositions: DOPC:DOPS:PI(3)P (91:6:3 mol%) (black line) and DOPC:DOPS:PI(3)P:Chol (61:6:3:30 mol%) (red line). A similar real-time response to the DOPC:DOPS:PI(3)P SLB was obtained with all non-Chol-containing vesicles. (B). Summary of acoustic signal changes observed upon the formation of SLBs of various composition on the SiO₂-coated QCM surface following the vesicle fusion technique. All SLBs were formed at a pH 6 with the exception of the Chol-containing SLBs, which were obtained at pH 4.6; Δf and ΔD measurements correspond to equilibrium. Error bars represent the mean $\pm$ standard deviation (S.D.) from independent experiments. The number of experiments for each lipid composition is listed in column ‘# Exp’.

The final signal changes obtained related to SLB formation were also studied and compared with previous reports. For most of the non-Chol containing vesicles, the SLBs gave similar signal changes in pH 6.0 (Bis-Tris) and pH 7.5 (Tris) buffers, i.e., an average of 24.19 Hz of Δf and 0.14×10^{-6} of ΔD . However, the formation of the binary DOPC:PI(3)P SLB, while it had a similar Δf (24.20 Hz), was accompanied by an almost double ΔD (0.24×10^{-6}). The Chol-containing SLBs (20 and 30%) were more difficult to produce and gave the best results in a pH 4.6 buffer (citrate), in agreement with previous studies [38], with corresponding signal changes in $\Delta f = 27.54$ Hz and $\Delta D = 0.9 \times 10^{-6}$. Moreover, previous QCM-based studies on the formation of SLBs also containing phosphatidylinositol (PIP) lipids reported values within the range of our results [37,38].

The table shown in Figure 2B summarizes the signal changes observed for each lipid composition, together with the corresponding acoustic ratios ($\Delta D/\Delta f$). The latter can be used for the qualitative comparison of the Chol-free and Chol-containing SLBs regarding the hydrodynamic behavior (coupling of water) affecting acoustic energy dissipation [38]. Based on the last two columns in this table, the differences in the viscoelastic properties are not significant among the various SLB formulations, apart from the Chol-containing SLBs, which exhibit a considerably higher acoustic ratio. This finding may be attributed to the Chol property to fill “spaces” between phospholipids within membranes, reducing intermolecular interactions and enhancing the membrane–water interaction sites through the higher separation of head groups [43]. In addition, the creation of liquid-ordered lipid microdomains [44] may also influence the coupling of water on the Chol-SLB surface.

As a last note, we considered the possibility that the higher acoustic ratio of Chol-containing SLBs may imply the presence of few intact vesicles on the surface. This is supported by our observation that Chol-containing vesicles proved difficult to fuse, and, in some cases (e.g., together with SM or PE lipids), did not fuse at all under the pH conditions tested (pH 4.6, 6.0, and 7.5). However, when our bilayers were tested for non-specific protein adsorption, no signal change was observed, indicating that the quality of the SLB was appropriate for performing protein studies.

3.3. EEA1 Binding to SLBs Containing PI(3)P

Following bilayer formation, the EEA1 protein was introduced and allowed to bind to the SLBs. Initially, we tested the specificity of the membrane–protein interaction depending on the lipid composition. While it is well-documented that EEA1 binds to PI(3)P-containing membranes via the C-terminal FYVE domains [13], this has not been verified in *in vitro* assays like the one we are using, where EEA1 is added to SLBs produced via fusion of PI(3)P-containing vesicles. Figure 3 shows the real-time binding curves of EEA1 to SLBs of various lipid compositions with and without PI(3)P.

Further evidence on the specificity of the binding to PI(3)P through the zing finger of the FYVE domain was produced by detecting a zero signal upon addition of the EEA1 incubated with 0.5 mM EDTA (Figure S3).

Based on the above results, we concluded that the SLBs produced on the QCM device surface can mimic properties of the EE membrane regarding the need for PI(3)P for specific EEA1 protein binding.

Moreover, we tested the impact of varying pH on protein binding and found that the membrane association of both EEA1 and EEA1-truncated mutants to the SLBs was highly affected by altering the pH from 7.5 to 6.0. This effect is depicted in Figure 4A for the full-length EEA1 binding to DOPC:DOPS:PI(3)P:DOPE. Our experiments showed that for all lipid compositions, pH 6.0 enhances the binding up to 3-fold (measured values at the plateau of Δf and ΔD). These results are in agreement with studies showing the pH dependency of the FYVE domain binding to PI(3)P-containing membranes and penetration

ability inside the lipid layer. FYVE binding is modulated by two adjacent His residues of the R(R/K)HHCRXCG signature motif [14,45]. The mutation of either His residue abolishes the pH sensitivity. Under pH 6.0, histidine residues are mostly protonated and are able to form a hydrogen bond to the 3-phosphate group of PI(3)P. The enhancement of binding of FYVE-containing proteins with membranes at a lower pH has been shown in both in vitro and in vivo assays [5,6,45], suggesting that pH-dependency is a general function of the FYVE finger family.

Given that the FYVE domain (with a theoretical pI of 8.7) is positively charged at pH 6.0, we also studied the effect of negatively charged lipids other than PI(3)P, and, specifically, DOPS, assuming that this could also affect EEA1 binding. Figure 4B shows that, indeed, the binding rate of 125 μ M of full-length EEA1 on SLBs of increasing DOPS content follow also an increase compared to non-DOPS-containing SLBs. However, only the 15% DOPS-SLB solution produced a final Δf that was significantly different ($\Delta f = 37$ Hz) to the Δf observed with low (6%) ($\Delta f = 13$ Hz) or no DOPS ($\Delta f = 7$ Hz).

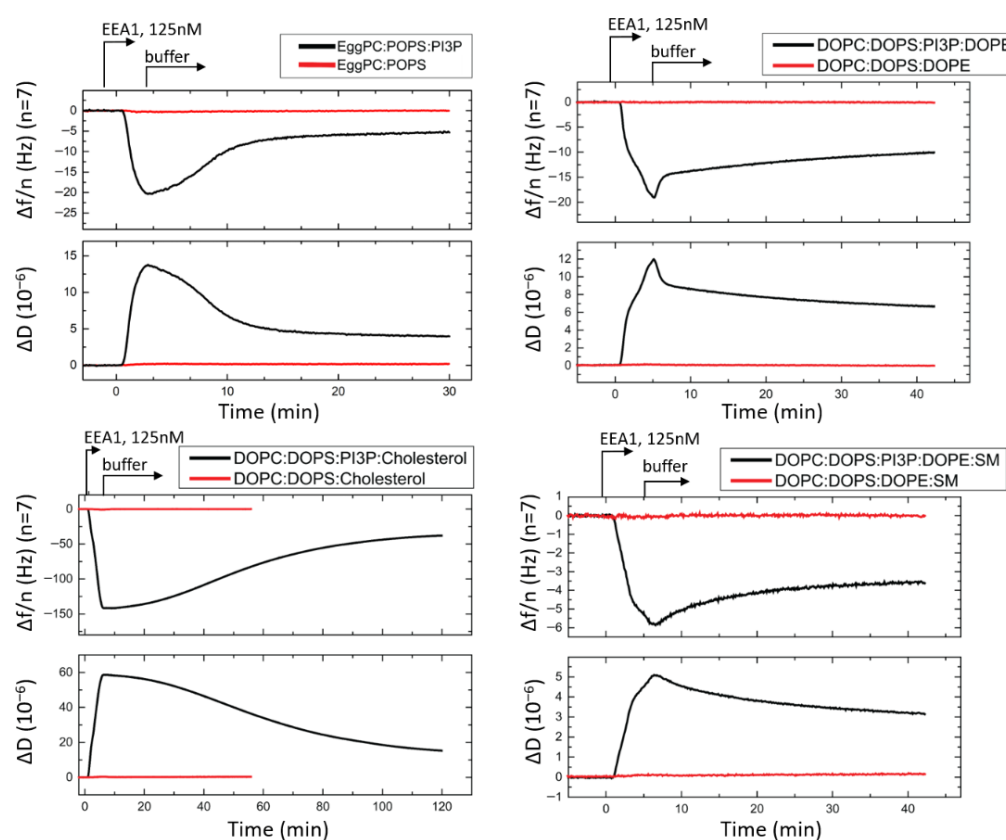


Figure 3. Real-time plots showing the changes in Δf upon EEA1 injection (125 nM). For all lipid compositions, a control SLB lacking PI(3)P was formed and tested for protein binding. The results verify the specific binding of EEA1 to PI(3)P. Red lines depict that EEA1 does not bind in the absence of PI(3)P. Black lines show EEA1 binding to PI(3)P-containing SLBs.

Based on these findings, we studied the real-time binding of various concentrations of full-length EEA1 or truncated mutants (Figure 1A) at pH 6.0 to SLBs consisting primarily of DOPC, DOPS, and PI(3)P, and, in some cases, DOPE, SM, and Chol. Figure 5A shows such an example of full-length EEA1 on DOPC:DO:PS:PI(3)P:Chol. Qualitatively, this response was the same for all the SLB compositions used in this study and all protein constructs, i.e., they all exhibited the same initial adsorption followed by a desorption step until equilibrium was reached. This two-step response has been reported before, during the study of the binding of the EEA1 FYVE domain on supported vesicles [6] as well as the

interaction of antimicrobial peptides with SLBs using the QCM-D technique [46,47]. The long desorption states might indicate the lack of an important factor for EEA1 binding, e.g., active Rab5, which is present in vivo on the EE membrane and which, together with PI(3)P, is responsible for optimal EEA1 binding on endosomes [12,20,48].

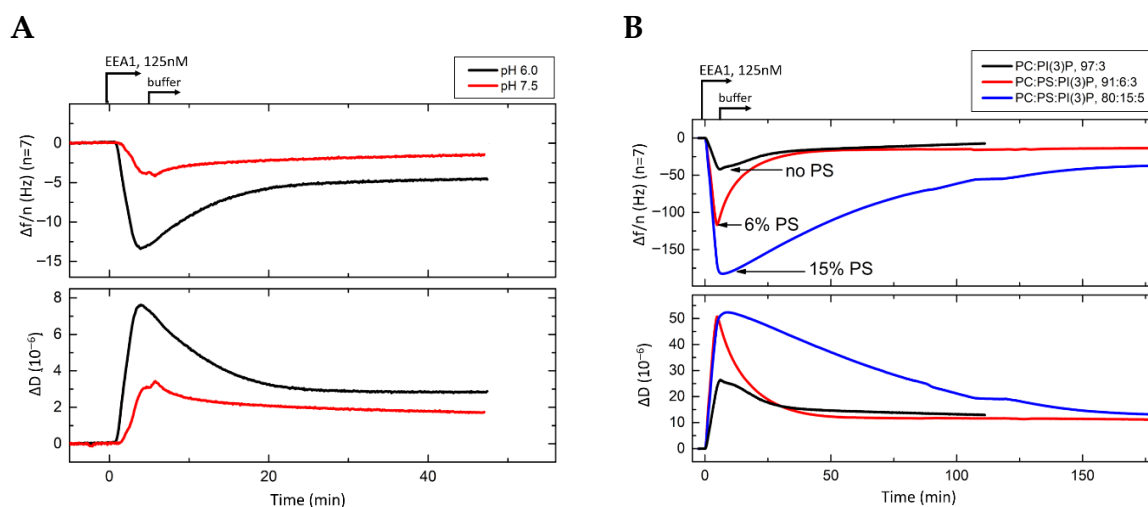


Figure 4. (A) Real-time plots showing binding of full-length EEA1 on DOPC:DOPS:PI(3)P:DOPE SLBs in both pH 6.0 (red line) and pH 7.5 (black line). A total of 125 nM of EEA1 protein solution was injected and the changes in Δf and ΔD were monitored. (B) Δf and ΔD real-time plots comparing the binding of 125 nM of the full-length EEA1 on SLBs with increasing amount of DOPS lipid.

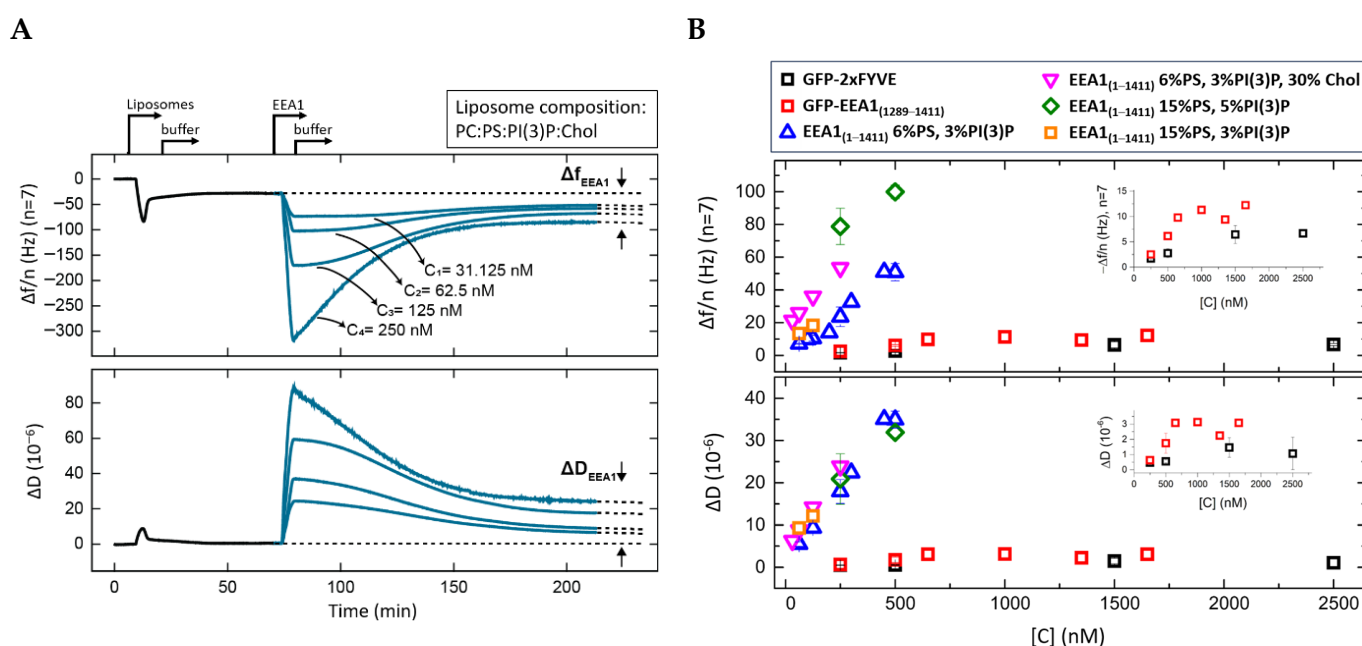


Figure 5. (A) Real-time binding of four different concentrations of EEA1 full-length protein to DOPC:DOPS:PI(3)P:Chol SLB, for a typical QCM-D experiment involving SLB formation from liposomes, buffer rinse (Bis-Tris, 150 mM NaCl, 2 mM MgCl₂, and 0.5 mM TCEP at pH 6), protein adsorption, and buffer rinse. Asymptotic shifts in the frequency (Δf) and dissipation (ΔD) for the protein relative to the bilayer (dashed lines) were calculated from each experiment and analyzed. (B) Summary of the asymptotic Δf and ΔD shifts for the different protein constructs. GFP-2xFYVE and GFP-EEA1 are plotted for comparison, SLB: PC:PI(3)P, 95:5 (mol%). Error bars represent the mean $\pm$ standard deviation (S.D.) from at least $n = 3$ independent experiments of EEA1 binding to different lipid compositions.

To compare the adsorption profiles between the different protein constructs and lipid compositions, we calculated the asymptotic shifts from the real-time QCM-D graphs, as illustrated in Figure 5A (dashed lines). Asymptotic shifts were calculated at each overtone and concentration by averaging the $\Delta f/n(t)$ and $\Delta D(t)$ signals corresponding to the SLB and the protein. Time points were selected to ensure signal stabilization across all EEA1 concentrations used. These averaging calculations were made over time periods of ~100–300 s for each step (SLB and protein) and the values were then subtracted one from another. The asymptotic shift values for the seventh overtone were plotted for the different protein constructs and lipid compositions as a function of the protein concentration. Due to limitations in the available amounts of the full-length EEA1 protein ($[EEA1]_{\max} = 500 \text{ nM}$), we could not reach signal saturation at the applied concentration range of EEA1 to obtain a binding isotherm. However, the other two proteins that were both available at higher amounts ($\max [GFP-EEA1]_{\max} = 1.5 \text{ }\mu\text{M}$ and $[GFP-2x\text{FYVE}]_{\max} = 2.5 \text{ }\mu\text{M}$) reached saturation above 600 nM (Figure 5B, inset). From Figure 5B, we can conclude that the amount of bound EEA1 depends primarily on the amount of Chol, PI(3)P, and DOPS lipids present in the underlying SLB. Vesicles consisting of PI(3)P and/or DOPS of 3 and/or 6%, respectively (i.e., lipid mixtures no 1, 2, 5, and 6 in Section 2.3), had a similar response in terms of bound EEA1 protein. The addition of 30% Chol enhanced the amount of EEA1 binding. Similarly, increasing the amount of the anionic DOPS to 15% leads to a small increase in protein binding, whereas the presence of 5% PI(3)P appears to enhance even further the amount of EEA1 bound to the SLB, leading to an even higher response than the one obtained with the Chol-containing SLBs. While a complete EEA1-binding isotherm could not be obtained within the applied concentration range, this was not the case for the two truncated proteins. Based on our results, the K_d of the two small versions of EEA1 is calculated to be ~500 nM. Although this value is higher than the previous estimate of a K_d (49 nM at pH 6.0) [6], the latter was calculated from a binding isotherm of an EEA1 FYVE domain mutant to support PI(3)P vesicles which, is different to our model membrane system, as well as the protein construct of the C-term FYVE domain. Lastly, it is noted that the observed dissipation changes in the bound full-length EEA1 protein on the various SLBs were the same in all cases.

In the absence of a plateau in full-length EEA1 lipid-binding events, we also considered the initial changes in surface coverage and allowed the comparison of EEA1 binding to different SLBs based on the initial binding rate (Figure 6A). For the analysis we used the method of the initial rates, described in Saha et al. [49] (see Supplementary Materials for the method of the initial rates of EEA1 binding). For this, we measured the slope of the first part of the association step of EEA1 on all lipid compositions used in this study. According to our results, the most significant differences were obtained for DOPC:PI(3)P SLBs and DOPC:DOPS:PI(3)P:Chol, as shown in Figure 6B. We explain this observation on the basis that the addition of Chol affects the phase state of the membrane bilayer (increases the liquid-ordered state) and, through local rearrangements/organizations of the lipid polar heads, the resulting recognition forces (electrostatic and H-bonds) change, affecting the reaction rate and binding constant. The presence of DOPS is also expected to increase protein binding due to the electrostatic interaction between the positively charged FYVE and negative PS at pH 6.0.

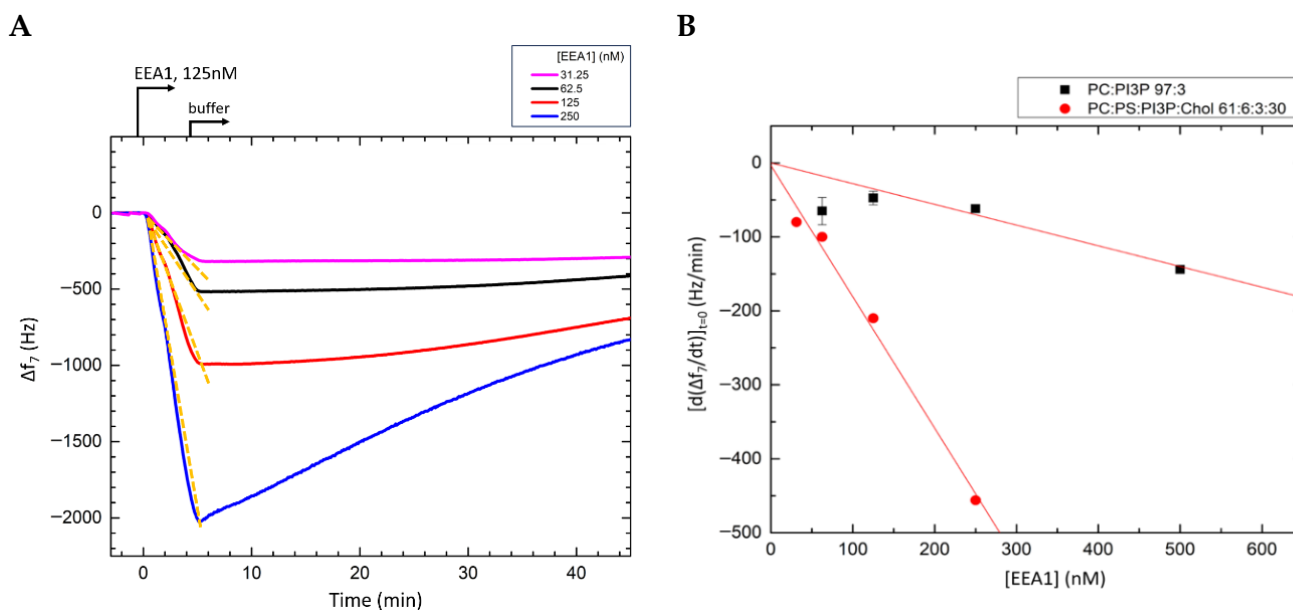


Figure 6. (A) Binding of EEA1 to DOPC:DOPS:PI(3)P:Chol (61:6:3:30 mol%) SLBs at different protein concentrations (62.5, 125, 250, and 500 nM). (B) Initial slopes (yellow lines in (A)) and equivalent data for DOPC:PI(3)P plotted vs. the four EEA1 protein concentrations used on two different lipid compositions (black: DOPC:PI(3)P; red: DOPC:DOPS:PI(3)P:Chol). Error bars represent the mean $\pm$ standard deviation (S.D.) from $n = 11$ independent experiments.

3.4. Viscoelastic Modeling

Asymptotic shifts in f and D between the protein and the SLB were calculated for each overtone (3, 5, 7, 9, 11, and/or 13). The process was followed for each protein (full-length EEA1, C-term EEA1, and 2xFYVE) and protein concentration by averaging the $\Delta f/n(t)$ and $\Delta D(t)$ signals corresponding to the SLB and to the protein at the plateau over time periods of ~ 100 – 300 s and subtracting one from another. The objective of the fitting was to find a set of parameters (ρ_f , h_f , J'_{ref} , J''_{ref} , β' , and β'') that reproduce the frequency and dissipation shifts observed experimentally. J'_{ref} and J''_{ref} are the values of elastic and viscous compliances at the reference frequency and β' and β'' are the respective power law exponents describing the frequency dependence of elastic and viscous compliances. Limits on the values of the exponents are known: $-2 < \beta' < 0$ and $-1 < \beta'' < 1$. In our case, the density and viscosity of liquid are set to those of water: $\rho_{\text{liq}} = 1.000 \text{ g/cm}^3$, $\eta_{\text{liq}} = 0.89 \text{ mPas}$; β' , β'' , and η'' for water are zero. Finally, densities of the layer and liquid were assumed to be equal. The EEA1/SLB system presents a challenge in that it consists of two layers with different elastic properties, an SLB, which excludes the aqueous media, and a protein layer, which is expected to be a strongly hydrated layer. Three different approaches were evaluated for the fitting of SLB/EEA1 data (typical experiment shown in Figure 7A). Viscoelastic modeling provides the ‘hydrodynamic thickness’, which represents the thickness of the protein layer including coupled water. The model fits the product of density and thickness ($\rho * h$), representing the total areal mass density. In our calculations, the density (ρ) of the protein layer was assumed to be constant. Given that the mass increases while the thickness remains relatively stable across different concentrations (Figure 7B), we conclude that the layer remains in a low-coverage regime. Under these conditions, the protein molecules do not reach the ‘brush regime’ density required for a significant intermolecular interaction, suggesting that the orientation is dictated primarily by the protein–lipid anchors rather than lateral crowding.

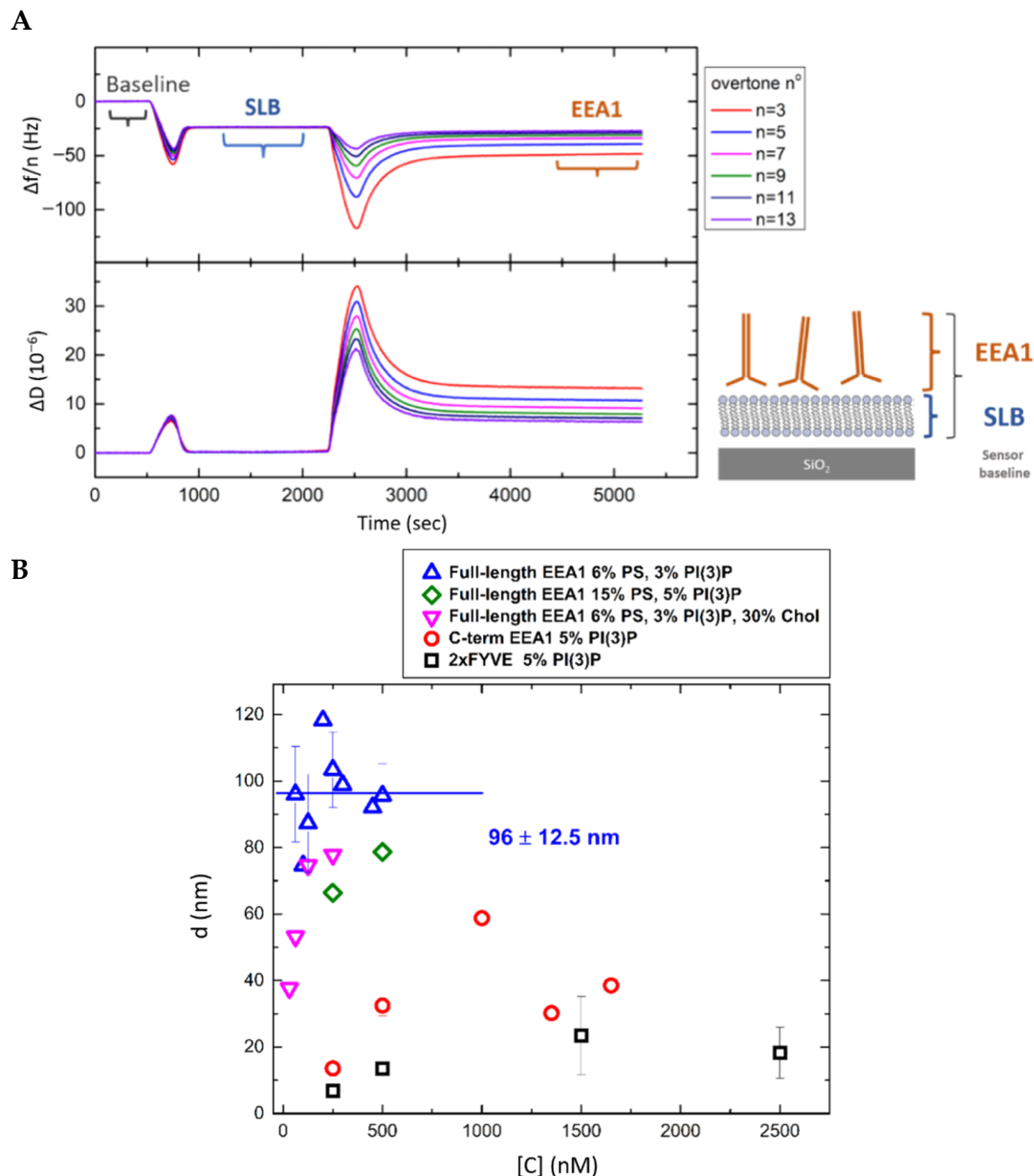


Figure 7. (A) Real-time experiments with different overtones used in the viscoelastic modeling ($n = 1$ – 13) of EEA1 protein layers. In this experiment, the SLB composition is DOPC:DOPS:PI(3)P, 91:6:3 (n/n) and EEA1 concentration is 125 nM. The schematic representation on the right (not in scale) shows the layers of SLB and EEA1. (B) Thickness of the full-length EEA1 films derived from viscoelastic one-layer model. Values are plotted as a function of EEA1 concentration. Thicknesses of C-term EEA1 and 2xFYVE are plotted for comparison. Error bars represent the mean $\pm$ standard deviation (S.D.) from at least $n = 42$ independent experiments.

Firstly, the shifts in f and D are calculated relative to the bare sensor in the buffer (e.g., Δf_{EEA1} subtracting Δf_{sensor}) and the results are fit with a five-parameter model. This approach, while yielding good-quality fits, combines the thickness and elastic properties of two different layers (SLB layer and EEA1 layer) into one and it is not clear how to separate them. Consequently, this approach was not pursued. Next, the shifts in f and D were calculated for the SLB relative to the sensor baseline (in the buffer) (e.g., Δf_{SLB} subtracting Δf_{sensor}). The SLB fitting data was used next for the fitting of the EEA1 layer relative to the SLB (e.g., Δf_{EEA1} subtracting Δf_{SLB}). The bilayer properties were fit in the first step (SLB layer), and the EEA1 properties were fit in the second step (EEA1 layer). Significant

differences were observed between Chol-free and Chol-PS-containing SLBs in terms of thickness, elastic compliance, and the frequency dependence of the elastic compliance, indicating different elasticities. In such a two-layers fitting strategy, not all SLB data could be fit properly and it was not possible to replace individual SLB fit parameters with the average ones. This indicates that the noise inherent in experimental data (in this case SLB data) affects the reliable determination of the fitting parameters [50].

Finally, the shifts in frequency between EEA1 and the SLB (Δf_{EEA1} subtracting Δf_{SLB}) are calculated and fit as one layer. This fitting strategy was also used for the other two constructs, C-term EEA1 and 2xFYVE. The results of this fitting strategy are shown in Figure 7B. The thickness of the EEA1 layer was averaged for all lipid compositions, except for the ones with Chol and high amounts of PS lipid. In Figure 7B, the thickness of full-length EEA1 appears to be independent of the EEA1 concentration in the solution and of SLB compositions that do not contain Chol or high amounts of PS lipid. Its value is 96 ± 12.5 nm. The end-to-end and contour length of EEA1 has been calculated previously [19]. The values follow Gaussian distributions with averages of 222 ± 26 nm and 195 ± 26 nm, respectively. When we calculated EEA1's max surface density on the SLB via Δf_{max} [22], this was no more than 40% (see Supplementary Materials, Section Surface Density Calculation), i.e., EEA1 does not correspond to a full monolayer thickness but rather to a dilute layer of protein molecules. Interestingly, measurements for the Chol- and PS-containing SLBs indicate that EEA1 thickness increases with EEA1 sample concentration and Δf . More information on the elastic and viscous behavior of the EEA1 layer can be found in Figure S4.

4. Conclusions

In the present study, the interaction of EEA1 with supported lipid bilayers mimicking the EE membrane has been investigated using the QCM-D technique. Our results confirmed the essential requirement of PI(3)P lipid for EEA1-specific binding on a model SLB membrane, which is similar to cellular *in vivo* studies [13]. Moreover, EE-mimicking SLBs with a high content of PS (15%) demonstrated 50% or more of EEA1 binding compared with membranes containing PI(3)P alone. However, a high PS content cannot compensate for the need for PI(3)P, since in the absence of the latter and presence of PS, no EEA1 binding was detected. This result indicates that while the electrostatic interaction with negatively charged PS lipids promotes EEA1 binding, this alone is not solely responsible for the process.

In addition to PI(3)P and PS lipids, when we included Chol in the SLB, further enhancement of EEA1 binding was observed. The analysis of the initial slopes of frequency changes for EEA1 on Chol-PS membranes revealed a strong difference compared to other lipid compositions with a 9-fold faster adsorption when Chol and PS were present. These findings provide evidence on the effect of Chol on EEA1 binding, while they also expand our understanding of the effect of certain lipids in the process. Interestingly, Chol has been shown to play a positive role in the clustering of Rab5 on an SLB [15]. It is unlikely that such an effect on EEA1 binding may be due to direct interactions between the lipid and the protein. A more likely explanation is that Chol may play a role in the modulation of lipid–lipid interactions, including PI(3)P, with consequent changes in the diffusion of the proteins on the plane of the membrane. Such a decrease in diffusion may contribute to protein–lipid and protein–protein interactions, thus stabilizing the association of the proteins with the bilayer.

While our QCM-D results and viscoelastic modeling provide strong evidence for the role of Chol in enhancing EEA1 binding through membrane-mediated effects, we acknowledge that the structural interpretation of protein clustering and membrane reorganization

would further benefit from the use of complementary techniques, such as high-resolution AFM imaging and nanomechanical mapping [51].

Our experiments also showed that EEA1 binding was highly influenced by the pH conditions, supporting the involvement of a histidine switch in regulating this interaction. This finding aligns with the existing literature [13,52] and underscores the multivalent binding mechanism employed by the EEA1 FYVE domain. Furthermore, the conservation of basic residues within the domain suggests that this electrostatic interaction is a common characteristic among FYVE domains [13]. Overall, the above findings emphasize the dynamic nature of the EEA1-PI(3)P interaction, including a pH-dependent control mechanism.

Finally, our acoustic data, together with the viscoelastic model analysis, indicate that EEA1 does not form a densely packed layer (brush) on lipid membranes, but a rather sparse layer of protein molecules. The results from the viscoelastic modeling show that the overall thickness of EEA1 layers was ~ 96 nm. This implies that EEA1 may not be fully extended but can also be flexible and/or tilted on the lipid membranes. However, it is also noted that at the current relatively low surface density of EEA1 on the membrane, a mixture of extended and collapsed molecules may exist, quantified with our model as an average value. Since Figure 5B shows that the binding of EEA1 has not reached saturation yet, adding higher amounts of EEA1 to produce a denser membrane layer would be important to evaluate the conformation of EEA1 at various surface coverages.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/membranes16020049/s1>, Table S1: Protein parameters; Table S2: Lipid composition of EEA1 (1) and of vesicles used in this study that produced a good SLB; Figure S1. Control experiment demonstrating that GFP tag alone doesn't bind to SLBs; Figure S2. Experimental attempt showing that addition of Triton detergent does not produce a bilayer; Figure S3: Real-time plot showing the changes in frequency (Δf) and dissipation (ΔD) upon EEA1 binding to PI(3)P-containing membranes. Black line shows signal changes upon 125 nM EEA1 injection, while red lines show the same amount of protein, first incubated with EDTA; Figure S4: Typical SLB formation and EEA1 adsorption experiment. Baseline, SLB, and EEA1 averaging time periods are indicated in light blue, dark blue, and red, respectively; Figure S5: Cholesterol-containing vs. cholesterol-free containing SLBs: experimental data and model fits. Data are shifted around the integer overtone orders for visibility; Figure S6. Parameters of the viscoelastic modelling as a function of sample concentration. J'_{ref} and J''_{ref} are the values of elastic and viscous compliances at the reference frequency and β' and β'' are the respective power law exponents describing the frequency dependence of elastic and viscous compliances. Refs [39,49] are cited in Supplementary Materials.

Author Contributions: F.P. Methodology, Investigation, Data Curation, and Writing—Original Draft; P.M.-G. Conceptualization, Funding Acquisition, Investigation, Methodology, Supervision, and Validation; J.L. Methodology and Supervision; M.Z. Conceptualization, Funding Acquisition, Resources, Validation, and Writing—Review and Editing; E.G. Conceptualization, Funding Acquisition, Project Administration, Supervision, Validation, and Writing—Review and Editing. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Human Frontier Science Program, HFSP/REF RGP0019/20.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The raw data supporting the conclusions of this article will be made available by the authors on request.

Acknowledgments: The authors would like to acknowledge I. Reviakine for performing the acoustic analysis with the viscoelastic model.

Conflicts of Interest: Author Janelle Lauer was employed by the company Arthrex. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:

BCA	Bicinchoninic Acid
Chol	Cholesterol
C-term	C-terminus/C-terminal
DOPC	1,2-dioleoyl-sn-glycero-3-phosphocholine
DOPE	1,2-dioleoyl-sn-glycero-3-phosphoethanolamine
DOPS	1,2-dioleoyl-sn-glycero-3-phosphoserine
EE	Early Endosome(s)
EEA1	Early Endosomal Antigen 1
GFP	Green Fluorescent Protein
MW	Molecular Weight
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PI(3)P	Phosphatidylinositol 3-phosphate
PI(4,5)P2	Phosphatidylinositol-4,5-bisphosphate
PIP	Phosphatidylinositol
PIP3	Phosphatidylinositol-3',4',5'-triphosphate
PS	Phosphatidylserine
QCM-D	Quartz Crystal Microbalance with Dissipation Monitoring
SLB	Supported Lipid Bilayer
SM	Sphingomyelin
ULV	Unilamellar Vesicle

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