Abstract: An ideal bioanalytical method would allow carrying out sensitive, selective, reproducible, fast, and in situ chemical measurements of the composition, structure and function of complex biological systems. Physical chemistry possesses advanced structure analyzing techniques which are suitable to solve, at a sub-molecular level, the structure of biomolecules. However, the prerequisite of the presence of the electrolyte solution limits the number of applicable structure analyzing techniques. Infrared spectroscopy is one of the most powerful analytical techniques used to identify the structure of biomolecules, monitor structural changes under various experimental environments and physical states. In addition, infrared reflection absorption spectroscopy (IRRAS) has been successfully applied to study supramolecular films at the solid|liquid interface. Assemblies of biomolecules belong to a particularly demanding because they bring specific for the maintenance of their functions experimental requirements which have to be taken into account planning in situ spectroelectrochemical experiments.

In this paper potential of in situ IRRAS under electrochemical control for studies of structural changes in assemblies of biomolecules such as lipid bilayers and protein films is described. Studies of different classes of biomolecules bring certain experimental conditions concerning the electrolyte composition, pH value, temperature or chemical nature of the solid support, which have to be fulfilled. Obviously, these conditions may significantly restrict the applicability of the in situ PM IRRAS. The selection of the solid substrate with required optical and electrical properties, the optical window and the electrolyte composition have to be adapted.
to the needs of biomolecules. Adjustment of experimental conditions as well as possibilities of the enlargement of the *in situ* PM IRRAS for studies of biomimetic assemblies is reviewed in this paper. Furthermore, experimental results reporting potential driven changes in models of cell membranes and protein films are reviewed. Pioneering studies aiming at the determination of structural changes in lipid membranes exposed to physiological electric fields and interacting simultaneously with proteins are described.

**Keywords:** Infrared Reflection-Absorption Spectroscopy, Spectroelectrochemistry, Lipid Membrane, Lipid-Protein Interaction, Protein Adsorption.

1 Introduction

Biomimetic studies are focused at a construction of assemblies of biomolecules that preserve their structure, recognition properties and reactivity. Functional supramolecular films of biomolecules find applications in biotechnology, bioenergetics and bioanalysis [1–4]. Application of biologically relevant assemblies brings the need of the development of preparation procedures of biomimetic films at solid surfaces. Investigation of the interface between biomolecules and a biomaterial is the focal point of biomimetic studies, because it determines the structure, stability and reactivity of a newly fabricated assembly.

Various moieties commonly present at biomolecules such as polar head groups of lipids, amino acids at proteins or nucleic acids bear a charge. Accumulation of charged molecules in an isotropic assembly of biomolecules present at a solid surface leads to the appearance of static electric fields at the interface. Static electric fields in the order of $10^7 – 10^9 \, \text{V m}^{-1}$, constitute the natural environment for lipids and proteins assembled at biological membranes [5]. Similar electric fields act at proteins interacting with a cell membrane both at its cytoplasmic and exoplasmic side. A voltage drop of $\sim 1.3 \times 10^7 \, \text{V m}^{-1}$ was determined across the ion channel in voltage dependent ion channel transmembrane proteins [6]. Due to the charge accumulated at the DNA molecule it is an interesting molecule to study the transport the electrical current in supramolecular assemblies of nucleic acids prepared on electrode surfaces. Some studies indicate that the DNA molecule transports the electrical current as effectively as semiconductors [7]. However, the confirmation of this finding requires intensive studies in this field. Electric fields acting at biomolecules may lead to proton dissociation, charge separation, cooperative alignment of dipoles, reorientation of the dipole in the molecule and even a transition to a state with a higher permanent dipole moment. Under these conditions the structure, orientation, hydration and hydrogen bond-
ing network at a molecule may differ from that in a bulk phase. Clearly, electric fields appearing at the phase boundary have a large impact on the structure and stability of assemblies of biomolecules fabricated at solid surfaces. Therefore, the effect of electric fields should be taken into account in biomimetic studies aiming at the determination of the structure and reactivity in functional supramolecular assemblies.

In order to approach this multidisciplinary and complex problem connecting surface chemistry with biology and material science advanced physicochemical and analytical techniques have to be used [8–10]. The deposition of biomimetic films on the electrode surfaces allows studies of the electric-field driven changes in these assemblies [9, 11]. Electrochemical techniques, however, are not sensitive to the structure of molecules adsorbed on the electrode surface. Determination of the structure of molecules adsorbed on electrode surfaces requires a use of spectroscopic and/or microscopic methods combined with electrochemical techniques. Among spectroscopic techniques, infrared spectroscopy (IRS) belongs to one of the most powerful bioanalytical techniques for studies of the molecular structure of biomolecules [12–15]. It gives the unique opportunity to analyze simultaneously each kind of organic species present in an assembly adsorbed on the electrode surface. In contrast to other spectroscopic techniques, a complex composition of a film limits neither the spectroscopic resolution nor sensitivity. Since various functional groups present at various molecules absorb the infrared light at specific frequencies, the influence of each component on the structure of the entire assembly can be studied simultaneously without the help of molecular probes.

Reflection based IRS techniques are commonly used to study molecular assemblies present at various interfaces. Infrared reflection absorption spectroscopy (IRRAS) techniques have been successfully applied in situ for studies of molecular films present at the electrode|electrolyte interface [16–21]. Application of IRRAS techniques to the electrochemical interface brings restricted experimental conditions. They concern the selection of a solid support with strictly defined optical and electric properties and the selection of an optical window. Moreover these two materials influence the composition of the electrolyte solution. In addition, aqueous solutions used in most of these in situ IRRAS experiments, absorb the IR radiation very strong contributing to a strong background signal which overlaps with a usually weak absorption from the adsorbed biomolecules. These experimental challenges of in situ IRRAS were solved by using either a cell in Kretchmann configuration and the phenomenon of total reflection of the IR light [22] or a thin electrolyte layer cell for experiments performed under condition of the external reflection of the IR beam [16]. In the last years attenuated total reflection infrared spectroscopy (ATR IRS) [23–28] and IRRAS tech-
Techniques [9, 11, 29, 30] have developed into powerful methods to study *in situ* the structure and its changes in biomolecules present in various supramolecular assemblies on the electrode surfaces. Obviously, studies of the structure and activity of biomolecules require a strict fulfilment of experimental conditions such as the composition, ionic strength and pH of the aqueous electrolyte solution, temperature or chemical nature of the used solid substrate. Clearly, all these experimental requirements restrict the application of IRRAS techniques for studies of the structure of biomimetic assemblies under electrochemical control. Studies of fragile biological samples and biomimetic assemblies bring specific experimental requirements, which have to be taken into consideration in *in situ* IRRAS experiments. In this paper, a brief introduction to IRRAS under electrochemical control (Section 2), limitations of IRRAS and possibilities of the extension of the application of the *in situ* IRRAS through modifications of the surface properties of the solid substrate, a selection of the optical window and electrolyte solution for studies of biomimetic films are discussed (Section 3). In the fourth section, the application of *in situ* IRRAS for structural studies of potential-driven changes in lipid bilayers [9, 30–36] and protein films [37, 38] is reviewed. Recent studies of the impact of lipid-protein interactions on the structure and stability of the membrane exposed to physiological electric fields are summarized and described [39–42].

## 2 *In situ* infrared reflection absorption spectroscopy at the electrolyte|electrode interface

Transmission infrared spectroscopy is not applicable for the analysis of thin organic films adsorbed on solid surfaces due to low intensities of the IR signals. In 1966 Greenler [43] published the theoretical fundamentals for the development of IRRAS. Greenler calculations showed that, upon reflection of the IR light from the gold|air interface, at grazing angles of incidence, the intensity of the *p*-polarized IR radiation is enhanced whereas the intensity of the *s*-polarized light is cancelled. The signal due to the absorption of the reflected *p*-polarized IR light by molecules adsorbed at the interface is enhanced. In contrast, the reflected *s*-polarized light contains no information about molecules adsorbed on the metal surface [43]. In the last decades IRRAS was expanded to study thin films adsorbed not only at the metal|gas interface [43] but also at the metal|liquid [18, 44, 45] and gas|liquid interfaces [46–48].
2.1 Application of IRRAS to the electrochemical interface: theoretical considerations and instrumentation approach

In the past 30 years IRRAS has developed into a powerful analytical tool to probe films adsorbed at the electrode|electrolyte interface [18, 22, 29, 49–51]. When IRRAS is combined with electrochemical experiments, the following considerations have to be taken into account: (i) a selection of a mirror for the IR radiation, which simultaneously serves as a working electrode, (ii) a strong absorption of the IR radiation by the electrolyte (usually aqueous solution) and (iii) the use of an IR optical window which is required to prevent electrolyte leakage. The experimental requirements mentioned above indicate that before an IR beam is reflected from the mirror surface the light has to travel through a few phases. Each phase has distinct optical constant, and thickness, and is assumed to be non-magnetic, and homogenous, with sharp, infinitely parallel boundaries [52, 53]. Figure 1 shows schematically the multilayer system of a spectroelectrochemical cell. Air, optical window, electrolyte, analyzed film and mirror have their complex frequency-dependent refractive indexes: \( \hat{n}_1, \hat{n}_2, \hat{n}_3, \hat{n}_4 \) and \( \hat{n}_5 \), respectively. The optical window is transparent to the IR light, thus the attenuation coefficient \( k \) \( k_2 = 0 \) and \( \hat{n}_2 = n_2 \). The thicknesses of air, IR optical window and mirror are assumed to be infinite because their thickness is much larger than the wavelength of the incoming radiation. Intermediate phases: the electrolyte and a film adsorbed on the mirror surface have finite thicknesses of \( d_3 \) and \( d_4 \), respectively (Figure 1).
The propagation, reflection and attenuation of the IR light in this stratified system are described by the classical electromagnetic light theory [52–54]. When an IR beam is incident at a phase boundary between two media (index 1 and 2), a part of the beam is reflected and a part is transmitted into the second phase. The incident IR beam has a wavelength $\lambda$ and is directed at an angle $\phi_i$ with respect to the surface normal (axis $z$ in Figure 1). The Fresnel equations describe the reflection ($r$) and transmission ($t$) coefficients of the parallel ($r^\parallel$, $t^\parallel$) and perpendicular ($r^\perp$, $t^\perp$) polarized incoming IR beam. They were derived from the law of the light reflection and the Snell’s law [53, 54]. Fresnel equations of reflection and transmission coefficients of the $p$-polarized light at the phase boundary between phase 1 and 2 are given by the following equations:

$$r^\parallel_{12} = \frac{\hat{\varepsilon}_2 \xi_1 - \hat{\varepsilon}_1 \xi_2}{\hat{\varepsilon}_2 \xi_1 + \hat{\varepsilon}_1 \xi_2}$$

$$t^\parallel_{12} = \frac{2\hat{\varepsilon}_2 \xi_1}{\hat{\varepsilon}_2 \xi_1 + \hat{\varepsilon}_1 \xi_2}$$

The Fresnel equations of the $s$-polarized light are given below:

$$r^\perp_{12} = \frac{\xi_1 - \xi_2}{\xi_1 + \xi_2},$$

$$t^\perp_{12} = \frac{2\xi_1}{\xi_1 + \xi_2},$$

where $\xi_j = \hat{n}_j \cos \phi_j$ and $\phi_j$ is the angle of incidence (reflection) at the phase $j$th and $\varepsilon_j$ is the dielectric constant of the $j$th medium. For a system with more than two phase boundaries the reflectivity and transmittance are functions of refractive indices, the angle of incidence IR light and the thickness of intermediate finite layers [52, 53, 55, 56]. In order to describe the Fresnel equations of a complex system with four phase boundaries (Figure 1), matrix algebra is used [29, 53, 57]. Characteristic matrices ($M_j$) describing the electromagnetic radiation propagating through $N$ phases with $N - 1$ phase boundaries for the $p$- and $s$-polarized IR beam of wavelength $\lambda$ are given below:

$$M^\parallel_j = \begin{bmatrix} \cos \beta_j & \frac{-i\xi_j}{\xi_j} \sin \beta_j \\ -i\xi_j \sin \beta_j & \cos \beta_j \end{bmatrix},$$

$$M^\perp_j = \begin{bmatrix} \cos \beta_j & \frac{-i}{\xi_j} \sin \beta_j \\ -i\xi_j \sin \beta_j & \cos \beta_j \end{bmatrix},$$

where $\xi_j$ is defined above and $\beta_j = \frac{2\pi \xi_j d_j}{\lambda}$ and $d_j$ is a finite thickness of a $j$th phase (here electrolyte and analyzed film). The multiplication of individual matrices of
Figure 2: 3-D plot of MSEFS of the $p$-polarized IR beam ($\lambda = 3.44 \mu m$) as a function of $D_2O$ (electrolyte) layer thickness and angle of incidence for the following multilayer system: CaF$_2$|$D_2O$|Au.

the entire stratified medium gives the characteristic matrix. The reflectivity and transmission coefficients, the mean square of electric field strength (MSEFS) and phase shift for a given $\lambda$ as a function of the angle of incidence $\phi_j$ and thickness $d_j$ of a finite layer of the $j^{th}$ phase can be calculated.

Considering a multilayer system shown in Figure 1 the MSEFS as a function of the angle of incidence and the electrolyte ($D_2O$) layer thickness ($d_3$) was calculated for the following experimental set-up: a CaF$_2$ equilateral prism as IR window; $D_2O$ as solvent and Au as mirror and electrode. Metallic surfaces are the most suitable mirror and electrode materials for in situ IRRAS experiments. Indeed monocrystalline and polycrystalline gold surfaces are most often used in IRRAS experiments under electrochemical control [8, 9, 11, 29, 34]. The beam convergence was set to $\pm 5\%$; comparable with the beam convergence in commercially available IR spectrometers. The wavenumber of the incoming IR radiation was set to $2900 \text{ cm}^{-1}$ ($\lambda = 3.44 \mu m$), corresponding to absorption modes of CH groups, the most often analyzed spectral region in organic molecules adsorbed on solid surfaces [12, 32, 51, 58–61]. Figure 2 shows the three-dimensional plot of the MSEFS as a function of the angle of incidence and electrolyte layer thickness.
Table 1: Calculated maximum MSEFS of the $p$-polarized IR light at 2900, 1600 and 1200 cm$^{-1}$ and the corresponding angle of incidence and electrolyte layer thickness in the following stratified media: equilateral prism as the IR window|electrolyte|Au.

<table>
<thead>
<tr>
<th>Window material</th>
<th>Wavenumber/cm$^{-1}$</th>
<th>MSEFS</th>
<th>Angle of incidence vs. \perp surface/°</th>
<th>Electrolyte thickness/μm Electrolyte</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaF$_2$</td>
<td>2900</td>
<td>3.7</td>
<td>55</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td>1600</td>
<td>2.2</td>
<td>68</td>
<td>2.2</td>
</tr>
<tr>
<td>BaF$_2$</td>
<td>2900</td>
<td>4.0</td>
<td>52</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>1600</td>
<td>2.6</td>
<td>60</td>
<td>3.3</td>
</tr>
<tr>
<td></td>
<td>1200</td>
<td>1.9</td>
<td>57</td>
<td>3.2</td>
</tr>
<tr>
<td>ZnSe</td>
<td>2900</td>
<td>5.7</td>
<td>28</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>1600</td>
<td>4.5</td>
<td>27</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>1200</td>
<td>3.0</td>
<td>26</td>
<td>4.0</td>
</tr>
<tr>
<td>Ge</td>
<td>2900</td>
<td>6.6</td>
<td>17</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>600</td>
<td>5.5</td>
<td>16</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>1200</td>
<td>5.8</td>
<td>16</td>
<td>5.0</td>
</tr>
<tr>
<td>Si</td>
<td>2900</td>
<td>6.5</td>
<td>17</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>1600</td>
<td>5.3</td>
<td>18</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>1200</td>
<td>4.8</td>
<td>18</td>
<td>3.2</td>
</tr>
</tbody>
</table>

The MSEFS has highest values either directly on the Au surface (electrolyte layer of 0.1 μm) at high angles of incidence, (ca. 80°) or when the electrolyte layer is ca. 4 μm thick and the angle of incidence is close to 60°. Calculations of the MSEFS, phase shift, reflectivity and transmittance coefficients in stratified systems composed of various IR windows, aqueous electrolytes and Au as the mirror were performed and are summarized in Table 1. Wavelengths used in calculations were set to characteristic values of group frequencies of most organic substances: 2900 cm$^{-1}$ for CH stretching modes (e.g. in hydrocarbon chains); 1600 cm$^{-1}$ for the absorption of amide groups (proteins), C=O stretching modes of lipids or ring deformation modes of aromatic compound and 1200 cm$^{-1}$ for P=O, C–O and C–C stretching modes of phospholipids or various glycoconjugates. Due to a strong absorption of the IR light by H$_2$O around 3000 and 1650 cm$^{-1}$, D$_2$O has to be used as the electrolyte solution.

Calculation results listed in Table 1 show that the highest enhancement of the reflected $p$-polarized light is obtained at the angle of incidence close to the critical angle of the window|electrolyte interface. The refractive indices of BaF$_2$ and CaF$_2$ at 1600 cm$^{-1}$ are equal to 1.44 and 1.38, respectively [62], giving an optimum angle of incidence of ca. 60°. The use of ZnSe as the IR optical window ($n = 2.42$ at 1600 cm$^{-1}$ [52]) causes a decrease in the optimal angle of incidence to 27°. Si
(n = 3.42) or Ge (n = 4.00) [52] have highest values of refractive indices and the optimum angles of incidence decrease to 15–18°. Table 1 shows that the thickness of the electrolyte layer, through which the IR light propagates, should not extend beyond 5–6 μm. An increase in the electrolyte thickness causes the decrease in the MSEFS at the mirror surface (Figure 2).

Each phase through which the IR light propagates influences the intensity of the IR beam reflected from the mirror surface and in consequence determines the quality of measured IRRA spectra. The performance of an in situ experiment at the electrochemical interface requires a proper adjustment of previously calculated parameters to a thoughtfully designed spectroelectrochemical cell. The main constructional challenge arises from the electrolyte strongly absorbing the IR radiation. Bewick overcame the problem of the strong IR absorption by the electrolyte designing a thin electrolyte layer spectroelectrochemical cell [16, 63]. In this cell a few micrometres thick electrolyte layer is sandwiched between an optical window and a flat IR mirror which also serves as a working electrode [16, 63]. The IR light is transmitted through the optical window and the thin electrolyte layer and finally reflected from the mirror (electrode) surface. This configuration of the cell is suitable for external infrared reflection techniques [16, 50, 52, 63]. The thin electrolyte layer in the spectroelectrochemical cell may affect the uniform distribution of the potential and current density at the working electrode. The resistance of the thin layer electrolyte causes slow response of the electrode to the potential change in the electrical double layer region.

In the last thirty years the basic design of the thin electrolyte layer cell has not changed. Few constructional modifications were made in order to improve the performance of the spectroelectrochemical cell [50, 64, 65]. Construction of the cell requires that the optical window and the mirror (electrode) lie parallel to each other and the separation between them is constant during the measurement. The body of the spectroelectrochemical cell is made either of glass [16, 29, 63, 66] or of polymers such as Kel-F or Teflon [64, 67]. Figure 3 shows schematically the spectroelectrochemical cell and the movable platform machined at the University of Oldenburg. The optical window (b) is connected with the spectroelectrochemical cell (a) via a Teflon holder (c) containing an O-ring that seals the cell. The glass cell is assembled into the Teflon holder (c) and supported via two PE holders (d). A disk Au electrode (diameter 10–15 mm) is attached to a gold wire and is inserted into a glass piston (e). The body of the cell is connected via a salt bridge with the Ag|AgCl reference electrode (f). The salt bridge is used to fill the cell with the electrolyte solution. The Pt foil counter electrode is built inside of the cell. The cell contains a small inlet and outlet to introduce an inner gas to remove oxygen from the electrolyte solution (g).
Figure 3: Scheme of the spectroelectrochemical cell with a moveable platform: (A) side view; (B) top view. The following parts are shown: (a) – the glass cell; (b) – optical window (prism); (c) – Teflon holder, with an O-ring, of the prism; (d) – PE holder of the cell; (e) – piston with the working electrode (Au disk); (f) – a slat bridge for the reference electrode; (g) – inlet and outlet for gas; (h) – holder attaching the cell to the platform; (i) – micrometric screw controlling the position of the piston (e); (j) – micrometric screw adjusting the position of the platform in the experimental chamber; (k) – rotating disc used to adjust the angle of incidence of the IR beam with respect to the cell position; (l) – platform floor attaching the cell into the experimental chamber purchased from Bruker. These schemes were kindly obtained from F. Roelfs, Mechanical Workshop, University of Oldenburg, 2009.
Main constructional problems of the thin electrolyte layer cell are due to the requirement of a parallel positioning of the prism and the metal electrode and maintenance of a constant thickness of the electrolyte layer during measurement. The first problem was solved by the use of a large piece of polished metal (disk or a metal foil) as a working electrode that is pressed against the prism. When the electrode approaches the prism it orients itself parallel to its surface. The immobilization of the electrode in the piston facilitates the positioning of the electrode against the prism surface (e). The piston with the working electrode is connected to a micrometre screw built into the movable platform on which the cell is placed (i). A micrometre screw changes slowly the distance between the electrode and the prism allowing precise adjustment of the thickness of the electrolyte layer. The thickness of the electrolyte layer is determined by the measurement of the reflection from the electrode surface and comparison with calculated reflectivity curves for the system under study. The spectroelectrochemical cell is fixed to the platform via a plastic holder (h). The platform is built on a rotating disc (k) which allows the adjustment of the angle of incidence between 0° and 90°. Finally, the entire platform with the cell is fixed in the experimental chamber via a commercially available sample holder (l) purchased from Bruker.

The IR absorption spectra from molecules adsorbed on the electrode surface can be obtained when calculated experimental parameters can be applied to a precisely machined spectroelectrochemical cell. Only such spectra provide structural details of the electrified interface [17, 21, 29, 51].

2.2 IRRAS techniques used in electrochemical experiments

IRRAS was successfully applied to probe the solid|liquid interface [21, 68, 69]. Its application to the electrochemical interface requires: (i) the use of a thin electrolyte layer cell in order to reduce the absorption of the IR radiation by the solvent [63]; (ii) the use of smooth reflecting surfaces in order to obey the laws of light reflection, which simultaneously conduct electrical current and can be used as working electrodes. This choice is limited to single crystal and polycrystalline metal electrodes. Due to constructional problems of the cell and adjustment of optical components before each experiment, a measurement of the reference spectrum from the electrolyte solution and the film adsorbed at the interface at exactly the same experimental conditions is not possible. Therefore, the background correction is done either by a potential [18, 70–73] or polarization modulation of the incident radiation [45, 64, 74]. When potential is modulated and IRRA spectra collected using p-polarized light the experimental techniques are called subtractively normalized interfacial Fourier transform
infrared spectroscopy (SNIFTIRS) or potential modulated infrared reflectance spectroscopy. In the case when IRRA spectra are collected at a given potential applied to the electrode and the polarization state of the incoming IR beam is modulated, the corresponding experimental technique is called polarization modulation infrared reflection absorption spectroscopy (PM IRRAS).

2.2.1 IRRAS techniques with the modulation of the electrode potential

In *in situ* IRRAS with the potential modulation *p*-polarized light is used to bring information on structural changes in species adsorbed on the electrode surface. Potential modulated infrared reflection absorption spectroscopy is applicable to study structural changes in electroactive molecules adsorbed on the electrode surface during the redox reaction as well as adsorption-desorption processes of inorganic ions and organic molecules with irreversible or very slow desorption process [70, 71]. In these cases spectra are collected at selected potentials of interest (*E*<sub>i</sub>) corresponding to reduction/oxidation of the adsorbed species on the electrode surface. A reference spectrum (*E*<sub>base</sub>) is collected at the end at potential at which one redox form is present in the film. Analogue procedure is used in SNIFTIRS which is applicable for studies of potential driven adsorption-desorption processes at which the desorption is reversible and the desorption potential is known [73, 75, 76]. In SNIFTIRS a reference spectrum is recorded at a base potential *E*<sub>base</sub> which corresponds to a film desorbed from the electrode surface. The sample spectrum is recorded at a potential of interest *E*<sub>i</sub> corresponding to a potential of the film adsorption.

In both cases a differential reflection-absorption spectrum (**ΔR**(ν)/**R**(ν)) is obtained. It is represented by the following equation [77]:

\[
\frac{\Delta R(\tilde{\nu})}{R(\tilde{\nu})} = \frac{R(E_i, \tilde{\nu}) - R(E_{base}, \tilde{\nu})}{R(E_{base}, \tilde{\nu})},
\]

where **R**(*E*<sub>base</sub>, *ν*) and **R**(*E*<sub>i</sub>, *ν*) are the reflectivity of the sample at the base potential *E*<sub>base</sub> and the potential of interest *E*<sub>i</sub>, respectively.

The resultant spectrum plots the difference between the absorption of the IR light by molecules at *E*<sub>base</sub> potential and absorption of these molecules at a potential of interest *E*<sub>i</sub>. Therefore, positive, negative and bipolar bands are observed [29, 70, 75, 77]. The presence of positive bands indicates that the absorption of the IR light by molecules at the *E*<sub>base</sub> originates from molecules desorbed from the electrode surface. The presence of negative bands in the differential spectrum indicates absorption of the IR light by molecules at the *E*<sub>i</sub>, thus originates from molecules adsorbed on the electrode surface. The appearance of bipolar
bands points to changes in the hydration, coordination, structure of a molecule between the $E_{\text{base}}$ and $E_i$ thus between desorbed-adsorbed (reduced-oxidized) states. SNIFTIRS was used to study the potential-dependent adsorption of inorganic ions from the electrolyte solution on the electrode surface and the structure of the electrical double layer [72, 76], as well as the potential-driven adsorption-desorption process of small organic molecules such as pyridine, L-phenylalanine or 2-mercaptobenzimidazole on the Au(111) electrode surface [78–80]. Changes in the orientation of electroactive molecules adsorbed on the electrode surface due to an oxidation-reduction reaction were also extensively studied by means of potential modulation IRRAS [24, 70].

2.2.2 Polarization modulation infrared reflection absorption spectroscopy

In polarization modulation infrared reflection absorption spectroscopy (PM IRRAS) an incident IR beam is modulated between the s- and p-polarization and thus the resulting reflection-absorption spectrum of species adsorbed on the metal|air interface is almost insensitive to the absorption of water vapour and CO$_2$ from the atmosphere [45, 51, 74, 81]. Russel et al. for the first time applied PM IRRAS to the electrochemical interface [74]. At the IR mirror surface the electric field of the s-polarized IR light is equal to zero, thus the absorption of the s-polarized light contains spectral information from the environment surrounding the sample (a background spectrum). By contrast, the electric field of the p-polarized light is enhanced at the mirror surface. Therefore, adsorbed molecules can absorb the light if the transition dipole vector of a vibrational transition has a non-zero component normal to the surface of the mirror [45, 74]. Due to the modulation of the light polarization the background and sample spectra are collected simultaneously. Each spectrum is collected at a selected potential applied to the electrode. The resulting reflection-absorption spectrum ($\Delta S(\tilde{\nu})$) is expressed by the following equation [47]:

$$\Delta S(\tilde{\nu}) = C \frac{(|R_s(\tilde{\nu}) - R_p(\tilde{\nu})|)}{(R_s(\tilde{\nu}) + R_p(\tilde{\nu})) + J_0(\phi_0)(|R_s(\tilde{\nu}) - R_p(\tilde{\nu})|)} J_2(\phi_0),$$  

(8)

where $R_s(\tilde{\nu})$ and $R_p(\tilde{\nu})$ are the reflectivity of the s- and p-polarized light, respectively, $J_0$ and $J_2$ are the zero and second order Bessel functions, $\phi_0$ is the maximum dephasing given by the photoelastic modulator and $C$ is a constant [44, 47]. Since $(|R_s(\tilde{\nu}) - R_p(\tilde{\nu})|) \ll (R_s(\tilde{\nu}) + R_p(\tilde{\nu}))$ and $\phi_0$ are equal or larger than $\pi$ the term $J_0(\phi_0)(|R_s(\tilde{\nu}) - R_p(\tilde{\nu})|)$ can be neglected. The second order Bessel function $J_2(\phi_0)$ is introduced by a photoelastic modulator and depends on the wavelength.
at which the dephasing was performed. This function contributes to the intensity of the measured spectrum and has to be subtracted [47, 51].

In the first in situ PM IRRAS study the potential-driven changes in a monolayer of CO molecules adsorbed on a platinum electrode were investigated [45, 74]. In this potential-dependent study of the adsorption process of cyanide ions on a silver electrode showed differences between the species adsorbed directly on the electrode surface and those dissolved in the electrolyte solution [82]. Later, structural aspects of the potential-driven adsorption-desorption process of amphiphilic organic films adsorbed on the gold electrode surface were investigated in detail [29, 30, 32, 51].

PM IRRAS technique is a quantitative analytical tool which allows detailed analysis of the molecular order in various forms of supramolecular assemblies present at the investigated interface [29, 30, 51, 83]. Due to the surface selection rule, the integral intensity of a given absorption band depends on two factors: (i) the surface concentration \( \Gamma \) of a given molecule in the film \( \Delta S \approx 2.3 \Gamma \epsilon \) and (ii) the orientation of the transition dipole vector of a given IR absorption in this anisotropic film [29, 51]. The integral intensity of a given IR absorption band is proportional to the square of the absolute value of the dot product of the transition dipole vector \( \vec{\mu} \) and the electric field vector of the \( p \)-polarized light \( \vec{E} \) according to the following equation [84, 85]:

\[
I = \int A d\nu \equiv \Gamma |\vec{\mu}|^2 \langle \vec{E} \rangle^2 \cos^2 \theta,
\]

where \( \theta \) is the angle between the vectors \( \vec{\mu} \) and \( \vec{E} \). In consequence some IR absorption bands may be enhanced while others may disappear from PM IRRAS spectrum. Figure 4 shows two limiting cases for the orientation of a hydrocarbon chain in a uniformly organized film of an amphiphilic molecule adsorbed on a mirror surface. Hydrocarbon chains are present in most amphiphilic molecules and therefore the analysis of their orientation is used below as an explanatory example. The transition dipole vector of the symmetric methylene stretching mode \( \nu_s(CH_2) \) lies in the bisector of the methylene group [12] as marked by the red arrow in Figure 4. The electric field vector is directed perpendicular to the mirror surface (blue arrow in Figure 4). As seen in Figure 4 these two vectors are oriented perpendicular (A) and parallel (B) to each other.

According to Equation (9), when the angle between \( \vec{\mu} \) and \( \vec{E} \) vectors is equal to 90° (Figure 4A) the integral intensity of the methylene stretching modes is equal to zero. In this case there is no coupling of the transition dipole and the electric field vectors. Since the transition dipole vector of the \( \nu_s(CH_2) \) mode lies perpendicular to the direction of the hydrocarbon chain, thus zero absorbance indicates that the hydrocarbon chain is oriented perpendicular to the solid surface. The
opposite situation is illustrated in Figure 4B. Parallel orientation of the $\vec{\mu}$ and $\vec{E}$ vectors causes their strong coupling and thus the intensity of the IR absorption band of the $\nu_s(\text{CH}_2)$ mode is enhanced. In order to provide the exact orientation of the $\vec{\mu}$ vector and in consequence the orientation of the investigated molecule in a film, a theoretical spectrum of randomly orientated molecules in the film has to be calculated [29, 51, 86]. The isotropic optical constants of molecules forming an isotropic molecular assembly are either available from the literature or have to be determined from an IR transmission measurement [29, 51, 87]. The PM IRRA spectrum is calculated for experimental parameters (thickness of the electrolyte layer, incidence angle, and optical components of the cell) identical with a previously performed \textit{in situ} experiment. The surface concentration and thickness of the analyzed film adsorbed on the electrode surface have to be determined by an independent method. Figure 5 shows calculated PM IRRA spectra in the symmetric methylene stretching mode for the angle $\theta$ between the $\vec{\mu}$ and $\vec{E}$ vectors equal to 0, 53.54 and 90°, representing their parallel, random and vertical orientation, respectively.

Figure 4: Limiting cases for the orientation of the methylene group in a molecule containing a hydrocarbon chain existing in the all-trans conformation adsorbed on a solid surface. The direction of the transition dipole vector of the symmetric methylene stretching mode (red arrow) and the direction of the electric field vector of the $p$-polarized light at the phase boundary (blue arrow) are shown in the figure.
Figure 5: Calculated PM IRRA spectra of the symmetric CH$_2$ stretching mode in a film when angle $\theta$ is equal to: (A) 0, (B) 54 and (C) 90$^\circ$.

The ratio of the integral intensity of a given band in the experimental spectrum ($A^{(E)}_{\text{exp}}$) to the calculated one from the random distribution ($A^{(Rand)}_{\text{cal}}$) is used to obtain the $\theta$ angle between $\vec{\mu}$ and $\vec{E}$ vectors in an isotropic film [85, 87]:

$$\cos^2 \theta = \frac{1}{3} \frac{\int A^{(E)}_{\text{exp}} \, d\nu}{A^{(Rand)}_{\text{cal}}}.$$  \hspace{1cm} (10)

The $\theta$ angle provides directly the orientation of the analyzed transition dipole vector with respect to the surface normal. When in a studied molecule the orientation of the $\vec{\mu}$ is known, the precise orientation of a particular group in an organized film can be provided. The spectrum shown in Figure 5B corresponds to the spectrum calculated from optical constants and represents a spectrum in which molecules have a random distribution. In this case the ($A^{(E)}_{\text{exp}}$) is equal to ($A^{(Rand)}_{\text{cal}}$) and thus, $\cos^2 \theta = \frac{1}{3}$ resulting in the $\theta$ angle equal to 53.54$^\circ$. Considering the example shown in Figure 5A, when the $\vec{\mu}$ and $\vec{E}$ vectors are parallel to each other, the PM IRRA signal is enhanced and its integral intensity is three times higher than the intensity of the randomly distributed molecules in the film. The hydrocarbon chains have parallel to the substrate surface orientation. When the $\vec{\mu}$ of the $v_s$(CH$_2$) mode lies parallel to the substrate surface and makes a normal angle to the $\vec{E}$ vector, no
absorption is observed in the PM IRRA spectrum (Figure 5C). It corresponds to a vertical to the substrate surface orientation of the hydrocarbon chain.

3 Advancement of in situ IRRAS experiments to the requirements of biological and biomimetic samples

*In situ* analysis of the structure of biologically relevant supramolecular assemblies such as models of cell membranes, protein films or strands of nucleic acids are experimentally challenging because they bring new experimental requirements. The PM IRRAS applied to the electrode|electrolyte interface is an excellent tool for *in situ* determination of the impact of electrical potentials (surface charge) on the structure and reactivity of biomolecules deposited at a conductive surface [31, 34, 39, 88–90]. Obviously the combination of experimental conditions of the PM IRRAS under electrochemical control to experimental conditions required to maintain the biological activity of biomolecules limits of the application of this excellent analytical technique.

A proper selection of the electrolyte solution for these experiments has a crucial role. The stability of biomolecules cannot be affected by ions present in the electrolyte solution. The use of aqueous, buffered electrolytes with physiological pH and a constant temperature (usually 37 °C) is essential to preserve the physiological activity of a large number of biomolecules. Chloride ions are commonly present in body fluids. However, the Cl⁻ ions adsorb strongly on gold, platinum and silver surfaces [91–93]. These materials are usually used in electrochemical PM IRRAS experiments since they reflect the IR radiation and conduct current. Since the number of materials available as electrodes and mirrors for IR radiation is limited to metals such as Au, Pt, Ag, Cu one normally avoids the use of Cl⁻ in the supporting electrolyte solution [21]. In addition, some ions present in the electrolyte solution (e.g. ClO₄⁻, SO₄²⁻) or organic buffers (e.g. TRIS) adsorb the IR radiation. A strong absorption of the IR light by the electrolyte may overlap with the usually weak absorption originating from a biomimetic film and the IR absorption signal from the sample cannot be distinguished from the strong absorption by the electrolyte ions.

Another experimental limitation arises from the use of optical windows. The selection of an optical window may bring the following problems: (i) limited stability due to solubility problems which are strictly dependent on the composition and pH of the electrolyte, (ii) available frequency range, (iii) reflection of the
Modification of the Au surface leading to an increase in biocompatibility of the metal surface: (A) deposition of thin layer of oxide (TiO₂ or SiO₂) and (B) adsorption of a protein film.

s-polarized light from the window|electrolyte interface and therefore a decrease in the intensity of the measured signal.

In order to enlarge the application potential of the structure sensitive in situ IRRAS to the requirements of a biological material and its supramolecular assemblies (i) modifications of the electrode (mirror) surface ensuring biocompatibility [55, 56, 94], (ii) careful selection of the optical window material [95] for a particular biological sample and (iii) some modifications at biomolecules [66] have to be taken into account. Below these points are discussed in detail.

3.1 The electrode (mirror) surface

IR light-reflecting metallic surfaces used in IRRAS experiments are not biocompatible materials. The physiological activity of a biological material, in particular of a protein, adsorbed on surfaces of noble metals (Pt, Au) or stainless steel often changes [96, 97]. In consequence, these substrates are not suitable to produce implants, chips or biosensors [96, 98]. The biocompatibility of noble metal surfaces may be increased either by a deposition of a thin oxide layer [55, 56, 94] or by the adsorption of a stable film of organic molecules [37]. Figure 6 illustrates modifications of the electrode and IR mirror gold surface by: a thin layer of an inorganic oxide (Figure 6A) and by the adsorption of a stable biofilm (Figure 6B) aiming at an increased biocompatibility.

Titanium with its native oxide layer as well as silica belong to the most often used implant materials [97]. The adsorption of water and ions such as calcium, magnesium or phosphates on their surface ensures their high biocompatibility [96, 97, 99]. IR transmitting or absorbing substances such as silica, titanium, titanium oxides are not suitable for the IRRAS experiments. However, deposition of an ultra-thin film of these materials on IR reflecting material such as gold ensures enhancement of the electric field at their surfaces comparable to that of Au (Figure 7) [56].
In order to obtain high values of the MSEFS and fulfill the surface selection rule the thickness of the silica film must not exceed 90 nm (Figure 7) [56]. The thickness of the titanium layer with its 4 nm thick native titania deposited on the Au surface should not exceed 50 nm [55]. PM IRRAS was successfully used by Zawisza et al. [55, 56, 94] for the first time to characterize ultra-thin silicate oxide and titanium films deposited on the reflecting gold surface. The orientation, hydration and stability of lipid molecules in bilayers, serving as models of cell membranes, adsorbed on the Au|SiO$_2$ and Au|Ti|TiO$_2$ surfaces were described.

Biochemical modifications of implant surfaces aim at faster and easier acceptance of biomaterials by a host organism. In order to accelerate the bio integration process synthetic hydrophilic polymers, lipid vesicles and proteins from extracellular matrix can be deposited on their surfaces [1]. Collagen, the most abundant protein in the human body, forms stable films on implant surfaces [97]. The protein layer adsorbed on the implant surface provides a flexible and hydrophilic “coushion” for the adsorption of cells and models of cell membranes (Figure 6B). Collagen molecules self-assembled on the gold surface form stable ca. 6 nm thick films [37]. This modification of the Au surface was used to mimic the physiological environment for deposition of model lipid bilayers [100].
Despite the fact that material that fulfill the surface selection rule of IRRAS are limited to metals such as Au, Pt, Ag or Cu [21] nonmetallic substrates such as glassy carbon [101] and even glass [102] have been successfully applied in IRRAS. However, due to a low enhancement of the electric field vector of the \( p \)-polarized light at these nonmetallic surfaces they are rarely applied for structural studies of monomolecular films of biomolecules. Therefore, the synthesis of new biomaterials having suitable optical and electrical properties is highly required to increase the applicability of IRRAS. Recently, a hybrid titanium oxycarbide (TiO\(_x\)C\(_y\)) material was reported as a new conductive material that reflects the IR radiation [103]. At the TiO\(_x\)C\(_y\) surface the IR radiation is strongly reflected, a constructive interference between the incoming and reflected \( p \)-polarized light occurs at the angle of incidence close to 70° and a destructive interference between the incoming and reflected \( s \)-polarized light takes place at all angles of incidence. Thus, the surface selection rule of IRRAS is fulfilled. This material has been used as a new mirror in IRRAS for quantitative analysis of the molecular structure and orientation of arachidic acid molecules in Langmuir–Blodgett monolayers [103]. At the microscopic level the compact hybrid TiO\(_x\)C\(_y\) material is not homogenous [104, 105]. On its surface TiO\(_2\), TiC, C (graphite) and substoichiometric titanium oxides (TiO\(_2-\alpha\)) have been identified. TiO\(_2\) surface belong to the best known biomaterials and graphite is also used as a biocompatible electrode material therefore titanium oxycarbide hybrid has a large application potential as a new biomaterial. Since at the TiO\(_x\)C\(_y\) surface the surface selection rule of IRRAS is fulfilled and it has similar electrochemical characteristics to the glassy carbon electrode TiO\(_x\)C\(_y\) can be used in \textit{in situ} PM IRRAS studies of the structure of biomolecules adsorbed on its surface.

### 3.2 Selection of the IR window material

In \textit{in situ} PM IRRAS experiments the use of optical windows resulting in the highest enhancement of the \( p \)-polarized light at the electrode|electrolyte interface is preferred (Table 1). Therefore, BaF\(_2\) and CaF\(_2\) are most often used as optical windows in such experiments [33, 74, 86]. The use of the BaF\(_2\) prism requires the presence of the F\(^-\) ions in the electrolyte solution of pH > 7 in order to prevent dissociation of BaF\(_2\). The presence of fluoride ions in alkaline solution may affect the activity and structure of proteins. Similarly, the presence of F\(^-\) ions in studies of drug interactions with natural cell membranes or model lipid membranes may influence the drug activity leading to false results [106–108]. The usage of CaF\(_2\) as optical window limits the frequency wavenumber range below 1200 cm\(^{-1}\). Various biological substances such as nucleic acids, glycoconjugates or natural poly-
mers such as cellulose or heparin contain phosphate, sulphate and sugar moieties that absorb IR light in this spectral region and therefore, cannot be analyzed when CaF$_2$ is used as a prism.

ZnSe and Si optical windows provide a wide spectral region (3500–800 cm$^{-1}$) and are stable in various aqueous solutions. The optimum angle of incidence for the ZnSe and Si optical windows is close to 28° and 15°, respectively. The enhancement of the electric field vector of the $p$-polarized light is similar for ZnSe, CaF$_2$ or BaF$_2$ optical windows. However, due to a significant reflection of the $s$-polarized light from the ZnSe|water interface, intensities of IRRAS spectra are significantly lower than those recorded with CaF$_2$ or BaF$_2$ prisms [95]. Figure 8 shows calculated and experimental PM IRRAS spectra of a 1,2-dimyristoyl-sn-glycero-3-phosphatidylcholine (DMPC): cholesterol bilayer in the CH stretching mode region originating from the hydrocarbon chains at phospholipid molecules for equilateral BaF$_2$ and ZnSe prisms used in experiments.

When ZnSe is used as the prism, the intensities the CH stretching bands in the DMPC bilayer are ca. 10–12 times lower then when BaF$_2$ is used as the prism. However, the excellent signal to noise ratio of the PM IRRAS experiment allows spectra subtraction as shown in Figure 8B. If the surface concentration of an ana-
lyzed biomolecule increases, e.g. in protein film, the intensity of the IR absorption bands increases and use of ZnSe as optical window becomes less problematic.

### 3.3 Role of isotopic substitution in biomolecules in a complex biomimetic system

The main analytical problem in studies of biological samples arises from their compositional complexity. Methylene, methyl, carboxylic, amide, amine or hydroxyl groups are commonly present at biomolecules. The same functional groups, even if located at different molecules, absorb the IR light at the same or very similar frequencies. Consequently, different components of a biological system give overlapping absorption bands which cannot be easily deconvoluted. The analysis of methyl and methylene groups is the most critical, because they are present at the majority of biomolecules. The absorption bands of methylene and methyl groups are well separated from other characteristic group frequencies. Therefore, they are widely investigated [12, 109–114]. However, most lipid and protein molecules contain a large number of these groups making assignments to a particular molecular structure difficult.

One approach is the use of single component models [12, 32, 34, 110, 114]. However, multicomponent systems can be investigated after the isotopic substitution of the compound of interest [59, 66, 86, 115]. The replacement of H by D is a powerful tool in vibrational spectroscopy with very little change in the chemical nature of the system. The mass change causes ~700 cm$^{-1}$ red-shift of the CH stretching vibrations relative to the CD stretching vibrations.

For example a selectively per-deuterated hexaethylene glycol-terminated alkane thiol was synthesized to distinguish methylene groups of two parts of the molecule in IR spectroscopy [116]. The per-deuteration of the methylene groups at the alkane chain allowed an independent analysis of the reaction with bromine of two parts of the amphiphilic molecule adsorbed on the Au surface [66]. Due to the separation of the IR absorption bands of methylene groups changes in the orientation, packing and reactivity in the monolayer were analyzed separately.

Despite the fact that the synthesis of specifically deuterated molecules is expensive and time-consuming, the need for this kind of molecules in research is clear. For purposes of biomimetic research, a large variety of deuterated or per-deuterated lipid molecules is commercially available [117]. Figure 9 shows schematically possibilities of the use of per-deuterated lipids in IR studies of membrane models.

Figure 9A shows that the layer-by-layer deposition of each lipid monolayer allows distinguishing lipid molecules adsorbed in each leaflet [31, 40, 86, 118].
Figure 9: Possibilities of the isotopic substitution in the hydrocarbon chains region in models of lipid bilayers used in biomimetic studies: (A) differentiation between each layer; (B) differentiation between various lipid components in a lipid bilayer and (C) differentiation between two classes of biomolecules such as proteins and lipids.

In this way information concerning the asymmetry in the molecular arrangement of lipid molecules in two leaflets is gained. Due to an isotopic substitution various components of a lipid bilayer can be differentiated as shown in Figure 9B. The structure, conformation, orientation and physical state in different components of a model lipid membrane are independently analyzed [39]. Two-component stratified systems composed of an adsorbed protein layer (e.g. collagen) and a lipid bilayer containing deuterated acyl chains allows distinguishing IR signals originating from two different kinds of biological molecules: a lipid and the protein (Figure 9C). The isotopic substitution offers an elegant solution for spectroscopic studies of multicomponent and complex biomimetic assemblies.

4 PM IRRAS studies of biomimetic films under electrochemical control

Biomolecules form well-defined and complex supramolecular architectures in which a long-range order is highly preserved. Due to a well-defined structure, long-range order and presence of charged residues and ions in their vicinity static electric fields in the order of $10^7$–$10^9$ V m$^{-1}$ act at lipids and proteins at cell membranes [5]. As mentioned earlier a deposition of biomimetic assemblies on electrode surfaces allows their exposition to physiological electric fields. Thus, application of the PM IRRAS to biomimetic assemblies deposited on electrode surfaces opens a unique possibility to determine in situ potential induced changes of the structure and orientation of molecules in a biomimetic film. In the last years, potential induced changes in the structure of lipid molecules in versatile models of cell membranes have been investigated using PM IRRAS under electrochemical control [8, 30–34, 83, 88, 118–121]. These results are summarized in Section 4.1.
Less is known about the impact of the interaction of a protein with a cell membrane on its structure under physiological electric fields [39–41, 89]. The first results available in literature in this field are reviewed in Section 4.2. Proteins form another important class of biomolecules which adsorb on various surfaces. The amount and the structure of an adsorbed protein depend on the charge accumulated on a solid surface [122, 123]. In Section 4.3 in situ PM IRRAS studies of the potential dependent adsorption process of a protein film on the electrode surface are described [37, 38].

4.1 Application of in situ PM IRRAS for the determination of the structure of models of lipid membranes

Planar phospholipid bilayers are the most often used models of cell membranes. Vesicles spreading and combined Langmuir–Blodgett and Langmuir–Schaefer (LB-LS) transfer are predominantly used to fabricate lipid bilayers on electrode surfaces [8, 9, 31–34, 88, 118, 124]. Numerous electrochemical, in situ PM IRRAS and AFM studies show clearly that pure phospholipid bilayers prepared on the Au electrode surface by vesicles spreading have poorly defined structure [8, 9, 32, 120, 125]. Defects are present in these bilayers and the hydrocarbon chains at phospholipid molecules adapt a large tilt with respect to the electrode surface [8, 120]. In order to increase the molecular-scale order in phospholipid bilayers obtained by vesicles spreading addition of cholesterol is necessary [9, 11, 32, 88, 126]. Phospholipid molecules in bilayers prepared by LB-LS transfer are closer packed, uniformly oriented, and show a higher degree molecular-scale order than lipid molecules in bilayers prepared by vesicles spreading [9, 31, 33, 120, 127, 128]. Structural studies of potential induced chains in lipid bilayers prepared by vesicles spreading and LB-LS transfer were recently reviewed by Lipkowski [9]. Summarizing, the LB-LS transfer is superb method of the preparation of single- and multi-component lipid bilayers over the vesicles spreading. Below, structural studies of the potential impact on the structural changes in symmetric single-component and asymmetric multi-component lipid bilayers prepared by the LB-LS transfer are described.

As the first example potential induced changes in the structure and orientation in phospholipid molecules in single-component bilayers deposited on the Au electrode surface and composed of DMPC, 1,2-dimyristoyl-sn-glycero-3-phosphatidylethanolamine (DMPE) and 1,2-dimyristoyl-sn-glycero-3-phosphatidylserine (DMPS) are reviewed. These three phospholipids have the same hydrophobic part, namely two saturated 14C atoms long myristoyl chains. Polar head groups contain either a zwietterionic moiety: phosphatidylcholine
In Situ IRRAS of Biomimetic Films

(PE), phosphatidylethanolamine (PE) or a negatively charged phosphatidylserine (PS). Lipid bilayers were prepared using the LB-LS transfer at the surface pressure 42–50 mN m\(^{-1}\) and investigated at room temperature [33, 35, 36]. Electrochemical studies show that in all investigated bilayers the lowest values of the capacitance were observed at potentials close to the potential of zero charge (pzc) of the Au electrode [33, 35, 36]. However, the value of the capacitance minimum depends on the nature of the polar head group and is equal to 2–3 \(\mu F\) cm\(^{-2}\) in the DMPE [35], 6–8 \(\mu F\) cm\(^{-2}\) in the DMPC [33] and of 9–10 \(\mu F\) cm\(^{-2}\) the DMPS bilayer [36]. After desorption from the Au surface (at \(E < -0.90\) V vs. Ag/AgCl reference electrode), phospholipid bilayers can be reversibly readsorbed upon the application of more positive potentials (\(E > -0.4\)). Since acyl chains in these lipids are identical, the electrochemical properties and therefore the orientation of each kind of the lipid molecule in the bilayer depend on the chemical nature of the polar head group.

PM IRRAS under electrochemical control was used to determine potential-induced changes in the orientation, hydration and packing of phospholipid molecules during the adsorption-desorption process of phospholipid bilayers. Despite the fact that acyl chains at the three lipids are the same, the PM IRRA spectra in the CH stretching modes region, representing absorption bands originating from the hydrocarbon chains, show large differences. Frequencies of the methylene stretching modes indicate that the hydrocarbon chains at the DMPC molecules exist in a liquid ordered phase [33] while the hydrocarbon chains in the DMPE and DMPS molecules in a gel state [35, 36]. Intensities of the methylene stretching modes and their changes due to the applied potential, as described in Section 2.2, reflect the orientation of hydrocarbon chains in lipid bilayers. The tilt angles of the acyl chains in phospholipid bilayers with respect to the Au electrode surface are collected in Table 2.

Figure 10 shows the PM IRRAS spectra of the C=O stretching mode at the ester carbonyl group in different phospholipid bilayers as a function of the electrode potential. In all bilayers intensities of all \(\nu(C=O)\) modes are significantly lower than the intensity of randomly distributed molecules in the bilayer thick film. This indicates that ester carbonyl groups tend to lie parallel to the bilayer plane and thus Au surface (see Table 2).

Moreover, Figure 10 shows clearly that the position of the \(\nu(C=O)\) mode depends strongly on the phospholipid forming the membrane as well as on the potential applied to the Au electrode. According to the literature the shape and number of deconvoluted absorption bands of the entire \(\nu(C=O)\) mode reflect the degree of hydration of the ester carbonyl group at the used phospholipid [129, 130]. The high frequency mode at 1743 cm\(^{-1}\) corresponds to dehydrated ester carbonyl groups, the mode around 1728–1730 cm\(^{-1}\) indicates that carbonyl groups
Table 2: Tilt angle of the acyl chains and the ester carbonyl groups at the DMPC, DMPE and DMPS bilayers adsorbed and desorbed from the Au electrode surface.

<table>
<thead>
<tr>
<th>Composition/</th>
<th>Adsorbed state</th>
<th>Desorbed state</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB-LS transfer pressure/mN m⁻¹ [Reference]</td>
<td>Tilt chains/°*</td>
<td>Tilt C=O ester group/°*</td>
</tr>
<tr>
<td>DMPC/42 [33]</td>
<td>26</td>
<td>78–80</td>
</tr>
<tr>
<td>DMPS/48 [36]</td>
<td>18</td>
<td>ca. 75</td>
</tr>
</tbody>
</table>

* Tilt angle is given with respect to the surface normal.

make hydrogen bonds to water whereas the presence of a third mode around 1715–1722 cm⁻¹ indicates a formation of intramolecular hydrogen bonds. As seen in Figure 10A the ν(C=O) mode in the DMPE bilayer is dominated by the high frequency component at 1744–1740 cm⁻¹, indicating a weak hydration of the ester group [35]. Neither the position nor the intensity of the ν(C=O) mode changes with the potential applied to the Au electrode. This result indicates that the hydration and orientation of the ester group in the DMPE bilayer is not affected by the adsorption-desorption process. However, when DMPC and DMPS lipids form the bilayer both, the overall shape and position of the ν(C=O) band depend on the applied potential (Figure 10B, C).

Figure 10B shows that in the DMPC bilayer a negative potential shift, leading to the bilayer desorption from the Au surface causes a 12 cm⁻¹ blue-shift of the ν(C=O) mode (Figure 10B) [33]. This result indicates that the ester groups at DMPC molecules are dehydrated in the desorbed state and well hydrated when the phospholipid molecules are directly adsorbed on the Au surface. An opposite behavior is observed in the DMPS bilayer. In other words, a negative potential shift leads to an increase in of the ν(C=O) mode around 1720–1730 cm⁻¹ (Figure 10C) [36]. Clearly, the ester carbonyl groups are better hydrated in the desorbed state. The presence of a weak mode around 1712 cm⁻¹ indicates a formation of intramolecular hydrogen bonds between DMPS head groups.

Figure 11 summarises PM IRRAS spectroelectrochemical studies of phospholipid bilayers [33, 35, 36] and illustrates the molecular scale order found in the DMPE (A), DMPC (B) and DMPS (C) bilayers in the adsorbed and desorbed state, respectively.
Figure 10: The PM IRRAS spectra in the C=O stretching mode region of the ester carbonyl group in LB-LS transferred bilayers on the Au(111) electrode surface: (A) DMPE copied with permission from [35], Copyright American Chemical Society 2013, (B) DMPC and (C) DMPS copied with permission from [36], Copyright Elsevier 2014.
Independently of the potential applied to Au electrode the hydrocarbon chains at zwitterionic DMPE molecules adapt an extended all-trans conformation and make a small angle of 16–18° vs. surface normal (Figure 11A). The DMPE bilayer contains a very low number of defects [35]. The PE group occupies the area of 0.38 nm² [131] which exactly corresponds to the area of two fully stretched and vertically oriented hydrocarbon chains. The size matching allows a tight packing of phospholipid molecules in the bilayer and ensures a stable molecular orientation which is not affected by the potential applied to the Au electrode (Figure 11A). By contrast, the structure and orientation of lipid molecules in DMPC and DMPS bilayers are dependent on the potential applied to the Au electrode (Figure 11B, C). The PC head group, depending on the access of water, occupies an area of 0.48–0.54 nm² [131, 132]. The desorption-adsorption process of the DMPC bilayer is connected with changes in the hydration of the polar head group and the tilt of the hydrocarbon chains. In the bilayer adsorbed directly on the Au surface the tilt of hydrocarbon chains (25°) provides the average area of ca. 0.48 nm² per
the PC moiety [40]. Thus, water molecules have a direct access to the polar head groups, which is confirmed by a good hydration of the bilayer (Figure 11B). Desorption of the bilayer leads to a more vertical to the Au surface orientation of the hydrocarbon chains and therefore to a decrease in the average area per the DMPC molecule. Simultaneously, the polar head groups become dehydrated. Structural rearrangements in the desorbed DMPC bilayer result in a tight molecular packing of DMPC molecules, similar to that found in the DMPE bilayer. By contrast, the acyl chains at DMPS molecules in the bilayer adsorbed directly on the Au surface are oriented almost normal to the electrode surface (Table 2, Figure 11C) [36]. The entire polar head at the DMPS molecule is well hydrated. The average area of the PS head group varies between 0.51 nm$^2$ and 0.47 nm$^2$ in the presence of Na$^+$ and Ca$^{2+}$ in the electrolyte solution, respectively [133]. Electrostatic interactions between PS head groups and cations from the electrolyte solution as well as the molecular area of PS comparable to the area of two hydrocarbon chains facilitate a tight packing of the DMPS molecules in the adsorbed bilayer. Moreover, lipid bilayers are directly adsorbed on the electrode surface at positive charges accumulated on its surface, what in the case of a negatively charged DMPS molecule facilitates charge-charge interactions stabilizing further a tight packing of lipid molecules in the film (Figure 11C). An increase in the negative charge accumulated at the electrode surface induces repulsions with DMPS molecules destabilizing the molecular scale order in the bilayer and leads to the bilayer desorption from the Au surface.

Natural cell membranes have a more complex lipid composition than a single component phospholipid bilayer. Next to phosphoglycerides, sterols, glycolipids and sphingomyelin are present in a cell membrane. Lipids present in the cell membrane have different composition and concentration in the exoplasmic and endoplasmic leaflet of the membrane, respectively. Each cell membrane has a lateral and transverse asymmetry, influencing its functions, stability and structure at the molecular scale. Therefore, new protocols of the preparation of asymmetric model lipid bilayers and bioanalytical approaches aiming at the determination of their structure are highly required. LB-LS transfer was successfully used to prepare asymmetric lipid bilayers containing glycolipids either in the inner or in the outer leaflet [39, 89, 119]. These two examples are described below.

Asymmetric bilayer lipid membranes (BLM) containing phospholipid (DMPC), sterol (cholesterol) in the inner electrode facing leaflet and DMPC, cholesterol and glycolipids (gangliosides G$_{M1}$ or G$_{Dia}$) in the outer electrolyte facing leaflet were prepared on the gold electrode surface [39, 89, 119]. The capacitance of these model membranes ranges from 1.7 to 4.0 $\mu$F cm$^{-2}$ depending on concentration and net charge of the ganglioside introduced into the outer leaflet of the bilayer [30, 39, 40]. PM IRRAS studies under electrochemical control show
clearly that the orientation of phosphatidylcholine molecules in both leaflets of
the bilayer is affected by the addition of ganglioside molecules to the outer leaflet.
The use of a per-deuterated phospholipid (d_{54}-DMPC) allowed simultaneous
analysis of the physical state and orientation of hydrocarbon chains at phospho-
lipid and ganglioside molecules, respectively [30, 39, 40, 89]. In both leaflets of
the model bilayer the acyl chains at the perdeuterated d_{54}-DMPC exist in a gel
state, in a large extend adopt an all-trans conformation and have a small tilt of ca.
14–18° with respect to the surface normal [30, 40]. Interestingly, the orientation
of the acyl chains at the DMPC molecules is not affected by the potential applied
to the Au electrode. By contrast to the DMPC molecules, hydrocarbon chains
at the ganglioside (GM1) exist in a liquid state [39]. The estimated average tilt
of hydrocarbon chains is close to 38° vs. surface normal and is significantly
higher than the tilt angle at DMPC molecules. In addition, the orientation of
hydrocarbon chains at the ganglioside changes with the potential applied to the
Au electrode. Due to a tight packing of hydrocarbon chains the average area per
phospholipid molecule is equal to 0.40 nm^2 and is significantly smaller than
0.46 nm^2 occupied by the phosphatidylcholine head group [131]. Thus, the PC
head groups adapt a space saving zig-zag orientation [39, 89].

A broad absorption mode appears in the PM IRRA spectra of BLM in 1150 and
1000 cm\(^{-1}\) spectral region. It is composed of several overlapped absorption bands
that originate from the symmetric phosphate stretching mode, and the \(v(CO[P])\)
stretching modes at the DMPC, and \(v_{as}(COC)\) stretching modes at the ganglioside
molecules in the bilayer adsorbed on the Au electrode surface and is shown in
Figure 12.

This broad absorption mode was deconvoluted into the IR absorption bands
centred at the following wavenumbers: 1119 ± 2, 1096 ± 2, 1088 ± 2, 1071 ± 1,
1062 ± 1, 1054 ± 2, 1042 ± 2 and 1020 ± 2 cm\(^{-1}\). Due to a large number of
overlapped bans, the deconvolution procedure leads to an error in the estimation
of the integral intensities of individual \(v_{as}(COC)\) and \(v_{s}(PO_{2}^{-})\) and \(v(CO[P])\)
modes. Interestingly, the most prominent difference between PM IRRA spectra of
the lipid bilayer adsorbed directly on the Au surface and of randomly distributed
molecules is an increase in the intensity of the overall band at wavenumbers be-
low 1065 cm\(^{-1}\). Integral intensities of these bands are by a factor 2.5–1.5 higher
than corresponding intensities in the spectrum of randomly oriented molecules.
In PM IRRAS, three times higher integral intensity of an IR absorption band than
in a randomly distributed film indicates parallel orientation of the electric field
and transition dipole vectors. Quantum chemical calculations aiming at the de-
scription of various IR frequencies at the sugar residues at the polar head group
of the GM\(_1\) ganglioside have been performed [134]. They show that the transition
dipole vectors of the \(v_{as}(COC)\) stretching modes expected around 1130, 1065, 1057
Figure 12: PM IRRAS spectra of asymmetric BLM containing DMPC and cholesterol (7 : 3 mole) in the electrode facing leaflet and DMPC, cholesterol and GD_{1a} ganglioside (5 : 3 : 2 mole) in the electrolyte facing leaflet LB-LS transferred onto the Au electrode surface at \( \pi = 40 \text{ mN m}^{-1} \) at (A) \( E = 0.2 \text{ V} \), (B) \( E = -0.8 \text{ V} \) and (C) a calculated spectrum for randomly distributed molecules. In the deconvoluted spectrum black lines: phosphate stretching and grey lines \( \nu_{as} \text{(COC)} \) modes.

and 1040 cm\(^{-1}\) lie parallel to the plane of the most inner \( \beta1\text{-1'}\text{Gluc-}\beta1\text{-3Gal} \) sugar rings [134]. Summarizing, described above PM IRRAS results when confronted with computational results, indicate that the inner \( \beta1\text{-1Glu-}\beta1\text{-3Gal-}\beta1\text{-4-GalNAc} \) sugar residues at the GD_{1a} ganglioside in the bilayer adsorbed directly on the Au electrode surface have preferred orientation along the surface normal. At negative potentials (\( E < -0.6 \text{ V} \) (vs. Ag/AgCl)), desorption of the lipid bilayer from the electrode surface is connected with a more random orientation of ganglioside molecules. These changes are irreversible. The readorption of the lipid film on the Au electrode surface does not lead to spectral characteristics of the freshly prepared asymmetric bilayer.

Figure 13 summarizes above described results and illustrates the molecular scale order in the asymmetric BLM as a function of the electrode potential. In a freshly prepared bilayer the hydrocarbon chains at the phospholipid molecules are well ordered and oriented almost vertically to the Au substrate surface. The polar head groups of PC adapt a zig-zag orientation. The ganglioside molecules
Figure 13: Molecular scale order in the asymmetric BLM prepared by LB-LS transfer at the surface pressure 42 mN m$^{-1}$ containing initially in the bottom electrode facing leaflet DMPC : cholesterol (7 : 3 mole) and in the outer electrolyte facing leaflet DMPC : cholesterol : GM1 (5 : 3 : 2 mole) on the Au electrode surface in a function of the potential applied to the electrode.

are present only in the outer layer and the inner sugar moieties at their large polar head groups tend to be oriented vertically to the Au substrate (Figure 13).

At negative potentials ($E < -0.65$ V vs. Ag/AgCl) asymmetric bilayers are desorbed from the Au electrode surface. By contrast to symmetric phospholipid bilayers, in asymmetric BLM the potential driven adsorption-desorption process is irreversible [8]. Electric fields are known to accelerate the formation of defects in thin organic films [135] what in the asymmetric bilayer may lead to the formation of pores [8, 39]. In addition, upon desorption, both sides of the membrane have for the first time a direct contact to the electrolyte solution. In situ PMIRRAS studies show that the tilt of the hydrocarbon chains at DMPC molecules is not dependent on the electrode potential. Changes in the orientation and structure of the entire supramolecular assembly are induced by reorientations of ganglioside molecules. In the BLM for the first time desorbed from the Au surface, large head groups at ganglioside molecules gain flexibility and may turn towards the second leaflet, as illustrated in Figure 13.

An asymmetric lipid bilayer containing glycolipids in the electrode facing leaflet was prepared on the Au electrode surface [30, 119, 136]. Figure 14 shows schematically the molecular scale order in the fabricated in the floating bilayer lipid membrane (fBLM). First the Au surface was modified by a monolayer of 1-thio-β-D-glucose (β-Tg) in order facilitate a sugar-sugar interaction with the glycolipid molecules and stabilize the lipid bilayer on the solid surface [119]. Next, a three-component monolayer containing up to 30% mole GM1 ganglioside, 30% mole cholesterol and corresponding concentration of DMPC was transferred by LB withdrawing onto the modified Au electrode. Finally, the second leaflet containing DMPC and cholesterol (7 : 3) was prepared by LS transfer.
Figure 14: (A) Schematic representation of the molecular-scale order in an fBLM on the 1-thio-β-glucose self-assembled on the Au(111) electrode surface. Composition of the bottom leaflet DMPC : cholesterol : GM1 (4 : 3 : 3 mole) and of the outer leaflet DMPC : cholesterol (7 : 3 mole). Chemical structures of molecules used to prepare the fBLM: (B) GM1 ganglioside, (C) cholesterol, (D) water, (E) 1-thio-β-glucose and (F) DMPC. Copied with permission from [119] Copyright, American Chemical Society, 2013.

In a fBLM both sides of the membrane have contact to aqueous environment, being an excellent model for incorporation of transmembrane proteins [119]. PM IRRAS under electrochemical control was used to study the orientation of lipid molecules in the fBLM. The hydrocarbon chains at DMPC molecules have an average tilt of $12^\circ$–$8^\circ$ with respect to the surface normal [136]. Almost vertical to the Au surface orientation of the hydrocarbon chains at phospholipid molecules in the fBLM indicates that the PC polar head groups adapt a space saving zigzag packing. Clearly, the orientation of DMPC molecules asymmetric bilayers containing gangliosides either in a bottom or in the outer leaflet is very similar [39, 40, 89, 119, 136]. The analysis of the PM IRRRA spectra shows however small differences between these two bilayers. In other words, the hydration of the intermediate ester group at DMPC is affected by the molecular packing. Due to the presence of the aqueous environment at both sides of the fBLM the maximum of
absorption of the $\nu(C=O)$ band is red shifted ($1725$ cm$^{-1}$ in the adsorbed state) indicating a good hydration of ester group $[30]$. In the bilayer adsorbed directly on the Au surface the maximum of absorption of the $\nu(C=O)$ band is located at $1731$ cm$^{-1}$ $[40, 89]$. Described above results show that fBLM are stable on the Au electrode surface and that indeed water is present on both sides making them attractive models for the incorporation of transmembrane proteins.

Models of lipid membranes with lateral and transverse asymmetry containing gangliosides mimic very closely the structure of natural cell membranes and show different electrical properties than pure phospholipid bilayers. Distinct electrochemical properties reflected different molecular scale structure and orientation of lipid molecules in these two models of the lipid membrane. In situ PM IRRAS studies show that the largest difference between asymmetric lipid membranes and phospholipid bilayers arises from the orientation of the phospholipid molecules in these two kinds of membranes. In symmetric single component phospholipid bilayers the hydrocarbon chains at phospholipids molecules have a larger tilt angle with respect to the surface normal than in the asymmetric membranes. Interestingly, in symmetric bilayers the orientation of hydrocarbon chains is dependent on the potential applied to the Au electrode. It is worth to underline that asymmetric lipid membranes are very fragile and undergo irreversible structural changes when they are exposed to external impulses such as change of the magnitude of electric fields acting at molecules present at the interface $[8, 30, 39]$ or temperature change $[137]$.

4.2 Application of in situ PM IRRAS for the determination of the structure of a model membrane interacting with proteins

Lipid molecules build a fluid matrix of biological membranes which host a large variety of proteins and constantly experience interactions with them. Proteins associated with cell membranes have developed various ways of interacting with lipid molecules $[138–142]$. These interactions depend on the structure and physiological functions of proteins. Transmembrane and peripheral proteins are buried in the lipid matrix. Globular and fibrous proteins from the cytoplasm and extracellular matrix usually attach to the membrane surface. The lipid-protein interactions appear either as nonspecific interactions or specific interactions of variable strength $[138, 142, 143]$. Nonspecific lipid-protein interactions assume a random contact between the protein and lipid molecules. They are often followed by molecular rearrangements, firm binding, and turn into specific interactions. Attractive van der Waals forces, electrostatic forces and hydrogen bonds are in-
volved in specific lipid-protein interactions [138, 139]. Following specific lipid-protein interactions have been described in the literature [138–142]: (i) protein binding to a residue at the polar head group of a lipid (e.g. saccharide moiety, carboxylic moiety), (ii) electrostatic interactions between charged groups at a polar head group of a lipid and protein molecules, (iii) anchoring of a hydrophobic fragment of a protein into a lipid membrane, (iv) insertion of a fragment of a lipid molecule into the protein structure (e.g. a hydrocarbon chain), (v) insertion of a transmembrane or peripheral protein into the lipid part of the membrane and (vi) aggregation of monomers of transmembrane proteins. The lipid-protein interactions may lead to phase transitions at hydrocarbon chains of lipid molecules, change the thickness of the membrane, the water content in the membrane or the net charge in the polar head group region. Moreover, both macroscopic and microscopic properties of a biological membrane such as permeability to ions, capacitance, lateral composition, concentration, orientation and physical state of lipid molecules and the activity and structure of associated proteins can be affected by drugs, changes in electrolyte composition and concentration or the static electric field [144, 145].

A combination of IRS, sensitive to the structure of lipid and proteins molecules, to electrochemistry testing macroscopic properties of model membranes such as redox activity, membrane capacity or permeability to ions, giving a possibility to monitor simultaneously potential-dependent changes in lipid bilayers arising from the lipid-protein interaction [39–42, 146] or redox activity of a protein embedded in the membrane [147–150]. Despite the fact that spectroelectrochemistry provides essential information concerning the structure of molecules at the sub-molecular level, the structure of the entire system exposed to physiological electric fields, these studies are rather rare. Predominantly, they concern surface enhanced infrared absorption spectroscopic studies under electrochemical control of transmembrane proteins, involved in redox-reactions, embedded into models of lipid membranes [24, 147–150]. Below results reporting the application of PM IRRAS under electrochemical control for studies of structural changes in lipid bilayers experiencing specific interactions with proteins are summarized [39–42].

4.2.1 Influence of the incorporation of ion channel forming proteins on the structure of lipid bilayers under electrochemical control

In a biological cell membrane transmembrane proteins interact with surrounding lipid molecules. Lipid molecules surrounding a protein are called lipid annulus [142]. The orientation and physical state of the hydrocarbon chains at lipid
annulus are affected by the interaction with the hydrophobic part of a transmembrane protein. Biomimetic \textit{in situ} studies of usually large transmembrane proteins are challenging due to complex preparation protocols of an active protein embedded into a lipid matrix. Small antibacterial peptides are able to form channels that span the lipid membrane, being smile models of transmembrane proteins. The opening and closing of transmembrane ion channel proteins depends directly on the potential drop across the lipid membrane making this system attractive for spectroelectrochemical studies [146]. Recently, the structure of the lipid bilayer with embedded two ion channel-forming peptides: gramicidin [41] and alamethicin [146] were investigated as a function of changing potentials. In lipid membranes gramicidin folds in a right handed $\beta^{6.3}$ helix with 6.3 amino acid residues per turn [30]. Gramicidin monomers may form dimers which span the membrane. The dimers are stabilized by hydrogen bonds formed between $N$-ends of peptides. \textit{In situ} PM IRRAS studies showed that gramicidin monomers introduced initially into the DMPC bilayer exist predominantly in the helical dimer ($\beta^{6.3}$) conformation [41]. The orientation of the acyl chains at the DMPC molecules and of the helix of gramicidin depend on the potential applied to the Au electrode. In the LB-LS DMPC bilayer containing 10\% mole of gramicidin adsorbed directly on the Au electrode the hydrocarbon chain of DMPC molecules make an average angle of ca. 35° vs. surface normal. This value of the tilt angle is high comparing with 25° reported to pure DMPC bilayers adsorbed on the Au electrode surface [33]. Analysis of the amide I’ mode at 1634 cm$^{-1}$ show that the long axis of the gramicidin helix embedded into the DMPC bilayer makes an angle of ca. 25° vs. surface normal [41]. Clearly, the larger inclination of the hydrocarbon chains in the bilayer containing gramicidin indicates that the membrane is thinner than a single-component DMPC bilayer. The thinning of the membrane is connected with the reorientation of annulus phospholipid molecules allowing a better matching of the hydrophobic parts of the lipid and peptide molecules. At negative potentials the bilayer desorption releases the stress at lipid and peptide molecules leading to almost perpendicular to the membrane plane orientation of the hydrocarbon chains at DMPC and gramicidin helices [41].

\subsection{4.2.2 Influence of the interaction of an anchoring protein on the structure of lipid bilayers under electrochemical control}

Proteins from the cytoplasm or extracellular matrix developed different ways of interactions with lipids present in the cell membrane. Some proteins are equipped in a hydrocarbon chain that is able to anchor a protein into the hydrophobic parts of the cell membrane [142] Recoverin belongs to Ca$^{2+}$-dependent proteins which
contain a myristoyl chain at the N-terminus. This flexible chain anchors the protein into the hydrophobic part of the membrane [151, 152]. PM IRRAS under electrochemical control was used to study potential induced changes in the structure of the lipid membrane and recoverin during the lipid-protein interaction [40]. The analysis of the PMIRRA spectra of symmetric DMPC : cholesterol bilayers (7 : 3) interacting with native recoverin show large spectral changes in the CH stretching modes region of the lipid membrane as well as in the amide I mode region of the protein. Intensities of the CH\(_2\) stretching modes in DMPC bilayers without and with attached recoverin change with the potential applied to the Au electrode. The average tilt angles of the acyl chains calculated from corresponding PM IRRRA spectra are shown in Figure 15.

Upon binding of recoverin, the order in the hydrophobic hydrocarbon chain region of the DMPC bilayer decreases. In the bilayer adsorbed in the Au surface, the large inclination of the acyl chains (ca. 55° vs. surface normal) provides an average area of 0.67 nm\(^2\) per lipid molecule [40]. This large area indicates a separation of the polar head groups of phosphatidylcholine molecules and the presence of water between them. The separation of the PC head groups is required in order to anchor recoverin into the hydrophobic part of the membrane. The analysis of the amide I band of the protein show irreversible changes with potential indicating a potential driven denaturation of the protein at negative potentials [40].

Figure 15: Average tilt of the acyl chains, calculated from PM IRRRA spectra, at the DMPC molecule in bilayers without (circles) and with attached recoverin (filled circles) adsorbed at the Au electrode surface vs. potential.
4.2.3 Influence of the interaction of a protein binding to a polar head group at a lipid molecule on the structure of lipid bilayers under electrochemical control

A specific interaction of a protein with lipid membrane often involves a protein binding to a polar moiety present at a hydrophilic part a lipid molecule at the membrane surface. A large number of proteins interact with sugar groups present on the exoplasmic surface of eukaryotic cell membranes [153, 154]. The carbohydrate-protein interactions involving binding to sugar groups are usually specific but the strength of these interactions varies from a strong irreversible binding of toxin proteins [155, 156] to a weak and reversible binding of sialic acid binding immunoglobulin-like lectins (siglec) [157]. The five subunits of cholera toxin bind specifically to GM₁ gangliosides on the cell surface [158]. Upon binding, the toxin translocate across the membrane and initiates infection via enzymatic reactions [159]. In order to prevent the infection, eukaryote have developed a large variety of transmembrane proteins such as siglecs which, reversibly and weakly bind to gangliosides on the cell surface [157, 160, 161]. Experimental detection of structural changes in the lipid membrane induced by the carbohydrate-protein interactions occurring at the cell surface is challenging due to a large diversity of sugar moieties at glycoconjugates and varying strength of these interactions. Recently, PM IRRAS was used to study the impact of a strong (cholera toxin B) [42] and a weak (myelin-associated glycoprotein MAG, siglec IV) [40] carbohydrate-protein interactions on the stability and structure of the membranes exposed to physiological electric fields. Interestingly, the binding of both proteins stabilizes the bilayer adsorbed on the Au surface but has little effect on the orientation of the lipid molecules in the bilayer.

In order to elucidate the impact of the MAG-glycolipid interaction on the structure of the entire lipid membrane, due to advantage of isotopic substitution, each layer in the lipid bilayer was analyzed separately. Figure 16 shows the PM IRRA spectra of the electrolyte and electrode facing leaflets of an asymmetric bilayer in the presence and absence of siglec (MAG) protein in the electrolyte solution [40].

Frequencies of the CH₂ stretching modes indicate that the hydrocarbon chains exist in a gel phase. The integral intensities of the methylene stretching modes in both leaflets of the bilayer are very weak and change neither with the applied potential nor in the presence of the interacting protein (Figure 16). A small decrease in the intensity of the CH stretching modes in the outer leaflet exposed directly to the interaction with the protein corresponds to the change in the tilt angle by 2°. This change is very small and comparable with the error of the experiment.
Figure 16: The PM IRRAS spectra in the CH stretching modes region of the following layers of the bilayer (A) electrolyte facing DMPC : cholesterol : GD_{1a} ganglioside layer (5 : 3 : 2) and (B) Au electrode facing DMPC : cholesterol (7 : 3) at potentials indicated in the figure; dashed lines: spectra of the bilayer in electrolyte solution 0.1 M NaF in D_2O and solid lines: spectra of the bilayer in electrolyte containing 2 μM MAG; copied with permission from [40] Copyright Wiley, 2011.

The binding of MAG [40] and cholera [42] proteins to ganglioside molecules at the surface of the lipid bilayer is clearly visible in the appearance of strong amide I’ modes in the PM IRRAS spectra. In situ PM IRRAS demonstrated the sensitivity to detect weak interactions of siglec with gangliosides present in the lipid bilayer [40]. This weak and specific interaction between MAG and GD_{1a} requires
a proper orientation of the binding site on the membrane surface. This interaction was expressed by the appearance of a new amide I absorption band at 1617 cm\(^{-1}\) indicating changes in the secondary structure of the \(\beta\)-sheet at the binding site. This band appears only when the specifically interacting ganglioside is present in the lipid bilayer. Moreover, the specific interaction occurs at potentials corresponding to the adsorbed bilayer. At negative potentials, the protein makes contact with the lipid membrane; however, no specific binding is observed. Since siglecs are supposed to play an important role in cell-cell signalling and signal transduction, changes of the potential across the membrane seem to be responsible for the binding and intercellular communication.

In the case of the strong binding of cholera toxin to the GM\(_1\) at the membrane surface the potential-induced reorientation of the helical structure of the toxin was observed [42]. As shown in Figure 17, the potential-induced change in the orientation of \(\alpha\)-helices corresponds to the opening and closing of the channel in the cholera toxin B subunit.

This change occurs at potentials corresponding to the desorption of the bilayer from the Au electrode surface and is not induced by changes in the orien-
tation of lipid molecules in the bilayer but by a change in the environment at the metal surface [42].

4.3 Application of in situ PM IRRAS for the determination of the protein structure

Adsorption of proteins on solid surfaces from their aqueous solutions has an important biological and technological meaning [97, 123]. At surfaces of biomaterials a successful bio integration process depends to a large extent on the adsorption of proteins on their surfaces [1, 97]. The adsorption of blood plasma proteins such as albumin, immunoglobulins or fibrinogen is of great importance at the surface of blood-contacting implant materials [162]. In the case of bone-substituting implants the adsorption of proteins from the extracellular matrix such as collagen, elastin, fibronectin or laminin is a crucial step in the bio integration process [1, 97].

The adsorption of proteins depends on properties of a solid surface, the nature of a protein and the solution conditions such as the concentration of the electrolyte, ionic strength, pH or temperature. The adsorption process may lead to changes in the secondary structure of the adsorbed protein [123, 162]. In order to understand, at the molecular level, the adsorption process of proteins the application of surface characterizing analytical techniques sensitive to the protein structure in situ at the solid|liquid interface is required. Indeed, IRS is a superb technique allowing studies of the secondary structure of proteins and its changes [14, 15, 163, 164]. PM IRRAS is sensitive to the structure of species adsorbed at a solid surface and it has been used to study the adsorption of proteins at stainless steel|air [162] and gold|air interfaces [165, 166]. The analysis of the amide I modes of PM IRRAS spectra of albumin adsorbed on the stainless steel surface show that the increase in the surface concentration of the adsorbed protein leads to a gradual loss of the helical structure and unfolding of the adsorbed protein [162]. Fibrinogen is a large dimer protein composed of two hydrophobic outer domains (D) which are bound to a central hydrophilic domain (E) via two coiled \( \alpha \)-helix coils [167]. In a native fibrinogen the content of \( \alpha \)-helix secondary structure is close to 30–37% and \( \beta \)-sheet to 21–30%. Figure 18 shows the content secondary structure elements (\( \alpha \)-helix and \( \beta \)-sheet) determined from the analysis of the amide I mode of PM IRA spectra of fibrinogen adsorbed on the stainless steel surface [162].

In fibrinogen adsorbed on the implant surface the content of \( \alpha \)-helical structure remains at same level as in the native protein and is equal to 30% (Figure 18A). In parallel, the protein adsorption leads to a decrease in the content of the \( \beta \)-sheet secondary structure elements from ca. 30% in the native form to only 12% in the
Figure 18: (A) Changes in the content of the $\alpha$-helix (circles) and $\beta$-sheet (triangles) secondary structure elements in fibronectin films adsorbed on the surface of the stainless steel from aqueous solutions of different protein concentrations obtained from the analysis of the PM IRRAS spectra. (B) Orientation analysis of the fibronectin molecule in the adsorbed state. Copied with permission from [162] Copyright, American Chemical Society, 2007.

adsorbed form. Clearly the loss of the $\beta$-sheet structure elements is not connected with a transition to the $\alpha$-helical structure elements. The intensity of the amide I mode originating from the $\alpha$-helical structure was used to discuss the orientation of the protein in the adsorbed state. Coiled coils connecting three globular domains of the protein contribute predominantly to this mode. A flat orientation of the fibrinogen molecule (Figure 18B upper panel) would cause that the transition dipole moment of the amide I mode, which is orientated perpendicular to the $\alpha$-helical coil, adapts a parallel to the surface orientation. According to the surface selection rule of IRRAS [54] it would lead to a cancellation of the IR signal in the PMIRRAS spectrum. A bent configuration of the adsorbed fibrinogen molecule, as illustrated in Figure 18B, corresponds to the appearance of the amide I signal from the $\alpha$-helical structure. Concluding, the PM IRRAS serves as a good analytical tool to study structural changes accompanying the protein adsorption process. Ex situ application of this technique does not provide any impact concerning the effect of the value of the surface charge on the substrate surface on the adsorption process. However, electrostatic protein-protein as well as protein-surface interactions influence the amount of adsorbed protein, its orientation on the solid surface and thus the activity and secondary structure of the adsorbed protein Therefore, studies of the adsorption process of proteins as a function of the surface charge (electrical potential) is of large scientific interest.
Figure 19: Charge density vs. potential plots of the unmodified Au electrode (unfilled circles) and of the Au electrode modified by a freshly prepared collagen film (filled circles) in 0.1 M NaF electrolyte solution. Inset: The PM IRRAS spectra in the amide I mode region of collagen films recorded at $E = 0.4$, $0.0$ and $-0.9$ V applied to the Au electrode surface.

Recently, PM IRRAS under electrochemical control was used to study the potential (surface charge) dependent adsorption of collagen molecules on the Au electrode surface [37, 38]. The adsorption of collagen molecules at the Au surface depends strongly on the applied potential, thus on the charge density accumulated at the electrode surface. As seen in Figure 19 two adsorption states are clearly seen in the collagen film at the Au surface [37].

One adsorption state is observed at small positive charges ($\sim 3 \mu$C cm$^{-2}$; II) and the second at negative charges ($\sim -15 \mu$C cm$^{-2}$) accumulated on the electrode surface. When the surface charge on the Au electrode $\sigma_M < -20 \mu$C cm$^{-2}$ the protein film is destabilized and desorbs from the Au surface. Interestingly, collagen type I molecules adsorb strongly on the Au surface at moderate positive and negative charges accumulated on the electrode surface. By contrast, many globular proteins adsorb strongest on metal surfaces at a potential corresponding to the potential of zero charge [168]. This unusual adsorption behavior of collagen is due to its uniqueness among proteins structure. A collagen molecule is composed of three coiled $\alpha$-helical polypeptide chains [169, 170]. Each polypeptide chain contains a repeat unit composed predominantly of glycine-proline-hydroxyproline amino and imino acids, respectively. A fraction of imino acids (ca. 15–20%) present at the native collagen molecule is replaced by ionisable amino acids such as lysine,
arginine, glutamic or aspartic acid [171]. In a physiological solution (pH = 7), due to the excess of basic over acidic residues, a positive net charge is accumulated at the collagen molecule [172, 173]. Charged residues are distributed unevenly along the 300 nm long collagen triple helix.

The inset to Figure 19 shows the PM IRRA spectra in the amide I' mode of collagen films at various potentials applied to the Au electrode. Neither the position, nor the shape of the amide I' mode in adsorbed collagen are affected by potential. Clearly, potential applied to the Au electrode surface does not cause any change in the secondary structure of the adsorbed collagen molecule. No changes in the intensity of the amide I' mode indicate that adsorbed collagen molecules adopt a stiff, rigid orientation which is not dependent on the charge accumulated on the Au surface. An independent in situ ellipsometric study showed that the potential induced transition at \( E = 0.05 \text{ V vs. } \text{Ag/AgCl} \) (Figure 19) is due to changes in the content of water in the protein film [37]. At negative charges accumulated on the Au electrode surface the content of water in the film increases. In order to compensate the net negative charge cations from the electrolyte solution are attracted to the electrode surface, leading to swelling of the polymer film by the electrolyte and its desorption from the metal surface. Interestingly, collagen molecules in the adsorbed state show a large stability against changes of the potential at the protein-electrode phase boundary [37] as well as temperature [38].

The first in situ PM IRRAS studies of protein films provide valuable information concerning the structure of an adsorbed protein and its changes caused by external impulses such as electric potential and temperature. It is an excellent experimental method to elucidate in situ the secondary structure of a protein and its changes caused by the adsorption process.

## 5 Conclusions

The development of new experimental strategies aiming at the description of the structure, molecular organization and activity of biomolecules in their biomimetic assemblies will remain a challenge and a source of new insights in the next years in the field of interface science, physical chemistry, biophysics and biological chemistry. The large advantage of the use of in situ PM IRRAS arises from the possibility of the structural analysis of various components in a supramolecular assembly exposed simultaneously to external impulses such as changing electric fields, temperature or binding agents. Due to the possibility of the combination of IRRAS techniques to electrochemistry, changes of the macroscopic parameters characterizing an adsorbed film such as surface coverage, capacitance, charge density,
rate and mechanism of a redox reaction can be described to structural changes, at a sub-molecular level, accompanying these processes.

Since most biological and pathological processes involve cell membranes, studies of models of lipid membranes are very important to understand their functions and reactions taking place at their surfaces. Results described in this paper show clearly that not only the physical state of hydrocarbon chains at lipid molecules but also their degree of hydration, formation and breaking of hydrogen bonds at polar head groups are a function of the potential drop across the membrane. Recognition of these changes is essential to study multicomponent lipid systems as well as versatile lipid-protein interactions. Continuation of these pioneering works may bring us to a better understanding of the biology, chemistry and physics of the lipid-protein interplay in nature.

In addition, application of PM IRRAS to studies of the solid-liquid interface opens the possibility to study the bio integration process and to describe, at the molecular level, changes taking place at the interface between biological molecules and biomaterials. In parallel this technique allows investigating in situ molecular aspects of the process of adsorption of proteins at solid surfaces.

According to the author knowledge, biologically and technologically important supramolecular assemblies of nucleic acids have not yet been investigated using in situ PM IRRAS. Assemblies of nucleic acids find various applications as sensors, chip or drug screening devices, in which the knowledge of their reactivity and sensitivity as a function of a molecular organization in a film is highly required. Since DNA and RNA fragments may form organized films on solid substrates and are often deposited on the gold surface, their assemblies provide an interesting research material for PM IRRAS studied with electrochemical control.

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References