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Atom-scale depth localization of biologically important chemical elements in molecular layers

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In nature, biomolecules are often organized as functional thin layers in interfacial architectures, the most prominent examples being biological membranes. Biomolecular layers play also important roles in context with biotechnological surfaces, for instance, when they are the result of adsorption processes. For the understanding of many biological or biotechnologically relevant phenomena, detailed structural insight into the involved biomolecular layers is required. Here, we use standing-wave X-ray fluorescence (SWXF) to localize chemical elements in solid-supported lipid and protein layers with near-Ångstrom precision. The technique complements traditional specular reflectometry experiments that merely yield the layers’ global density profiles. While earlier work mostly focused on relatively heavy elements, typically metal ions, we show that it is also possible to determine the position of the comparatively light elements S and P, which are found in the most abundant classes of biomolecules and are therefore particularly important. With that, we overcome the need of artificial heavy atom labels, the main obstacle to a broader application of high-resolution SWXF in the fields of biology and soft matter. This work may thus constitute the basis for the label-free, element-specific structural investigation of complex biomolecular layers and biological surfaces.

In the present work, we demonstrate that light, biologically relevant chemical elements such as P and S can be localized also in Bragg reflection configuration and thus with atom-scale resolution. This approach enables the label-free, high-resolution, element-specific structural investigation of biomolecular layers. We work with representatives of the three most important classes of biomolecules: lipids, saccharides in the form of glycolipids, and proteins. Lipids with phosphatidylcholine (PC) headgroups are among the most abundant lipid classes in eukaryotic cells and the dominant class in animals (17). Glycolipids bearing a sulfate group, such as the sulfoglycolipid 3-O-sulfo-D-galactosyl-β-D-glucopyranosylceramide (SGC), are found in eukaryotic cell membranes, especially in the nerve system and in photosynthetic membranes (18, 19). Element-specific studies as presented here can thus reveal the structural details of biological multicomponent surfaces and devices, I-00133 Roma, Italy.

Author contributions: E. Schneck, R.F., D.N., G.F., and J. Daillant designed research; E. Schneck, E. Scoppola, D.N., G.F., and J. Daillant performed research; J. Drnec, C.M., and R.F. contributed special experimental tools; E. Schneck and J. Daillant analyzed data; and E. Schneck, G.F., and J. Daillant wrote the paper.

The authors declare no conflict of interest.

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Significance

Interfacial molecular layers are a major component of all biological matter and also play key roles in most biotechnological applications. The understanding of important biological processes involving molecular layers typically relies on detailed structural insight. We demonstrate that standing-wave X-ray fluorescence enables the label-free, element-specific structural investigation of molecular layers at atom-scale resolution perpendicular to the interface. The present work establishes a promising approach to the comprehensive structural analysis of complex biological and biotechnologically relevant surfaces.
membranes. The protein human serum albumin (HSA), on the other hand, is the most abundant protein in the human blood serum and plays an important role in protein adsorption to biomaterial surfaces and in the course of foreign body reactions (20). The structural investigation of HSA interaction with surfaces is therefore important for the rational design of protein resistant and thus biocompatible surfaces (21).

Results and Discussion

Fig. 1A and B shows the chemical structures of the molecules of which the studied solid-supported layers are composed: SGS, the phospholipid 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), the lipopolymer 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-5000] (PEG-lipid), and HSA. SGS (Fig. 1A, Top) roughly consists of a sulfated galactose headgroup connected to two hydrophobic fatty acid tails via a compact linker region. DSPC (Fig. 1A, Middle) has a similar architecture, but the headgroup is formed by phosphocholine comprising one P atom. PEG-lipid (Fig. 1A, Bottom) is similar to DSPC but the tertiary amine of choline is replaced by an amide-bonded PEG chain comprising 114 monomers. HSA (Fig. 1B) is a globular protein with about 600 amino acids and mass \( m_{\text{HSA}} = 67 \text{ kDa} \) and comprises 41 S atoms contained in its cysteine and methionine amino acids (www.uniprot.org/uniprot/P02768). The center of mass position of the S distribution approximately coincides with the one of the whole molecule (22).

The planar multilayers used for the SWXF experiments had 20 repetitions of Al (10 nm)/Ni (10 nm) alternating layers on top of sapphire single crystal wafers, so that the terminal layer is Al, which forms a thin layer of amorphous aluminum oxide at the outer surface. The measurement geometry is schematically illustrated in Fig. 1C. The X-rays via photoelectric ionization induce characteristic fluorescence of the target elements. Fluorescence spectra (see Fig. S1 for a representative example) were measured for various incident angles \( \theta \) in angular scans around the multilayer Bragg angle \( \theta_B = 0.645^\circ \). For each \( \theta \), the amplitudes of the fluorescence peaks were determined. The angle-dependent fluorescence intensity of target element \( j \), \( I_j(\theta) \), is proportional to the spatial integral over the product of the elemental density profile perpendicular to the interface, \( \rho_j(z) \), and the known angle-dependent standing wave (SW) intensity \( \Phi(\theta, z) \)

\[
I_j(\theta) = A \int_{-\infty}^{\infty} \Phi(\theta, z) \rho_j(z) dz. \tag{1}
\]

Eq. 1 thus allows reconstructing \( \rho(z) \) from the angle-dependent characteristic fluorescence. \( A \) is a prefactor determined by fluorescence yield and detection efficiency, and in general also depends on the incident angle (7). In Eq. 1, we safely neglected the depth dependence of the fluorescence attenuation, because in the present study the target elements are confined in nanometric layers. For a given incident angle \( \theta \), \( \Phi(\theta, z) \) follows from the interfacial scattering length density (SLD) profile and can be computed from a suitable slab model representation of the SLD via the phase-correct summation of all reflected and transmitted partial waves (23), as has been described previously (14). As shown in the inset of Fig. 2, close to the Bragg condition of the periodic structures, strong beam reflection occurs, giving rise to a strongly modulated SW with period \( \Lambda = d/n \), where \( d \) is the multilayer repeat and \( n \) is the Bragg peak order (\( n = 2 \) in the present work). The main panel of Fig. 2 shows SWs (solid, dashed, and dotted lines) computed for three different angles around \( \theta_B \) indicated with vertical straight lines in the inset. As \( \theta \) is increased through the strong reflection condition, the nodes and antinodes of the standing wave gradually move toward the interface by half a SW period (Fig. 2) and probe structures with high spatial resolution. With \( \Phi(\theta, z) \) at hand, the angle-dependent fluorescence intensities of the target elements, \( I_j(\theta) \), were then modeled according to Eq. 1 using a suitable parameterization of \( \rho_j(z) \)

\[
\rho_j(z) = \rho_{\text{max}} e^{-(z-c_j)/\sigma_j^2}. \tag{2}
\]

In this Gaussian representation, the amplitude \( \rho_{\text{max}} \), the center position \( c_j \), and the width \( \sigma_j \) are adjustable fitting parameters. The weak angle dependence of the prefactor \( A \) in Eq. 1 arises due to geometrical effects and was approximated linearly in a
narrow interval around the Bragg angle, as $A(\theta) = 1 + (\theta - \theta_0)C$ with an adjustable fitting parameter C.

Column B of Figs. 3 and 4 and column A of Fig. 5 show angle-dependent $K_s$ fluorescence intensities $I_s(\theta)$, from P, S, and K atoms in the biomolecular layers studied together with the simulated intensities according to the best matching model parameters. It is seen that the shapes of the experimental data are well captured by the simulated intensities. The well-defined positions of the minima and/or maxima in the curves, together with the intensity levels on the two sides of these extrema, encode the center position $\theta_0$. The associated uncertainty was estimated as $\pm 2 \text{ Å}$ (Supporting Information and Fig. S2). The width $\sigma_j$ is primarily encoded in the relative amplitude of the modulation of $I_s(\theta)$ with respect to the “baseline” further away from the Bragg condition. When $\sigma_j$ is much smaller than $\Lambda$, in practice $\sigma_{\text{min}} \sim \Lambda/10$ (Supporting Information and Fig. S3), then the amplitude of this modulation saturates and no longer sensitive to the precise value of $\sigma_j$. In the present work, $d \approx 200 \text{ Å}$ and with $n = 2$, we obtain $\Lambda \sim 100 \text{ Å}$ and $\sigma_{\text{min}} \sim 10 \text{ Å}$. Finally, $\rho_{\text{max}}$ merely acts as a scale factor. The best-matching values of $z_j$ and $\sigma_j$ are summarized in Table 1.

**Lipid Single Monolayers.** Fig. 3 A and C, Top, schematically illustrates the chemical details and the architecture of a single SGS monolayer on top of bare Al oxide. As a result of the Langmuir–Blodgett (LB) transfer, the hydrophilic monosaccharide headgroup is oriented toward the solid surface. Fig. 3B, Top, shows the angle-dependent $S$, $K_s$, and $P$ fluorescence intensity $I_s(\theta)$ from the SGS sulfate layer together with the modeled intensity. The intensity minimum is located at $(\theta - \theta_0) \sim 0.005^\circ$. This feature, together with the significantly different intensity levels on the two sides of the minimum, sharply defines the value for the center position of the $S$ distribution, $z_S$. Because the sulfate group is chemically attached close to the tip of the molecule and can be assumed to be in close contact to the surface, we use the obtained value as zero position of our coordinate system, $z_S = 0$ (Fig. 3D, Top).

Fig. 3, Middle, summarizes the results obtained with a single DSPC monolayer on Al oxide. The $P$, $K_s$, and $S$ fluorescence intensity $I_s(\theta)$ (Fig. 3B, Middle) from the phosphogroups in the DSPC monolayer (Fig. 3 A and C, Middle) is an excellent match to the one of the S $K_s$ fluorescence from the SGS monolayer. However, the minimum is located very close to the Bragg condition, $(\theta - \theta_0) \sim 0.002^\circ$, and thus shifted slightly but significantly with respect to $I_s(\theta)$ of the SGS monolayer. Moreover, the intensity levels on the two sides of the minimum are quite similar. The best-matching center position of the $P$ distribution according to these features is $z_P = 4 \pm 2 \text{ Å}$ (Fig. 3D, Middle). The obtained height difference of about $\Delta z \sim 4 \text{ Å}$ between the DSPC phosphate layer and the SGS sulfate layer appears plausible in view of the different headgroup structures of the two molecules: while the sulfate group in SGS is located at the tip of the headgroup, the phosphate group in DSPC is not the terminal moiety of the molecule. Instead the terminal $(\text{CH}_3)_2\text{N}(\text{CH}_2)_{18}^+$ (choline) groups can act as spacer layer with a thickness of a few Ångströms between the substrate surface and the phosphate layer.

Fig. 3, Bottom, shows the results obtained with a DSPC monolayer incorporating 5 mol% PEG-lipid on Al oxide. The molecular organization of this DSPC/PEG-lipid mixed layer is not obvious a priori. Several scenarios with similar overall layer thicknesses may occur in principle. When deposited onto hydrophobic substrates in water, the used DSPC/PEG-lipid mixtures form dense lipid monolayers displaying a highly hydrated PEG brush on top of the hydrophilic headgroups (24). One may thus expect a similar architecture also on the solid surface, with a PEG layer between the Al oxide surface and the lipid monolayer. However, also intermixed phases of PEG and lipids (25), as well as the escape of PEG from the space between oxide surface and lipid layer (26), have been reported. In the present work, SWXF puts us in the position to accurately localize P atoms and thus directly discriminate between different scenarios. Fig. 3B, Bottom, shows the $P$, $K_s$, and $S$ fluorescence from the phosphogroups in the mixed DSPC/PEG-lipid monolayer together with the modeled intensity. With $z_P = 3 \pm 2 \text{ Å}$, the best-matching center position of the $P$ distribution is undistinguishable from that in the pure DSPC monolayer. This result clearly rules out the presence of any...
significant spacer layer formed by PEG between Al oxide and the lipid layer. Instead, it suggests an architecture in which the PEG portion is mainly on the backside of the lipid monolayer, as depicted schematically in Fig. 3C, Bottom. This structure can be attributed to the more favorable interactions of the positively charged Al oxide with the polar lipid headgroups, especially with the negatively charged phosphatidyl-ethanolamine moieties of PEG-lipid, than with PEG. As discussed in Supporting Information, only a small fraction of PEG chains may percolate the headgroup layer without affecting $z_{\text{P}}$. Note that the transfer of the mixed monolayer onto Al oxide was confirmed in independent ellipsometry measurements (Supporting Information). For all studied lipid single monolayers the obtained widths of the P and S distributions, $\sigma_P$ and $\sigma_S$, respectively, are below 10 Å. In fact, it can be assumed that the widths of these distributions are vastly dominated by the topographic roughness of the solid surface and therefore approximately coincide with its RMS roughness, $\sigma_{\text{RMS}} \sim 3$ Å (Fig. S4 and Table S1). For the illustration of elemental distributions in 3D, $\sigma_P = \sigma_S = \sigma_{\text{RMS}}$ was therefore assumed.

Lipid Double Monolayers. Fig. 4 shows the results obtained with a double SGS monolayer on top of a surface hydrophobically functionalized with octadecyltrichlorosilane (OTS). The sample architecture is illustrated in Fig. 4A and C. The two negatively charged headgroup layers are facing one another. Because for this sample the LS (Langmuir–Schaefer) and LB transfers were done from a 1 mM KCl solution, the negative charge is compensated solely by K+ counterions, which can be locally dispersed consistently by SWXF. Fig. 4B shows the S $K_x$ fluorescence from the sulfate groups along with the intensity of the K $K_x$ fluorescence from the counter ions. Both exhibit pronounced maxima near the Bragg condition. This behavior is in clear contrast to the S fluorescence intensity measured with SGS single monolayer (Fig. 3B, Top) and reflects that the elements of interest are located at a substantially different height above the solid surface, as implied by the different sample architecture. Strikingly, $I_S(\theta)$ and $I_K(\theta)$ for the SGS double monolayer peak at virtually the same angle, $(\theta - \theta_B) \sim 0.003^\circ$, indicating that the centers of mass of S and K have a common center of mass position to good approximation, which can be understood from the mirror symmetry of the double layer. The best-matching positions, $z_S = 58 \pm 2$ Å and $z_K = 59 \pm 2$ Å, are identical within the error. Their precise common value $z_{SK} \sim 58$ Å can be rationalized in the following way. Starting from the solid surface at $z = 0$, the dense OTS layer, including silane and alkyl portions has a thickness of $d_S \sim 25$ Å, as reported in previous reflectometry studies (27, 28). The thickness of the first (inner) monolayer of SGS with its C16/C17 alkyl chains and the saccharide headgroup can be estimated as $d_S \sim 20$–25 Å. For symmetry reasons the centers of mass of the S and K distributions must be located at the midplane between the two SGS surfaces. The remaining height difference $d_S = z_{SK} - (d_S + d_S)$ must thus be attributed to the separation between the two opposing monolayer surfaces, suggesting that the hydration layer between the surfaces has a significant thickness $D = 2d_S$. Strong repulsion between the negatively charged surfaces can be expected when only monovalent counter ions are available, giving rise to substantial swelling with water even at moderate (as much as 25%) air humidity. In fact, it was shown recently that the incorporation of negatively charged sulfoglycolipids at even small fractions dramatically extends the swelling range in glycolipid multilayers (29). Although the experimental data are well described by the model based on Eq. 2 with $\sigma_{\text{S}}$, it should be noted that in the studied double monolayer neither S nor K distributions can be assumed as unimodal. As explained above, the shape of an elemental distribution cannot be resolved when its width, or more generally, its SD is below a certain threshold $\sigma_{\text{min}}$. For a Gaussian distribution, SD and $\sigma_{\text{S}}$ coincide. For a more suitable, bimodal description of the S and K distributions, comprising two Gaussian peaks of individual width $\sigma_{\text{mid}}$ separated by a distance $\Delta z$, the criterion for the SD to be below $\sigma_{\text{min}}$ is $\Delta z \leq 2\sqrt{\sigma_{\text{min}}^2 - \sigma_{\text{mid}}^2}$. For $\sigma_{\text{min}} = 10$ Å and with the rough approximation that $\sigma_{\text{mid}}$ coincides with the topographic roughness of the substrate, $\sigma_{\text{mid}} = \sigma_{\text{RMS}}$, we obtain $\Delta z \leq 19$ Å (Table 1).

Protein Layers. Fig. 5B, Top, schematically illustrates HSA adsorbed to a bare Al oxide surface. Fig. 5A, Top, shows the $S$ $K_x$ fluorescence from the S atoms contained in HSA’s cysteine and

Table 1. Best-matching values of $z$ and $\sigma$ for the P, S, and K distributions as obtained in fits to the respective angle-dependent fluorescence intensities from the studied biomolecular layers

<table>
<thead>
<tr>
<th>Sample</th>
<th>Element</th>
<th>Center position (Å)</th>
<th>Width (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SGS monolayer on Al oxide</td>
<td>S</td>
<td>$z_S = 0$</td>
<td>$\sigma_S \leq 10$</td>
</tr>
<tr>
<td>DSPC monolayer on Al oxide</td>
<td>P</td>
<td>$z_P = 4 \pm 2$</td>
<td>$\sigma_P \leq 10$</td>
</tr>
<tr>
<td>DSPC monolayer with 5 mol% PEG-lipid on Al oxide</td>
<td>P</td>
<td>$z_P = 3 \pm 2$</td>
<td>$\sigma_P \leq 10$</td>
</tr>
<tr>
<td>SGS double monolayer on OTS</td>
<td>S</td>
<td>$z_S = 58 \pm 2$</td>
<td>$\Delta z \leq 19^*$</td>
</tr>
<tr>
<td></td>
<td>K</td>
<td>$z_K = 59 \pm 2$</td>
<td>$\Delta z \leq 19^*$</td>
</tr>
<tr>
<td>HSA on Al oxide</td>
<td>S</td>
<td>$z_S = 17 \pm 2$</td>
<td>$\sigma_S \leq 10$</td>
</tr>
<tr>
<td>HSA on OTS</td>
<td>S</td>
<td>$z_S = 34 \pm 2$</td>
<td>$\sigma_S \leq 10$</td>
</tr>
<tr>
<td>HSA on Al oxide under water</td>
<td>S</td>
<td>$z_S = 37 \pm 5$</td>
<td>$\sigma_S = 20 \pm 5$</td>
</tr>
</tbody>
</table>

*Distance between two peaks in a bimodal distribution.
methionine amino acids together with the modeled intensity. The intensity exhibits a minimum slightly below the Bragg angle \((\theta - \theta_B \sim -0.003^\circ)\) and a maximum significantly above the Bragg angle \((\theta - \theta_B \sim 0.01^\circ)\). These distinct features correspond to a center position of the S distribution at \(z_S = 17 \pm 2 \text{ Å}\). We recall that the center of mass of the S distribution and that of the whole HSA protein roughly coincide. The thickness \(d_{\text{HSA}}\) of a HSA layer with an S distribution centered at an altitude \(z_S = z_{\text{surf}}\) above the surface can thus be approximated as \(d_{\text{HSA}} \sim 2z_S\). For \(z_S = 17 \pm 2 \text{ Å}\) and \(z_{\text{surf}} = 0\), we obtain \(\Delta z = z_S - z_{\text{surf}} = 10 \pm 2 \text{ Å}\) and \(d_{\text{HSA}} \sim 30-40 \text{ Å}\). Note that in this procedure the width of the S distribution is not involved for the determination of \(d_{\text{HSA}}\). Serum albumin has been described as a prolate ellipsoid with major and minor axes of 145 and 40 Å, respectively (30), or as heart-shaped molecule with dimensions \((55 \text{ Å})^2 \times 90 \text{ Å}\) (31). The thickness obtained here thus indicates that HSA assumes a side-on configuration in which d coincides with the shorter molecular axes. Our result, \(d_{\text{HSA}} \sim 30-40 \text{ Å}\), is somewhat smaller than the reported length of the shorter molecular axis, further indicating that HSA experiences significant deformation on the surface as compared to the dry case (35). For the width \(\Delta z = 2\sigma = 34 \pm 2 \text{ Å}\) of the OTS-functionalized surface is even thinner than that on the bare Al oxide surface. In fact, more pronounced protein deformation on hydrophobic surfaces than on hydrophilic surfaces has been reported in several studies (32–34) and has been attributed to exposure of hydrophobic portions to the surface (35). For the width of the S distribution, we consistently obtain \(\sigma_z \lesssim 10 \text{ Å}\), corresponding to \(\text{FWHM} \leq 24 \text{ Å}\). However, our results are not always consistent with shorter \(\sigma_z\) when considering that S atoms are not evenly distributed over the HSA molecule but preferentially found in its interior (22).

Fig. 5B, Middle, illustrates HSA adsorbed to an OTS-functionalyzed surface. The S K\(_2\) fluorescence (Fig. 5A, Middle) exhibits a maximum slightly above the Bragg angle at \(\theta - \theta_B \sim 0.004^\circ\). The fit to the data yields \(z_S = 34 \pm 2 \text{ Å}\). The thickness of the OTS layer is \(25 \text{ Å}\) (see above). The surface to which HSA is adsorbed is thus located at \(z_{\text{surf}} = 25 \pm 2 \text{ Å}\) so that \(\Delta z = z_S - z_{\text{surf}} = 10 \pm 2 \text{ Å}\) and \(d_{\text{HSA}} \sim 2z_S \sim 20 \text{ Å}\), indicating that the HSA layer adsorbed to the OTS-functionalized surface is even thinner than that on the bare Al oxide surface. In fact, more pronounced protein deformation on hydrophobic surfaces than on hydrophilic surfaces has been reported in several studies (32–34) and has been attributed to exposure of hydrophobic portions to the surface (35). For the width of the S distribution, we consistently obtain \(\sigma_z \lesssim 10 \text{ Å}\), corresponding to \(\text{FWHM} \leq 24 \text{ Å}\). For the S distribution’s center position \(z_k \sim 37 \text{ Å}\). Due to the more scattered nature of the fluorescence intensities, we estimate the associated parameter error as \(5 \text{ Å}\). With \(z_S = 37 \pm 5 \text{ Å}\), the center position obtained under water is shifted away from the surface substantially with respect to the dry case \((z_S \lesssim 17 \pm 2 \text{ Å})\). Within the assumption of coinciding center of mass positions of the S distribution and of the whole protein, this indicates that the fully hydrated protein layer is as thick as \(d_{\text{HSA}} \sim 2\Delta z = 2\sigma_z = 60-80 \text{ Å}\). For the width of the S distribution, we consistently obtain \(\sigma_z = 20 \pm 5 \text{ Å}\), corresponding to an FWHM of \(48 \pm 12 \text{ Å}\). Absorption of serum albumin to Al oxide was previously investigated using zeta-potential and UV-Vis measurements, and a layer thickness of about 55 Å under water was reported and interpreted as side-on configuration (31). Thicker serum albumin layers of more than 100 Å and up to 70 Å were reported for mica/water (36) and air/water interfaces (37), respectively. Those results have been interpreted either as the result of tilted, or top-on configurations, in which the long protein axis is perpendicular to the surface, or as protein double layers. Our results point toward a tilted top-on configuration. Important aspects of the configuration of proteins adsorbed to solid surfaces, such as their height and lateral extension, can also be obtained by atomic force microscopy (AFM) (38, 39). However, this approach is only sensitive to the outer protein shape and not applicable to proteins adsorbed to soft interfaces (24). In contrast, SWXF can equally probe rigid and soft interfaces and due to its element specificity has the potential to yield a comprehensive picture of protein configurations. Future studies optimized for the simultaneous localization of S, the low P amounts in phosphorylated amino acids, and metal ligands, can serve to unambiguously identify the orientation and internal conformation of proteins adsorbed to surfaces.

In the present work, the surface densities of P and S were at the order of 1 atom/nm\(^2\). Although with the current methodology densities as low as 1 atom/10 nm\(^2\) (corresponding to one or several S atoms per protein in a monolayer, depending on the protein size) already appear to be sufficient, one may expect that methodological improvements will further enhance the sensitivity. Especially for the most interesting but also most challenging measurements involving solid/liquid interfaces (Fig. 5, Bottom) such improvements may include \((i)\) the use of thinner and chemically purer polymer films or nano-confinements and \((ii)\) the use of multi-layers with shorter periods and larger Bragg angles, thereby reducing beam absorption.

**Conclusions**

We showed that SWXF with planar solid multilayer substrates enables localization with near-Ångstrom precision of the light, biologically important chemical elements P and S in the direction perpendicular to an interface. By using this method, we structurally characterized nanometric layers of the most important classes of biomolecules. The measurements yield element-specific insight into the architecture of various lipid monolayer architectures and into the conformations of proteins adsorbed to planar surfaces under various conditions. The presented approach allows for the label-free investigation of complex biomolecular interfaces with great structural detail.

**Materials and Methods**

**Materials and Sample Preparation.** Sulfoglycolipids (SGS, 3-0-sulfato–galactosyll-1′-N-heptadecanoyl-o-erythro-sphingosine), phospholipids (DSPC, 1,2-distearoyl-sn-glycero-3-phosphocholine), and lipopolymers (PEG-lipid, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-(methoxy(polyethylene glycol)-5000)) were dissolved in chloroform at 2 mg/mL. In addition, a mixture of DSPC and PEG-lipid with a PEG-lipid mole fraction of 5% at 2 mg/mL overall concentration was used. HSA was dissolved in water at 1 mg/mL. Multilayer substrates were purchased from Xscitech. Further details about the materials are given in Supporting Information. The substrates were cleaned by washing with chloroform, acetone, ethanol, and water, followed by UV-ozone treatment.

In some cases the substrates were then rendered hydrophobic via covariant functionalization with OTS by immersion in freshly prepared solutions of OTS in hexadecane at a concentration of 1 mM for 1 h and subsequent rinsing in hexadecane and ethanol. This treatment resulted in water surface contact angles of >100°, in agreement with earlier studies dealing with the OTS functionalization of aluminum oxide surfaces (40). Single and double lipid monolayers on the surface of the multilayer substrates were prepared using the LB and/or LS transfer methods. For this purpose, lipid solutions (of SGS, DSPC, or DSPC/PEG lipid mixtures) in chloroform were first spread at the air/water interface in a Teflon Langmuir trough (Nima Technology) containing water or dilute aqueous salt solution. The amphiphilic lipid molecules immobilized at the interface were then compressed to a monolayer with a lateral pressure of 35 ± 1 mN/m. As can be seen in the compression isotherms shown in Fig. S5, the incorporation of 5 mol% PEG-lipid into DSPC had no significant influence on the area per lipid at this pressure. Single lipid monolayers were deposited via LB transfer onto bare Al oxide surfaces. For the preparation of double lipid monolayers, a first lipid layer was deposited onto OTS-functionalized surfaces via LS transfer. Subsequently the second layer was deposited via LB transfer on top of the first layer. HSA protein monolayers were prepared by letting HSA adsorb to bare or
OTS-functionalized substrates from aqueous HSA solution for 1 h followed by rinsing with water. In the last step, the HSA layers were either dried or covered with a thin water layer under a 4-μm-thick polymer film (Ultralene; SPEX SamplePrep). The water layer was stabilized by capillary forces and according to beam absorption measurements was no thicker than 1 μm.

**SWXF Experiments and Data Analysis.** SWXF experiments were carried out at the ID03 beamline of ESRF and at the DIFFABS beamlime of Synchrotron SOLEIL with 7.0-keV beam energy. The sample plane was oriented vertically. X-ray fluorescence was measured using a silicon drift detector (either Ketek AXAS or Hitachi Vortex) placed perpendicular to the sample plane about 3 mm from the surface. To improve signal statistics without the risk of beam damage to the samples, the scans were repeated several times with laterally shifted beam position on the sample surface. For this purpose in the first step the reference spectra were analyzed. The relevant reference spectra for biomolecular layers on bare substrates, on OTS-functionalized substrates, and at the solid-liquid interfaces, respectively, are the spectra of the bare substrate, of the OTS-functionalized substrate, and of the bare substrate under the polymer film. After energy calibration they were modeled as linear combinations of elemental spectra following ref. 41. In the next step, the spectra of the biomolecular layers were modeled while taking into account the elemental amplitudes in their respective reference spectra. The angle-dependent SW generated by the multilayers was computed using Sergey Stepanов’s X-ray Server (serg.jmcaps.ans.w.gov), based on the structural parameters obtained in the reflectivity analysis (Supporting Information and Fig. 56).

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Supporting Information

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X-Ray Fluorescence Spectra

The left panel of Fig. S1 shows the X-ray fluorescence spectrum of a solid-supported double SGS monolayer (Fig. 4). It exhibits the characteristic fluorescence peaks of the chemical elements of interest, S and K, as well as of some of the chemical elements found in the multilayer substrates and in the sample environment (Al, Ar, Ti, and Cr). Note that Ni is not visible because the incident beam energy is below its K edge. The right panel shows the same spectrum in the region of interest around the S and K peaks together with the reference spectrum of a bare OTS-functionalized multilayer substrate. The spectra are virtually identical apart from the S and K fluorescence originating from the double SGS monolayer itself.

Sensitivity of the SWXF Measurements to the Center Position $z_m$ of an Elemental Distribution

To illustrate the sensitivity of the SWXF measurements to the center position $z_m$ of the elemental distributions Fig. S2 exemplarily shows the angle-dependent P $K_s$ fluorescence intensity from a DSPC single monolayer (symbols) together with modeled intensities for various assumptions of $z_m$. The dotted line in each panel represents the best-matching value of $z_m$. The solid lines correspond to various shifts $\Delta z$, from the optimum, namely $\Delta z = +2$ Å (Top Left), $\Delta z = +4$ Å (Top Right), $\Delta z = -2$ Å (Bottom Left), and $\Delta z = -4$ Å (Bottom Right). It is seen that for $|\Delta z| = 4$ Å (Right), the deviation between experiment and model is substantial. For $|\Delta z| = 2$ Å (Left), the deviation can still be recognized but is visually at the limit of significance. $|\Delta z| = 2$ Å can therefore be considered a reasonable estimate for the uncertainty in the parameter $z_m$.

Sensitivity of the SWXF Measurements to the Width $\sigma_j$ of an Elemental Distribution

The width $\sigma_j$ of an elemental distribution is encoded in the shape of the angle-dependent fluorescence intensity, primarily in the relative amplitude of the modulation with respect to the baseline further away from the Bragg condition (see Fig. S3 for an exemplary set of fluorescence curves modeled for Gaussian elemental distributions with different widths). When $\sigma_j$ is much smaller than the standing wave period $\Lambda$, then the amplitude of this modulation saturates and is no longer sensitive to the precise value of $\sigma_j$. In the present work, in view of the limited statistics of the experimental data points and bearing in mind minor residual uncertainties in the background treatment, $\sigma_{\text{min}} \sim \Lambda/10$ can be considered a conservative estimate for the lower end of the $\sigma_j$ range that can be determined reliably. The standing wave period close to the Bragg condition is determined by the multilayer period $d$ and the order $n$ of the Bragg peak: $\Lambda = d/n$. In the present work, the multilayer period was $d \sim 200$ Å and a second-order Bragg peak was used ($n = 2$), so that $\Lambda \sim 100$ Å and $\sigma_{\text{min}} \sim 10$ Å.

PEG Chains in the Headgroup Layer in DSPC/PEG-Lipid Mixed Monolayers on Al Oxide

In the following, we derive that only a small fraction of the PEG chains in a DSPC/PEG-lipid mixed monolayer may percolate the headgroup layer without significantly altering the height of the lipid layer with respect to the solid surface. At a PEG-lipid mole fraction of 5% ($x = 0.05$), the PEG mass per lipid is $x m_{\text{PEG}} = 250$ g/mol (with $m_{\text{PEG}} = 5,000$ g/mol). The mass ratio between PEG and lipid is thus $x m_{\text{PEG}}/m_{\text{lip}} = 0.32$ (with $m_{\text{lip}} = 790$ g/mol). If we now safely assume that the density of the percolating PEG chains is at most as high as that of the lipids, the mass ratio can serve as a lower limit of the ratio between the thickness of the compact PEG layer $d_{\text{PEG}}$ and the thickness of the lipid layer $d_{\text{lip}}$, $d_{\text{PEG}}/d_{\text{lip}} \gtrsim 0.32$. For an estimated $d_{\text{lip}} = 2.5-3.0$ nm, we obtain $d_{\text{PEG}} \gtrsim 0.8$ nm. In other words, even for the comparatively low PEG-lipid mole fraction used, the PEG amount is too high to get accommodated in the headgroup region without significantly elevating the lipid layer. Nonetheless, a small fraction of PEG may still percolate the headgroup layer.

Transfer of DSPC/PEG-Lipid Mixed Monolayers onto Al Oxide

The complete transfer DSPC/PEG-lipid mixed monolayers was confirmed in independent ellipsometry measurements on planar Al substrates covered with native Al oxide before and after monolayer transfer. In the data analysis, monolayers were approximated as homogeneous layers of refractive index $n = 1.45$, which is typical for organic materials (The exact value is not important for the following considerations.). For DSPC alone, the thickness of the organic layer as determined by ellipsometry was $d_{\text{PEG}} = 2.8 \pm 0.5$ nm. For a DSPC/PEG-lipid mixture with 5% PEG-lipid, we obtained a significantly thicker layer with $d_{\text{PEG}} = 4.1 \pm 0.5$ nm. The ratio between these two values, $d_{\text{PEG}}/d_{\text{PEG}} = 1.5 \pm 0.3$, within the error is consistent with the ratio between the transferred amounts per unit area when a constant area per molecule is assumed. Expressed in terms of the mass, this ratio is $(m_{\text{PEG}} + x m_{\text{PEG}})/m_{\text{PEG}} = 1.32$. Here, $m_{\text{PEG}} = 790$ g/mol is the mass of the lipid anchor, $m_{\text{PEG}} = 5,000$ g/mol is the mass of a PEG chain, and $x = 0.05$ is the fraction of lipids carrying a PEG chain.

Topographic RMS-Roughness of the Multilayer Substrate Outer Surface

The surface topography of a cleaned multilayer substrate was measured by AFM with a cantilever of tip radius of ~10 nm. Fig. S4 shows the obtained height profile over a measurement area of $2 \times 2$ μm. Table S1 summarizes the characteristics of the topography.

Further Details About the Materials Used

Unless stated otherwise, all chemicals were purchased from Sigma and used without further purification. Water was purified and double-deionized (MiliQ). Sulfoglycolipids (SGS, 3-O-sulfo-6-galactosyl-β1-1'–N-heptadecanoyl-d-erythro-sphingosine), sphingophospholipids (DSPC, 1,2-distearoyl-sn-glycero-3-phosphocholine), and lipopolymers (PEG-lipid, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-5000]) were purchased from Avanti Polar Lipids. The design of the planar metal multilayer substrates used to generate the standing X-ray wave was guided by several simultaneous requirements. First, materials with strongly different electron densities were desirable to give rise to a strong Bragg reflection. Second, the materials used had to be free of fluorescence lines in the energy range relevant for the detection of the target elements. In addition, the period of the multilayer should be chosen to match the characteristic size of the system under investigation. Finally, the terminal surface had to be suited for the chemical functionalization with thin biomolecular layers.

Compression Isotherms of DSPC and DSPC/PEG-Lipid Mixtures at the Air/Water Interface

Fig. S5 shows compression isotherms of a pure DSPC monolayer and of a DSPC monolayer incorporating 5% PEG-lipid. The monolayers were compressed at a rate of 4 Å/2 min per lipid. In both cases, the area per lipid $A_{\text{lip}}$ was rescaled such that at
30 mN/m it corresponds to the literature value for DSPC, $A_{\text{lip}}$ (30 mN/m) $= 47 \text{ Å}^2$ (42). It is seen that, although PEG-lipid has a strong influence on the compression isotherm for low pressures ($\Pi \lesssim 20 \text{ mN/m}$), for higher pressures ($\Pi \gtrsim 20 \text{ mN/m}$) $A_{\text{lip}}$ is dominated by the largely incompressible lipid layer and therefore virtually unaffected by the presence of PEG-lipid. The lipid packing density at the transfer pressure of $\Pi = 35 \text{ mN/m}$ can therefore be assumed to be very similar in the presence of absence of 5% PEG-lipid.

**Reflectivity of the Multilayer Substrates**

Fig. S6 shows the measured X-ray reflectivity curve of a cleaned multilayer substrate together with the best-matching fit based on a 20-fold periodic structure with two layers (Al and Ni) per period on top of a semiinfinite sapphire support. The best-matching thickness parameters of Al and Ni layers are $d_{\text{Al}} = 9.93 \text{ nm}$ and $d_{\text{Ni}} = 9.71 \text{ nm}$, respectively. The best-matching RMS-roughness corresponding to the electron density transition between Al and Ni layers is $\sigma_{\text{Al/Ni}} \sim 1.3 \text{ nm}$.

![Double SGS monolayer X-ray fluorescence spectrum](image1)

**Fig. S1.** (Left) X-ray fluorescence spectrum of a solid-supported double SGS monolayer (Fig. 4) exhibiting distinct fluorescence peaks of the chemical elements of interest S and K, as well as of chemical elements found in the multilayer substrates. (Right) Comparison between sample and reference spectra in the region of interest, highlighting the S and K fluorescence from the double SGS monolayer itself.

![Angle-dependent PKα fluorescence intensity](image2)

**Fig. S2.** Angle-dependent $P_{\text{K}\alpha}$ fluorescence intensity from a DSPC single monolayer (symbols) together with modeled intensities (solid lines) for various shifts $\Delta z_p$ in the center position $z_p$ of the P distribution. The dotted line in each panel represents the best-matching value of $z_p$. 

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Fig. S3. Exemplary set of fluorescence curves modeled for Gaussian elemental distributions with different widths $\sigma$. $\Lambda$ is the standing wave period.

Fig. S4. Height profile of a cleaned multilayer substrate as obtained by AFM.
Fig. S5. Compression isotherms of pure DSPC and DSPC incorporating 5% PEG-lipid.

Fig. S6. X-ray reflectivity curve of a cleaned multilayer substrate (symbols) together with the best-matching fit (solid line).

Table S1. Characteristics of the surface topography according to the height profile in Fig. S4

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z range</td>
<td>6.352 nm</td>
</tr>
<tr>
<td>Mean</td>
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<tr>
<td>Raw mean</td>
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<tr>
<td>RMS (Rq)</td>
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<tr>
<td>Mean roughness (Ra)</td>
<td>0.244 nm</td>
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<tr>
<td>Box x dimension</td>
<td>4.016 μm</td>
</tr>
<tr>
<td>Box y dimension</td>
<td>4.016 μm</td>
</tr>
</tbody>
</table>