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Spontaneous Formation of Cushioned Model Membranes Promoted by an Intrinsically Disordered Protein

Yuri Gerelli,* Amanda Eriksson Skog, Stephanie Jepthah, Rebecca J. L. Welbourn, Alexey Klechkov, and Marie Skepö*

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ABSTRACT: In this article, it is shown that by exposing commonly used lipids for biomembrane mimicking studies, to a solution containing the histidine-rich intrinsically disordered protein histatin 5, a protein cushion spontaneously forms underneath the bilayer. The underlying mechanism is attributed to have an electrostatic origin, and it is hypothesized that the observed behavior is due to proton charge fluctuations promoting attractive electrostatic interactions between the positively charged proteins and the anionic surfaces, with concomitant counterion release. Hence, we anticipate that this novel “green” approach of forming cushioned bilayers can be an important tool to mimic the cell membrane without the disturbance of the solid substrate, thereby achieving a further understanding of protein—cell interactions.

INTRODUCTION

Solid-supported lipid bilayers (SLBs) are well-known systems extensively used to mimic cell surfaces and to develop biosensors.1–7 Despite successfully recreating some aspects of the cell membrane behavior, the supporting solid surface induces undesirable artifacts, for example, not reproducing the deformability nor the natural curvature of living cells, which indeed are properties that critically affect cellular adhesion processes, dynamics, and localization of transmembrane proteins. For example, the interaction of transmembrane proteins and nanoparticles with SLBs can be hindered by the presence of solid support, making SLBs’ suboptimal models for cell membranes. Due to these limitations, there have been attempts to reduce substrate effects by preparing floating bilayers,8,9 or using adsorbed or chemically grafted polymer cushions,10–15 which can support lipid bilayers either in the gel or in the fluid phase. The disadvantage of grafted polymer cushions is that their design makes it difficult to control polymer film thickness, density of functional groups, and homogeneity in both the lateral and the vertical direction of the lipid bilayer. In addition, these methods do not ensure optimal reproducibility with respect to the sample quality. First attempts of preparing nongrafted polymer cushions for lipid bilayers were reported already at the end of the 1990s by the groups of Israelachvili16–18 and Sackmann.19 Of particular interest are the results reported by Israelachvili’s group on the formation of polyethylamine (PEI)-supported bilayers. They described not only the formation of a bilayer on the top of a preformed PEI layer17,18 but also the possibility of incubating PEI in the presence of an already deposited bilayer to obtain the formation of a PEI cushion.19 For unknown reasons, this method has not been fully exploited in applications and biophysical studies. The possibility of using proteins instead of polymers to form a naturally hydrated cushion lipid bilayer on a solid substrate was described by Schuster and Sleytr.20–23 Histatin 5 (Hst5) contains 24 amino acids and is a histidine-rich, intrinsically disordered, multifunctional, cationic, saliva protein,24–26 known to act as the first line of defense against oral candidiasis caused by Candida albicans.27–29 The histatin family consists of 12 members, where Hst5 is the most potent with respect to antifungal activity,30 which has been ascribed to the high content of basic amino acids.31 The ionic strength has also been reported to play a role in its activity.32–34 At physiological conditions, Hst5 behaves as a random coil,35 although possessing some degree of polyproline II helical structure.36 Regarding the structure–function relationship, it has been hypothesized that a more ordered secondary structure might be induced upon interaction with charged surfaces.37–39 Since 7 of 24 amino acids are histidines, where the conjugated imidazole side chain has a pK_a value of approximately 6.0, one can expect that charge titration and charge regulation might play an important role.37–39 Over the years, the structure and function of Hst5 have been evaluated using a variety of different techniques; nevertheless, the mechanism is still not very well understood.

In this article, we present an elegant method to obtain a cushioned SLB as a result of lipid–protein interaction. Following the pioneering work of Israelachvili and co-workers, we show that upon interaction with preformed SLBs, Hst5...
crosses the bilayer and accumulates, in a highly hydrated state, between the solid substrate and the SLB, inducing a natural cushion that does not affect the structural integrity of the SLB. In addition, we show that electrostatics has a predominant role in Hst5–membrane interactions.

### Experimental Section

#### Histatin 5 Solutions

Hst5 was purchased from TAG Copenhagen A/S, Denmark, with a purity of 99% measured by high-performance liquid chromatography (HPLC). Prior to use, proteins were dissolved in 20 mM Tris with pH 7.4, set by HCl, and 10 mM NaCl, to a concentration of approximately 4 mg mL⁻¹. Thereafter, they were centrifuged using a concentration cell (Vivaspin 20, MWCO 1 kDa, Prod. No. VS2002, Sartorius Stedim Biotech GmbH, Germany, 2700 rpm, 4 °C). The sample was then rinsed with buffer 10 times its volume. The final concentration of the proteins in the solution was determined using a Nanodrop spectrophotometer operated at a wavelength of 280 nm. The extinction coefficient used for calculations was 2560 M⁻¹ cm⁻¹.

#### Vesicle Preparation

1-Palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-sn-glycerol-3-phospho-1-serine (POPS) were purchased from Avanti Polar Lipids (Alabaster). Stock solutions were prepared in chloroform using the following lipid molar ratios (% POPC/%POPS): 100:0, 91:9, and 60:40. These ratios are indicated in the acronyms of the lipid mixtures as PC%POPC/PS%POPS. The use of partially deuterated lipids is also indicated in the acronyms (usually as d31).

The chloroform was evaporated under nitrogen flow to form lipid films. Any remaining chloroform was dried under vacuum overnight. The lipid films were treated with five cycles of freezing in liquid nitrogen and thawing at 40 °C, using a water bath. The lipids were then rehydrated in either Milli-Q water for the pure zwitterionic lipids or a 500 mM NaCl solution for charged lipid mixtures, to reach a final lipid concentration of 1 mg mL⁻¹. Small unilamellar vesicles, SUVs, were prepared in 500 mM NaCl, and (iii) rinse the charged SUVs. The most efficient way to obtain high-quality charged SLBs on silicon dioxide involved the following steps: (i) fill the flow module with a 500 mM NaCl solution, (ii) inject SUVs already prepared in 500 mM NaCl, and (iii) rinse the flow module with Milli-Q water after an incubation time of 60 min. Following this protocol, reproducible high-quality PC₃₅PS₃ and d₃₁PC₉₁d₃₁PS SLBs were obtained. The need for high NaCl concentration also demonstrates that the main interaction force disturbing vesicle adhesion and therefore impacting the SLB quality is of electrostatic nature. In fact, at pH 5, which is the reference pH for Milli-Q water, both silicon dioxide and SUV surfaces carry negative charges that are not sufficiently screened by the counterions naturally present. The use of a 500 mM salt solution screened the electrostatic interactions and thereby favoring vesicle adhesion to the surface. The application of the osmotic shock promoted the fusion of the vesicles. A longer rinsing step allowed then to remove any unbound lipid material.

For depositions on sapphire surfaces, no protocol development was necessary, since zwitterionic and charged vesicles fused spontaneously without any necessity of the osmotic shock nor the use of salt solutions. This can be most likely explained in terms of electrostatics the sapphire surface being positively charged in the experimental conditions applied.

#### Neutron Reflectometry

NR experiments were performed using, as solid substrates, either a silicon single crystal (8 × 5 cm² surface, 1.5 cm thick, cut along the 111 plane, polished with 3 Å root-mean-square (RMS) roughness) or randomly oriented sapphire blocks, with the same dimensions and nominal roughness as the silicon ones. Substrates were cleaned by soaking them in chloroform, acetone, and ethanol/ultrasound was used to improve the cleaning efficiency of the solvents. Substrates were kept in each solvent for 15 min, rinsed with Milli-Q grade water, and dried before being immersed in the next solvent.

To remove any remaining organic contaminants and to make the surfaces hydrophilic, the cleaning procedure was followed by the exposure of the substrates to air-plasma for 2 min (Harrick Plasma). For the silicon crystals, this treatment promotes the formation of hydroxyl groups on the surface of the silicon oxide layer, which is naturally present on their surface. Substrates were then assembled into water-filled solid/liquid (S/L) cells prior to their use. S/L cells were provided by the Institut Laue-Langevin (ILL, Grenoble, FR) and were composed of a water reservoir made of PEEK in contact with the surface of the substrate and by two metallic plates allowing for precise temperature regulation (0.1 °C accuracy by water baths). The water reservoir was 0.5 mm thick with a 7 × 4 cm² area. The water reservoir was equipped with inlet and outlet valves, allowing the exchange of water subphase and the injection of lipid and protein solutions. The controlled exchange of the aqueous solution necessary to apply the contrast variation method was performed using an HPLC pump.

NR measurements were performed on three different neutron reflectometers: INTER47 (ISIS, U.K.), Super ADAM,48 and D1749 (both at the ILL, FR). On INTER and D17, NR measurements were performed in time-of-flight mode using wavelengths from 2 to 20 Å and two angular configurations, θ = 0.7 and 2.3° on INTER and θ = 3.3° on D17.
0.8 and 3.0° on D17, thereby covering a Q-range from ~8 × 10⁻³ to ~0.3 Å⁻¹. In the framework of specular reflectometry, Q is the component of the exchanged wave vector defined along the direction normal to the sample surface, and it is expressed as

$$Q = \frac{4\pi}{\lambda} \sin(\theta)$$  

(2)

Super ADAM was operated in monochromatic mode, with a fixed wavelength $\lambda = 5.21$ Å ($\Delta\lambda/\lambda = 0.005$). To cover a Q-range comparable to those covered on INTER and D17, the incident and detector angle $\theta$ was varied stepwise between 0° and 5.0°. The corresponding Q-range was 0–0.21 Å⁻¹. The acquisition time was changed point-by-point to account for the inherent decrease of reflected intensity for increasing angle values. During the measurements, the collimation configuration was kept constant. This led to the overillumination of the sample surface for 0° < $\theta$ < 0.9°. Because of the presence of a plastic o-ring and being a portion of the total substrate surface not exposed to the water solution (i.e., not covered with a sample), the data corresponding to this angular region were not analyzed being considered "contaminated" by reflections originated from outside of the sample region. Analysis was thus performed only for Q > 0.04 Å⁻¹.

NR data were measured for the bare substrates and for the lipid bilayers before and after protein incubation. Extensive rinsing was performed before the measurement of the postincubation curves. All of the measurements were performed at 20 °C. Raw data were converted into reflectivity curves, R(Q), using MANTID⁵⁰ (INTER), pySared (Super ADAM), and COSMOS (D17) routines. To obtain a detailed structural characterization of the sample, NR data were collected using the contrast variation method.⁵¹ To increase the accuracy of the modeling, data were measured using both hydrogen- and deuterated bilayers. Reflectivity curves were collected exposing the samples to water subphases with different D/H-buffer ratios. In particular, 100% D-buffer, 100% H-buffer, and a 38:62 D/H-buffer mixture (named silicon matched buffer, SIM-buff) were used. The SIM-buff was characterized by a scattering length density (SLD) value matching that of the silicon crystal. Data sets collected for the deuterated and hydrogenated bilayers were analyzed simultaneously using the lipid plugin provided by the Aurore software application.⁵² The global analysis of NR curves measured in different contrast conditions resulted in a set of scattering length density profiles, SLD(z). These profiles describe the distribution of chemical species along the vertical direction with respect to the bilayer surface and are directly connected to the model chosen. In brief, the model consisted of a series of layers, each described in terms of scattering length density, thickness ($\delta$), buffer volume fraction ($f_b$), and interfacial roughness ($\sigma$). The model for bare substrates consisted of an infinite layer with the SLD of the bulk material (silicon or sapphire), by an oxide layer (for silicon solely) and by an infinite bulk aqueous layer. When a bilayer was present, five additional layers were used to describe the aqueous cushion between the bilayer and the solid substrate, with the headgroups and the tails of the bilayer leaflet facing the solid substrate and of the leaflet in the proximity of the aqueous bulk phase. After protein incubation, different scenarios were evaluated to determine the more appropriate model. It showed that it is not necessary to increase the number of layers in the model; different curves could be successfully analyzed by simply allowing changes of the SLD values of the existing layers to account for the presence of protein molecules. It is worth mentioning that the total SLD of a layer composed of $N$ chemical species can be calculated as

$$\text{SLD} = \sum_{j=1}^{N} \phi_j \text{SLD}_j$$  

(3)

where $\phi_j$ ($\sum_{j=1}^{N} \phi_j ≡ 1$) and SLD, are, respectively, the volume fraction and the SLD of the jth molecular species in a given layer. Equation 3 was also used to account for the presence of buffer molecules within the layers. In this case, $\phi_j$ was named $f_{b,j}$ in the model and in the text. Details of the modeling procedure for SLBs in the absence and presence of proteins are given elsewhere.⁵³,⁵⁴

**Modeling of POPS and Histatin 5 for the Analysis of Contrast Variation NR Data.** To properly analyze NR data collected in different D/H-buffer mixtures, the effect of the exchange of labile protons in the POPS headgroup and the Hist5 sequence had to be evaluated. The first effect could be taken into account by modifying the scattering length of the PS headgroup using the lipid plugin provided by the Aurore software. The SLD values used for modeling lipid molecules are reported in Table S1.

Changes in the SLD value of Hist5 upon contrast variation were evaluated using the Biomolecular Scattering Length Density Calculator provided by ISIS.⁵⁵ The results are shown in Figure 1 as a function of the buffer SLD. The linear dependence between the buffer SLD (SLD_b) and the Hist5 SLD (SLD_Hist5) was exploited to account for Hist5 SLD changes in terms of “effective” changes in the buffer fraction within the layer regions containing Hist5. In fact

$$\text{SLD}_{\text{Hist5}} = a \times \text{SLD}_b + \text{SLD}_{\text{Hist5}}^0$$

(4)

where $a = 0.22$ and $\text{SLD}_{\text{Hist5}}^0 = 2.40$ were determined from the linear fit shown in Figure 1. In this way, the total SLD of a hydrated layer SLD containing histatin 5 could be written as

$$\text{SLD} = (1 - f_{b,j}) \text{SLD}_{\text{Hist5}}^0 + (a - a f_{b,j} + f_{b,j}) \text{SLD},$$

(5)

where $f_{b,j}$ is the volume fraction of buffer in the layer of interest. Equation 5 was used for the global fit of curves originated from the same sample and measured in different contrast conditions. In this way, the correct amount of hydration water and protein present in each layer of the model could be evaluated.

**RESULTS AND DISCUSSION**

Partially charged model membranes were formed on solid substrates by vesicle fusion,⁴²,⁴³ using mixes of negatively charged phospholipids and zwitterionic lipids, with charge ratios of 9 mol % (PC91PS9) and 40 mol % (PC60PS40), corresponding to surface charge densities of approximately −25 mC m⁻², resembling that of the common fungal membrane,⁵⁴ and −110 mC m⁻², respectively. Control measurements were performed on zwitterionic SLBs composed of POPC lipids only. In addition, partially deuterated phospholipids, d₈POPC and d₈POPS, were used to enhance the resolution of the analysis for NR experiments.

In all experiments, the temperature was set to 20 °C, and a 20 mM Tris buffer at pH 7.4 was utilized. To investigate the
effect of the electrostatic interactions, four different ionic strengths were evaluated by the addition of NaCl: 10, 80, 140, and 500 mM, corresponding to Debye screening lengths, \( \kappa^{-1} \), of 3.01, 1.06, 0.8, and 0.4 nm.

QCM-D measurements were performed to screen the optimal conditions for Hst5 adsorption to the bilayers in terms of buffer composition, pH, and electrostatic interactions. SLBs composed of POPC or PC91PS9 were deposited by the vesicle fusion method directly in the flow modules of the E4 instrument. The behavior of frequency shifts and dissipation factors upon injection and fusion of the vesicles was perfectly in agreement with the one expected for a single SLB55 (\( \Delta F \approx -25 \) Hz, overtones overlapping and \( D < 1 \times 10^{-6} \)). All of these features indicated that high-quality SLBs were obtained. Systems resulting in different behaviors were discarded after the careful evaluation of the discrepancies. The injection and incubation of Hst5 molecules were performed as described in the previous paragraph. Changes in \( \Delta F \) and \( \Delta D \) were monitored (i) after incubation but before rinsing and (ii) after rinsing. To better compare QCM-D traces upon protein addition, \( \Delta F \) and \( \Delta D \) values were all reset to zero before Hst5 injection. Selected QCM-D traces are shown in Figures 2 and 3 for PC91PS9 and POPC samples, respectively, at both low and high ionic strengths.

Figure 2. Normalized QCM-D traces for a negatively charged bilayer at two NaCl concentrations. Frequency shifts (blue) and dissipation factors (red) for a PC91PS9 sample at low (10 mM NaCl, open symbols) and high (500 mM NaCl, full symbols) salt concentration. The baseline corresponding to the signal due to an SLB (\( \Delta F = -25 \) Hz) was set to 0. The injection of Hst5 and rinsing with pure buffer are indicated by the two dotted lines. Data are shown for the 11th overtone, the most sensitive to thin films.

QCM-D experiments indicated that, at low ionic strength, Hst5 interacts with POPC and PC91PS9 bilayers. The change in frequency (and therefore in mass) was larger for the charged system, but they became equal after rinsing with the pure buffer. The extent of the adsorption strongly reduced for the PC91PS9 SLB upon increasing the salt concentration up to 500 mM. In this case, rinsing with pure buffer had no effect on the QCM-D signal. In the case of POPC, the adsorption was completely hindered at 500 mM NaCl. For all of the systems, the normalized frequencies of different overtones overlapped and dissipation factors were all smaller than \( 10^{-6} \), indicating that the systems still behaved like a solid film. Transient changes in the data recorded (as those visible in Figures 2 and 3) were caused by the flow while rinsing. By using eq 1, the adsorbed amount of Hst5 was quantified as \( \sim 0.8 \) mg m\(^{-2} \) before rinsing and as \( \sim 0.3 \) mg m\(^{-2} \) after rinsing for PC91PS9 in 10 mM NaCl. The behavior of the adsorbed amount as a function of the ionic strength of the solution emphasizes the role of electrostatic interactions, which is in line with the results reported by Hyltegren et al.\(^{37} \) for Hst5 adsorption to silica surfaces using ellipsometry. However, by simply using the QCM-D technique, it was not possible to determine the location of the adsorbed molecules. For this reason, NR measurements were performed. As discussed in the following, discrepancies between the adsorbed amount measured by QCM-D and the actual changes detected in thin films by NR were present. These differences might be explained by the different nature of the substrate surface, rough sputtered SiO\(_2\) in the case of QCM-D sensors and ultraflat native silicon oxide in the case of NR.

NR experiments were used to characterize, with a subnanometer resolution, the internal structure of solid-supported lipid bilayers before and after their exposure to solutions containing Hst5. Concerning pristine lipid bilayers, the scattering length density (SLD) profiles and structural parameters obtained from the analysis of the data are in full agreement with literature values of similar systems.\(^{55–57} \) Samples were measured in buffers with different H\(_2\)O and D\(_2\)O contents for application of the contrast variation method,\(^{46} \) and at different NaCl concentrations. NR allowed us to monitor changes taking place within the bilayer and in its proximity. In the case of purely zwitterionic SLBs, the analysis of NR data did not reveal any structural nor compositional changes upon SLB exposure to Hst5 solutions at any ionic strength (Figure S3).

Furthermore, no effect of Hst5 was detected for the PC91PS9 samples neither at 80 mM nor at 140 mM NaCl. On the contrary, at 10 mM NaCl, an increase in reflectivity at \( Q \approx 0.075 \times 10^{-6} \) Å\(^{-2} \), as well as a deepening of the first minimum at \( Q \approx 0.03 \times 10^{-6} \) Å\(^{-2} \), was detected in D-buffer after the addition of Hst5, indicating an increase of the total sample thickness at the silicon–water interface (Figure 4). Similar results were also observed in the SiM-buffer and H-buffer, which could be quantified through the global analysis\(^{55} \) of the NR data (Figures S5, S4, and S5).

Comparison between the respective profiles and related model parameters, procured before and after Hst5 injection, indicated the formation of a “gap” between the SLB and the substrate. At the same time, the structure of the bilayer itself
could be modeled using the same set of structural parameters used to describe the corresponding pristine SLB.

These results were further confirmed by NR experiments on partially deuterated 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DOPC) SLBs. On average, the thickness of the gap between the substrate and the SLB increased from 3.2 ± 0.4 to 21 ± 3 Å upon addition of Hst5, where the increased thickness corresponds to approximately twice the radius of gyration of the protein in a random coil conformation. The composition of this gap was obtained by the global fit of the NR curves, and it resulted to be 74 v/v % water and 26 v/v % Hst5 molecules, determined by considering the H-to-D proton exchange when buffers with different H2O/D2O contents were used (Figure 1). From the Hst5 volume fraction in the gap, the Hst5/lipid ratio was determined as 1:20 (by mol) assuming a molecular volume of 1224 Å3 for a protein molecule. We could therefore confirm that the gap was indeed related to the spontaneous formation of a protein cushion lifting up the bilayer. These results are in line with those reported in the earlier works of Majewski et al. and Wong et al., in which the formation of a polymer cushion (PEI) was observed underneath a preformed zwitterionic bilayer (1,2-di-myristoyl-sn-glycero-3-phosphocholine, DMPC). They found that in selected electrostatic conditions, PEI (positively charged) could penetrate the bilayer, probably because of a defect-mediated mechanism, promoting the formation of a ~4 nm polymer region between the bilayer and the substrate (quartz in their case, negatively charged). The PEI-enriched region resulted composed of 20 v/v % PEI and 80 v/v % D2O. These values are very similar to those described in the present work, obtained using Hst5 instead of PEI. The mechanism of interaction and the nature of the PEI–bilayer–quartz interaction potential was briefly discussed in terms of an electrostatic balance between the various components of the system. Opposite to the results shown in the present article, in high-salt conditions (larger screening effect), the authors reported a stronger PEI–bilayer interaction as compared to the one observed in low-salt conditions. Nonetheless, they indicated that a strong PEI–quartz electrostatic attraction could be the origin of the bilayer displacement.

For the Hst5 case, to elucidate the role of bilayer charge in the interaction, additional NR measurements were performed at physiological ionic strength for SLBs in which the POPC content was increased to 40 mol %. In this case, the data analysis indicated the presence of a 14 ± 1 Å thick cushion and an altered SLB internal structure (Figure S1). The presence of defects in these SLBs might have affected the mechanism and the extent of interaction, resulting in the observed changes. However, all of the results obtained by NR support the hypothesis that electrostatics plays a major role in driving the interaction between Hst5 and the membranes.

The influence of the surface properties of the solid substrate on the Hst5–SLB interaction has been further analyzed by NR on randomly oriented sapphire substrates (Al2O3) that are positively charged for the pH values adopted in this study. In contrast to silicon, the injection and incubation of Hst5 molecules did not affect the structure of the SLB nor did promote the formation of the cushion (Figure S6).

The presented results have a twofold implication. First, we could demonstrate the spontaneous formation of a highly hydrated cushion as a result of the interaction between negatively charged lipid bilayers and an intrinsically disordered protein such as Hst5 (Figure 6). The formation of the cushion is based on the ability of Hst5 to cross the bilayer without causing any structural changes in SLBs characterized by a charge density of biological relevance. The method reported does not require any prior modification of the
chemistry of the supporting interface nor the use of toxic solvents; hence, it can be considered “green.” This method allows us to overcome the main drawbacks currently affecting other methods used to decouple SLBs from substrates, especially on large surfaces as those used in NR.

Second, these findings have a clear biological relevance and, to our knowledge, this is the first report on the ability of HstS to penetrate and cross a supported membrane. This behavior resulted to be strongly influenced by the electrostatic interactions in the system, and by varying the SLB charge (from 9 to 0% and 40%) and the ionic strength between 10 and 500 mM NaCl, the HstS–SLB interaction could be tuned.

Regarding the underlying mechanism, it is known that the driving force for the adsorption of an oppositely charged macromolecule to a solid surface has an entropic origin, e.g., due to counterion release. On the contrary, the conformation of the adsorbed macromolecule depends on the charge distribution and on the properties of the surface such as charge density and distance between charged groups. When a lipid bilayer is deposited on a solid surface, the system consists of one solid surface and two fluid interfaces, with accompanying counterions. The pH and the ionic strength can be considered equal on both sides of the bilayer, since bilayers are permeable to ions and other small molecules, whereas, because of the confinement, the dielectric constant can be different in the substrate-SLB cushion and in the bulk solution, as recently reported.61–63 Hence, when HstS, which has the ability to regulate charge due to its high content of histidines, approaches the headgroups of the lipid bilayer facing the bulk solution, the charge of the protein will slightly increase, as proven by computer simulations.37,38 The length scales at which protonation is initiated, determined by simulations, and the thickness of the bilayer are of the same order of magnitude; hence, the protein recognizes the electrostatic field from the inner bilayer headgroups as well as the solid silica surface already in the bulk solution. The adsorption to the silica surface is most likely promoted by the counterion release, which induces an increase in osmotic pressure between the silica surface and the bilayer, and thereby a cushion is formed.

The results indicate that the protein adsorbs to the solid silica surface rather than to the lipid headgroups, since (i) the cushion is not formed if the bilayer is deposited on a positive surface and (ii) the QCM-D measurements have shown that approximately 41% more protein adsorbs to a pure silica surface in comparison to the bilayer comprising 9% charges. Nevertheless, because HstS possesses a polyelectrolytic behavior, one cannot exclude that the protein bridges, or induces, steric stabilization between the solid surface and the lipid bilayer. This can be motivated by the fact that the thickness of the cushion is twice the radius of gyration of the protein in a solution.33,38 In conclusion, we envision that this type of cushion will play an important role in the life science area, allowing the preparation of better cell membrane mimics.

ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.langmuir.0c00120. Molecular volumes and scattering lengths for the lipid samples; analysis of additional NR data (PDF)

### AUTHOR INFORMATION

**Corresponding Authors**

Yuri Gerelli — Partnership for Soft Condensed Matter, Institut Laue-Langevin, 38000 Grenoble, France; Department of Life and Environmental Sciences, Polytechnic University of Marche, 60131 Ancona, Italy; [orcid.org/0000-0001-5655-8298](https://orcid.org/0000-0001-5655-8298); Email: y.gerelli@univpm.it

Marie Skepo — Division of Theoretical Chemistry, Department of Chemistry, Lund University, SE-221 00 Lund, Sweden; LINXS—Lund Institute of Advanced Neutron and X-ray Science, SE-233 70 Lund, Sweden; [orcid.org/0000-0002-8639-9993](https://orcid.org/0000-0002-8639-9993); Email: marie.skepo@teokem.lu.se

**Authors**

Amanda Eriksson Skog — Division of Theoretical Chemistry, Department of Chemistry, Lund University, SE-221 00 Lund, Sweden; Partnership for Soft Condensed Matter, Institut Laue-Langevin, 38000 Grenoble, France

Stephanie Jephthah — Partnership for Soft Condensed Matter, Institut Laue-Langevin, 38000 Grenoble, France

Rebecca J. L. Welbourn — ISIS Pulsed Neutron Facility, Rutherford Appleton Laboratory, Harwell Science and Innovation Campus, STFC, Didcot, Oxford OX11 0QX, United Kingdom; [orcid.org/0000-0003-4287-506X](https://orcid.org/0000-0003-4287-506X)

Alexey Klechikov — Department of Physics and Astronomy, Uppsala University, SE-75120 Uppsala, Sweden

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.langmuir.0c00120

**Notes**

The authors declare no competing financial interest.

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