In Situ Infrared Attenuated Total Reflection (IR ATR) Spectroscopy: A Complementary Analytical Tool for Drug Design and Drug Delivery

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Abstract. A comprehensive summary of basic relations for quantitative IR ATR spectroscopy of isotropic and oriented samples is given. Experimental requirements for detection of sub-monolayer quantities in aqueous environment are discussed. New instrumental developments such as a single-beam-sample-reference (SBSR) attachment, and FTIR modulation spectroscopy at low frequencies are presented. Examples of in situ experiments with tertiary-amine local anesthetics interacting with planar, immobilized lipid bilayers are discussed. Partition coefficients of the total amine, as well as of the protonated and deprotonated forms have been determined. Structural alterations, especially of lipids, were detected upon interaction. Adsorption isotherms periodically induced changes of the state of the sample.

Introduction

In the past decade, Fourier-transform IR (FTIR) spectrometers have replaced dispersive instruments because of their better performance in nearly all respects [1]. The problem of background compensation e.g. could be reduced significantly, enabling routine measurements even in aqueous environment. Therefore, a growing interest in IR membrane spectroscopy was observed from the biophysical, biochemical, and biomedical viewpoints [2–39] as well as from the biosensor part [40][41]. The works cited here have to be considered as typical examples just available to the author at present. Since the aim of this article is presenting ATR techniques for in situ experiments as well as the theoretical background for quantitative analysis and orientation measurement, rather than reviewing papers published so far, the reader is also referred to the reference lists of cited papers. In most of these papers conventional transmission (T) technique has been used, perhaps because for simple applications sample preparation and data interpretation may be easier than with ATR technique. The latter, however, has significant advantages over T spectroscopy, especially with respect to structure determination under in situ conditions.

Sect. 2 gives a comprehensive introduction into the principles of ATR spectroscopy, whereas Sect. 3 deals with experimental considerations, such as sensitivity requirements, background compensation and modulation spectroscopy. Sect. 4 gives a summary of typical applications like preparation of model membranes and living cells for in situ experiments, interaction of local anesthetics with lipid bilayers, determination of partition coefficients between membrane and aqueous environment, as well as temperature modulation. The latter enables access to conformational equilibria of polypeptides and lipids. Besides of utmost background compensation, modulation spectroscopy enables kinetic analysis, too, provided that the circular modulation frequency \(\omega_m\) and the rate constants of excited reactions are of the same magnitude. The situation is optimum, when the response of the sample is fully reversible [42–46]. Estimates reveal that signal to noise (S/N) enhancement by this technique should be sufficient to get information on the molecular structure of a substrate molecule bound to its specific receptor under in situ conditions. Such data are of predominant interest in drug design.

2. Principles of ATR Spectroscopy

2.1. Basic Phenomena

Fig. 1 shows a schematic comparison of the conventional transmission (a) and the internal reflection (b) technique. The latter is achieved by placing the sample
material in close contact with the optically transparent internal reflection element (IRE) of higher refractive index and working above the critical angle \( \theta_c \).

Under these conditions, it follows that an electromagnetic field still exists in the rare medium beyond the reflecting interface. This field exhibits the frequency of the incoming light, but the amplitude falls off exponentially with distance \( z \) from the surface.

\[
E = E_o e^{-\alpha z}
\]

(1)

\( d_p \) denotes the depth of penetration and is given by

\[
d_p = \frac{\lambda_1}{2\pi (\sin^2 \theta - n_1^2)^{1/2}}
\]

(2)

\( n_p \) stands for the ratio \( n_p/n_1 \) of the refractive indices of the rarer medium \( n_p \) and of the internal reflection element \( n_1 \). \( \lambda_1 \) denotes the wavelength \( \lambda_{\text{air}}/n_1 \) in the latter. According to Eqn. 2, the penetration depth amounts to the order of magnitude of the wavelength \( \lambda_1 \) of the IR radiation in the IRE. Fig. 2 schematically represents the situation when a thin film (e.g. a bilayer or a protein layer) is immobilized at the surface of the IRE. This is a typical set-up for in situ membrane and biosensor studies. The displacement \( D \), the so-called Goos-Hänchen displacement [48], is also in the order of \( \lambda_1 \).

The ‘evanescent wave’ exhibits another interesting feature, namely the existence of electric field components along all three axes of the rectangular coordinate system attached to the ATR plate (Fig. 3). In contrast, the incident plane wave has only electric field components perpendicular to the direction of propagation. By means of a polarizer, two distinct directions of the incident electric-field vector may be chosen, namely parallel (\( ||, pp \)) and perpendicular (\( \perp, vp \)) to the plane of incidence (\( x, z \) plane). For geometrical reasons (see Fig. 3) it follows that \( E_\parallel \) results in the \( E_x \) and \( E_y \), and \( E_\perp \), the \( E_z \) component of the evanescent field.

The relative electric field components in the rarer medium 2 at \( z = 0 \) (\( 1,2 \) interface), i.e. the ratio between the electric-field components \( E_{o1}, E_{o2}, E_{o3} \), and \( E_{oA} \), are given by Fresnel's equations [49]. Eqsns. 3–5 hold exactly for non-absorbing media 1 and 2, but they are good approximations for weak absorbing samples [46][47]. This condition is fulfilled for all types of experiments reported in this article [46].

The relative electric field components in a thin film of thickness \( d < < d_p \) (see Fig. 2) are given by:

\[
n_{th} = \frac{n_i}{n_p}
\]

(3)

\[
E_{o01} = \frac{2\cos \theta (\sin^2 \theta - n_1^2)^{1/2}}{(1 - n_1^2)^{1/2} [1 + n_3^2 \sin^2 \theta - n_3^2]^1/2}
\]

(3)

\[
E_{o02} = \frac{2\cos \theta (\sin^2 \theta - n_2^2)^{1/2}}{(1 - n_2^2)^{1/2} [1 + n_3^2 \sin^2 \theta - n_3^2]^1/2}
\]

(4)

\[
E_{o03} = \frac{2\cos \theta (\sin^2 \theta - n_3^2)^{1/2}}{(1 - n_3^2)^{1/2} [1 + n_3^2 \sin^2 \theta - n_3^2]^1/2}
\]

(5)

where \( n_1, n_2, n_3 \) denote the refractive indices of IRE, film, and bulk environment, respectively. The penetrating ray is displaced by the distance \( D \) [48].

\[
T = I_2/I_1 = 10^{-A} = 10^{-\alpha \cdot d}
\]

(7)
The meaning of the symbols are transmission (\(T\)), intensity before (\(I_0\)) and after (\(I_2\)) the sample, absorbance (\(A\)), molar absorption coefficient (\(e\)), molar concentration (\(c\)), and sample thickness (\(d\)), respectively. To apply Lambert-Beer's law to ATR data, one has to introduce a hypothetical thickness \(d_h\) of a sample which would result in the same absorbance of a given band via transmission as obtained with the real sample thickness \(d\) in the ATR experiment. The quantity \(d_h\) was introduced by Harrick [47] and referred to as 'effective thickness'.

For the limiting cases 'bulk medium 2' (\(d = \infty\)), and 'thin layer' (\(d < d_h\)) one obtains

\[
\begin{align*}
\frac{1}{d} &= \frac{\cos \theta}{d_p} - \frac{E \cos \theta}{2} \text{ and} \\
\frac{d}{d_h} &= \frac{1}{\cos \theta} \frac{n_g}{n_1} - \frac{E \cos \theta}{2} \text{ respectively (8)}
\end{align*}
\]

It should be noted that \(d_h\) for a bulk medium increases linearly with wavelength \(\lambda\) (see Eqn. 2), whereas \(d_h\) for a thin film does not depend on \(\lambda\). The effective thickness for an intermediate sample thickness \((d = d_h)\) may be calculated by using Eqn. 6. For more details, on effective thickness, see [46]. As a consequence of the application of Fresnel's equations, simple application of Lambert-Beer's law to ATR data requires polarized incident light [46].

\[
T_h = 10^{-e \cdot \lambda \cdot d_h} = 10^{-A_h}, \text{ and}
\]

\[
T_z = 10^{-e \cdot \lambda \cdot d_z} = 10^{-A_z}
\]

2.3. Quantitative Analysis of Oriented Samples

In the case of an oriented sample, the absorbance depends not only on the number of particles per volume (or area) but also on the structure of the sample. For that reason, quantitative analysis of an oriented sample means both, determination of concentration and orientation.

2.3.1. Orientation Measurements

For a comprehensive discussion of orientation measurements in transmission spectroscopy, see [50–52].

2.3.2. Theory of Integrated Absorbance of Oriented Samples

The integrated absorbance is proportional to the square of the scalar product between the transition dipole moment \(\vec{M}\) and the electric field \(E\) of radiation. For the case of excitation of one quantum of the \(k\)-th normal mode \(q_k\) from the ground state, \(\vec{M}\) is proportional to the change \((\delta E / \delta p_{q_k})\) of the molecular dipole moment \(\vec{M}\) with respect to the normal mode displacement \(q_k\) [53].

\[
A(\vec{M}, E) = \int e \cdot |\vec{M}| \cdot \vec{M} \cdot E \cdot \cos(\vec{M}) \cdot E \cdot \cos(\vec{M})
\]

Eqs. 11 and 12 form the basis of orientation measurements.

2.3.3. Ultrastructure

Two problems have to be solved in order to get structural information. First, the relative orientation of the ATR plate fixed coordinate system \((x,y,z)\) and the molecular coordinate system \((a,b,c)\) must be known. This is equivalent to the knowledge of the ultrastructure of the molecular entity. Secondly, the knowledge of the orientation of the transition dipole moment \(\vec{M}(M_x,M_y,M_z)\) within the molecule is a prerequisite for molecular-structure analysis. This requirement often limits the application of the analytical procedure described above to typical vibrations of functional groups, unless more details on the direction of \(\vec{M}\) are available via normal coordinates analysis [53].

The angles \(\alpha, \beta, \) and \(\gamma\) are the Eulerian angles, enabling description of the mutual position of the two coordinate systems. Transformation of \((M_x,M_y,M_z)\) into \((M'_x,M'_y,M'_z)\) is performed by the matrix Eqn. 13.

\[
\begin{pmatrix}
M'_x \\
M'_y \\
M'_z
\end{pmatrix} =
\begin{pmatrix}
\cos \beta \cos \gamma \cos \alpha + \sin \beta \sin \alpha \\
-\sin \beta \cos \gamma \cos \alpha + \cos \beta \sin \alpha \\
-\cos \gamma \sin \alpha
\end{pmatrix}
\]

Thus, if \((M_x,M_y,M_z)\) are known from experience or from normal coordinates analysis [53] \((M'_x,M'_y,M'_z)\) are calculated by coordinate transformation (13) and inserted into Eqsns. 11 and 12. The electric-field components are given by Eqsns. 3–6. Obviously, this procedure results in a quantity which is proportional to the integrated absorbance, however, for most samples this is only a fractional contribution, since the entity of molecules exhibit a certain distribution with respect to the Eulerian angles, which is typical for a given ultrastructure. For defination of ultrastructures see [45][46]. Typical features of biomembranes are random arrangement of molecules around a space-fixed axis normal to the plane of the membrane \((z\) axis) and free rotation around the molecular axis \((c\) axis) as well as fluctuation of the angle \(\beta\) between molecular axis and space-fixed axis (Fig. 4). This ultrastructure is referred to as liquid crystalline ultrastructure (LCU) and is characterized by isotropy with respect to \(\alpha\) and \(\beta\), whereas \(\gamma\) exhibits a distribution around a mean value \(\gamma(0^\circ \leq \gamma < 90^\circ)\).

The fluctuation of the molecular axis \((c)\) with respect to the space fixed axis \((z)\) is described by the distribution function \(f(\gamma)\) [51][52][54].

2.3.4. Dichroic Ratio and Order Parameter

The relevant parameter in most optical orientation measurements is the dichroic ratio \(R\)

\[
R = \frac{A_1}{A_0} = \frac{\int A_1(\theta) d\theta}{\int A_0(\theta) d\theta} \quad (14)
\]

\(A_0\) and \(A_1\) denote the peak absorbances of a given band for parallel (\(0\)) and perpendicular (\(\perp\)) polarized incident light, respectively. Following the path described above, the axial absorbances \(A_0, A_{\perp}\) and \(A_{\parallel}\) can be calculated for a given ultrastructure. The procedure has been described in details for transmission spectroscopy by Zbinden [51] and Michl and Thulstrup [52]. Adaptation to ATR spectroscopy is straightforward and has been done selecting the y axis [55] and the z axis [45][46] as space-fixed axis of a LCU. In the latter case, the axial absorbances are given by
samples aligned along the z axis. The relevant quantity for orientation measurement was reported to be \( f = \frac{1}{2} (3 \cos^2 \beta - 1) \) and referred to as 'order parameter'. \( \beta \) denotes the mean angle between the z axis and the molecular axis. From the definition of the order parameter (Eqn. 18), which is the same as in [38], it follows that the expression \( f = \frac{1}{2} (3 \cos^2 \beta - 1) \) can only result for a distribution function \( \delta(y-\beta) \) (see Eqn. 18). This means, however, that any molecular fluctuation is excluded, which is equivalent to setting the order parameter \( S = \frac{1}{2} \) (Eqn. 19). Analysis of polarized ATR spectra of LCU systems by the formalism given in [38] may, therefore, lead to significant systematic errors. Let e.g. \( R_1 \) and \( R_2 \) be the dichroic ratios of equal functional groups at different positions in a molecule. \( R_1 = R_2 \) only means same orientation for both groups, if \( S_1 = S_2 \). If, however, \( S_1 \neq S_2 \), which means

\[
R_{\text{ATR}}(\text{LCU}) = \frac{E_{2\text{ox}}^2}{E_{2\text{ox}}^2} + \frac{E_{2\text{oy}}^2}{E_{2\text{oy}}^2} \left( 1 + S(3\cos^2 \Theta - 1) \right)
\]

Finally, it should be mentioned that structural features revealed by Eqns. 15–17 and Eqn. 20 are expressed by the orientation function

\[
\sigma = S(3\cos^2 \Theta - 1)
\]

As a consequence, in a LCU picture as described above determination of both, \( \Theta \) and \( N \) is not possible by a single dichroic ratio of a given band. It requires the analysis of the dichroic ratios of at least two absorption bands \((1,2)\) resulting from the same functional group. Since in this case \( S_1 = S_2 = S \), and in a given (assumed) orientation \( \Theta_1 \) and \( \Theta_2 \) are related for geometrical reasons, it is possible to determine both, the order parameter (fluctuation) and the mean spatial orientation of the corresponding functional group. Good estimates of the direction of \( M(M_1, M_2, M_3) \) (see Fig. 4) are known for a number of functional groups [45], such as \( \nu_1(\text{CH}_2), \nu_2(\text{CH}_2), \delta(\text{CH}_2), \gamma_1(\text{CH}_3), \gamma_2(\text{CH}_3); \nu_{30}(\text{COO}^-), \nu_{31}(\text{COO}^-); \nu_{32}(\text{PO}_3), \nu_{33}(\text{PO}_3) \) etc. Combination of Eqns. 20 and 21 results in 2.3.5. Dichroic Ratio of Overlapped Bands

Different Degrees of Ordering

A situation often found in polypeptide- and hydrocarbon-chain spectroscopy is a superposition of typical absorption bands (e.g. amide I, II, CH stretching, bending, rocking). This leads to an overall dichroic ratio which is experimentally accessible. It depends on the orientation of each associated functional group. Separation by any line-shape analysis procedure is mostly not possible. A good estimation of the amount and orientation of one component can be obtained, however, when mole fraction and orientation of the other components are known [59–61]. Eqn. 23 is at least a good tool to calculate the dichroic ratio of a composed band i.e. to assess an

\[
R_{\text{ATR}}(\text{LCU}) = \frac{E_{2\text{ox}}^2}{E_{2\text{ox}}^2} + \frac{E_{2\text{oy}}^2}{E_{2\text{oy}}^2} \left( 1 + \sigma \right)
\]

In a recent paper, Hübner and Mantsch [38] have derived a formalism for the analysis of polarized ATR spectra of LCU expected molecular conformation with respect to its consistency with experimental data.
where $R_{\text{iso}}$ denotes the dichroic ratio of an isotropic sample. Obviously, in the dichroic difference spectrum $A^* (\theta)$, all absorption bands resulting from isotropically oriented transition dipole moments are eliminated. Eqn. 24 has been used for the subtraction of the random part of protein spectra [2][28][60]. Details on the practical procedure are given in [46].

### 2.4. Determination of Surface Concentration

The concept of 'effective thickness' (see above) is maintained also for oriented samples. However, one has to take into account that the effective thickness may become zero (no absorbance), if the angle between the transition dipole moment $M$ and the electric-field vector $E$ is $90^\circ$, and maximum for parallel alignment of the two vectors. Weighting is achieved by multiplying the axial effective thickness of an isotropic sample by factors depending on the ultrastructure of the sample.

In the case of LCU with predominant alignment along the z axis, the axial absorbances $A_x, A_y,$ and $A_z$ are given by Eqns. 15–17 with $A_x = A_y + A_z$ and $A_z = A_x - A_y$. $A_x$ and $A_z$ are experimentally available. Obviously, the expressions within brackets are the typical features of LCU/z. The weighting factors for axial effective thicknesses of an oriented sample are, therefore, given by

$$W_\gamma = 1 - \frac{1}{2} S (3 \cos^2 \Theta - 1) = 1 - \frac{1}{2} \sigma$$

for peak absorbances, or integrated absorbances (band areas), respectively. $\sigma$ denotes the integral molar absorption coefficient. Use of integrated absorbances is recommended in all cases, where band broadening or narrowing must be expected, e.g. due to conformational changes. $N$ denotes the number of equal functional groups per molecule, and the number of active internal reflections, respectively.

In the case of thin layers ($d \leq d_{\text{el}}$, Fig. 2), it is often more adequate to use the surface concentration $\Gamma$ instead of the molarity $c$. $\Gamma$ is obtained from Eqns. 29a, 29b simply by multiplication by the layer thickness $d$.

$$\Gamma = cd$$

### 3. Experimental Considerations

#### 3.1. Sensitivity Requirements for in situ Measurements

**Optimum Number of Internal Reflections**

Considering the symmetric CH$_2$ stretching ($\nu_2$(CH$_2$)) band of a phospholipid monolayer, one should expect an absorbance of about 0.2% per internal reflection. From this point of view, 100 or even more internal reflections would be desirable. However, these mono and submonolayer absorbances have to be detected in aqueous environment which itself is a strongly absorbing medium (see Fig. 5). The latter requires as few internal reflections as possible. Optimization [46] resulted in $N = 20–40$ for a germanium IRE, and 10–20 for a ZnSe IRE (angle of incidence: $\Theta = 45^\circ$).

#### 3.2. Significance of Liquid Water Compensation

The vibrational spectrum of liquid water (see Fig. 5 and [46]) is commonly the most intense part of the background to be compensated in IR spectroscopy of biological systems. Unfortunately, the amide-I band of peptides and proteins, which are sensitive to the secondary structure are completely overlapped by the H$_2$O bending ($\nu$(H$_2$O): $\sim 1640$ cm$^{-1}$). In many papers published so far, access to amide I was performed by subtraction of the background by means of the spectrometer software. Structural information was then obtained by application of Fourier self-deconvolution and/or curve-fitting techniques, to separate the amide-I band into components typical for secondary structural elements, such as $\alpha$ helix, pleated sheet, $\beta$ turns, random. These results, however, are of little confidence, because bulk water and bound water exhibit different peak wavenumbers (up to 20 cm$^{-1}$) and different molar absorption coefficients (up to a factor of 10) [46]. As a consequence, one should be aware that bound water is an integral part of the sample. It cannot be subtracted, at least not by manual manipulations with a bulk water reference spectrum. For a more detailed discussion of this fundamental problem, see [46].

#### 3.3. ATR Attachment

**Single-Beam-Sample-Reference (SBSR) Technique**

As a consequence of the required minimum number of internal reflections for monolayer spectroscopy on the one hand, and of the strongly absorbing background on the other hand, the spectral regions of OH stretching ($\sim 3400$ cm$^{-1}$) and OD stretching ($\sim 2500$ cm$^{-1}$) are completely blocked, depending on whether H$_2$O or D$_2$O is used as solvent. The transmittance...
of the corresponding bending vibrations (\(\Delta H_2 O\) : ~1640 cm\(^{-1}\); \(\Delta D_2 O\) : ~1200 cm\(^{-1}\)) is only a few percent under these conditions. A significant compensation of such strong bands is a prerequisite for \textit{in situ} spectroscopy, and requires a good reproducibility of the optical path of the IR beam in the multiple internal-reflection element (MIRE), especially for corresponding sample and reference measurements. Moreover, a well-defined angle of incidence and number of internal reflections are important for quantitative analysis. We have achieved best results with dispersive [60] and Fourier transform (FT) instruments [46][61][62][105] by a single trapezoid MIRE (Fig. 6) for sample and reference, as well.

Using parallel incident light focused to the entrance face of the crystal by means of a cylindrical mirror, it was possible to place the sample, \(\varepsilon\) at the lower half, and the reference at the upper half of the MIRE. The IR beam is directed alternatively through sample and reference side. In dispersive instruments, a rotating chopper wheel with two rows of different numbers of holes (see Fig. 7) was used in order to label sample and reference beams with different frequencies.

Because sample and reference are measured with a single beam without cell displacement, this method is referred to as Single-Beam-Sample-Reference (SBSR) technique. The most obvious advantage of this attachment is the lack of problems with atmospheric \(H_2 O\) and \(CO_2\) absorptions. SBSR spectra exhibit also a better long-term stability than generally achieved in the single-beam mode [46]. Furthermore, SBSR technique enables \textit{ad hoc} changes of the reference environment without problems, since there is no need for separate reference spectra which might require detachment of the ATR cell. The latter would be necessary in the single-beam (SB) mode, leading to a decrease of the accuracy of background compensation.

3.4. Modulation Spectroscopy

3.4.1. Introduction

A situation often encountered in chemical and biological IR spectroscopy is the detection of small structural differences on a molecular level, induced in a system by the change of external parameters, as the concentration of an agent, temperature, irradiation, electric field, etc. Conventionally, this information is obtained by measuring the IR spectra at two different parameter settings and then calculating the difference spectrum. The smaller this difference is, however, the more critical is the influence of system instabilities occurring between the two measurements. They decrease with decreasing time delay. SBSR technique, as described above, can reduce such instabilities considerably; however, best results are achieved in all cases in which modulation spectroscopy can be applied.

3.4.2. Requirements for the Applicability of Modulation Spectroscopy

The chemical or biological system to be investigated must allow a reversible or quasi-reversible periodic stimulation. If an external parameter is modulated by the circular frequency \(\omega_m = 2\pi \nu_m\), all molecules of the sample which depend on this parameter will exhibit a concentration modulation of the same frequency, whereas all inert molecules remain unaffected. The use of phase-sensitive detectors (quadrature demodulation) enables a selective observation of the stimulated process in real time, whereas all inert components are suppressed (real time difference spectroscopy). Moreover, if the ‘time constant’ of the stimulated process is comparable to, or larger than the stimulation period, a phase lag and a decrease of the amplitude will result, which both are typical for the kinetics of the process.

3.4.3. Theoretical Remarks

Consider a sample with concentration \(c\), which may be influenced by a periodic modulation of an external parameter with circular frequency \(\omega_m\). The resulting concentration modulation may then be described by the Fourier series Eqn. 31:

\[
c(t) = \bar{c}_i + 2c_1 \sin(\omega_m t + \phi_1) + 2c_2 \sin(2\omega_m t + \phi_2) + \ldots = \bar{c}_i + \left[ 1 + \rho_1 \sin(\omega_m t + \phi_1) + \rho_2 \sin(2\omega_m t + \phi_2) + \ldots \right],
\]

where \(\bar{c}_i\) denotes the mean concentration (DC term) and \(2c_2\) the corresponding Fourier coefficient of the \(k \cdot \omega_m\) term. The degree of modulation is defined by

\[
\rho_1 = \frac{2c_1}{\bar{c}_i} \quad (0 \leq \rho_1 \leq 1)
\]
It should be noted that, if the stimulation is harmonic or rectangular, \( \rho_2 \) should only deviate from zero, if nonlinear reaction steps (other than first-order kinetics) occur in the chemical process.

According to Lambert-Beer’s law, the light intensity \( I_s \), after passing the sample \( S \) is given by

\[
I_s = I_0 \cdot T_S = I_0 \cdot 10^{-\sigma \alpha_0 d} = I_0 \cdot 10^{-a_0} (33)
\]

where \( T_S \) and \( A_S \) denote transmittance and absorbance of the sample. Introducing Eqn. 31 into 33, one obtains after reorganization

\[
I_s = I_0 \cdot T_{S0} \cdot T_{S1} \cdot T_{S2} \cdot \ldots (34)
\]

\( T_k \) \((k = 1, 2, \ldots)\) denotes the transmittance corresponding to the modulated responses of the frequency \( k \cdot \omega_m \).

\( a_0 \) and \( a_\alpha \) denote abs. transmittance and absorbance of sample, and reference beams, labelled by \( \alpha_0 \) and \( \alpha_\alpha \). M: Monochromator. D: Detector. PA: Preamplifier. PSD: Phase-sensitive detectors for demodulation of carrier frequencies \( \alpha_0 \) by means of oscillator OSC. \( I_s, I_R \) intensities of sample, and reference. A: wavelength. Relevant output signals are digitized (DIG) and stored in a computer.

4. Applications to Drug/Membrane Interaction Studies

4.1. Preparation of Immobilized Bilayer Membranes

Model membranes used for drug interaction studies are phospholipid bilayers immobilized at the surface of the MIRE (Fig. 8). For technical reasons, the preparation must be performed in two independent steps [62]. The first monolayer is transferred by means of the Langmuir-Blodgett technique [69]. The transfer from the air-water interface is achieved by withdrawing the ATR plate perpendicularly through the compressed monolayer at constant pressure and constant velocity (Fig. 9).

The monolayer coated dry plate is then mounted in a liquid sample cell which has been hydrodynamically optimized for flow-through experiments. For completion of the bilayer, a vesicular solution (1 mg of lipid/ml of buffer, sonication under \( N_2 \) flow at 35-40°C) is circulated through the ATR cell (Fig. 10) resulting in spontaneous adsorption of phospholipid molecules.

The procedure was optimized based on electron micrographs (F. Kopp et al., to be published) leading to very stable and reproducible lipid bilayer membranes. No lipid loss was detectable by pumping a buffer solution in a closed cycle during 12 h (1.9 ml/min). Recently, corresponding mixed POPC/POPG bilayers have been prepared for melittin interaction studies [27].

4.2. Preparation of Double Bilayer Assemblies

It was shown earlier [72][73] that membrane fragments with enriched Na,K-ATPase isolated from rabbit kidney adsorb spontaneously to black lipid membranes (BLM) of POPC. It turned out that these membrane fragments adsorb to a DPPA/POPC bilayer under the same conditions as they do to POPC BLM. The result is a double bilayer assembly as shown in Fig. 8. This procedure offers new possibilities for in situ studies with integral membrane...
that the absorbance spectrum of 100-mM bulk DIBU has to be scaled by 0.37 instead of 0.50 in order to match the absorbance of 10-mM DIBU interacting with the membrane. This reveals unambiguously that about 2/3 of the absorbance of DIBU results from membrane-bound LA. Quantitative analysis by Eqs. 8, 9, and 25-30 results in a surface concentration of total DIBU (LA_{tot}) of 7.8 \times 10^{-6} \pm 0.4 \times 10^{-6} mol/m^2.

**4.3. Immobilization of Membranes, Proteins and Living Cells**

For dissolved substances ATR, spectroscopy requires minimum concentration of $c_{\text{min}} = 1-10$ nm, depending on the molar absorption coefficient. The sensitivity is drastically enhanced upon immobilization of the sample at the MIRE/water interface. Two possibilities have been described in Sect. 4.1 and 4.2. A variety of applications such as adsorption of lipid membranes, adsorption or chemical immobilization of proteins as well as living cells (e.g. erythrocytes) are enabled by silanization of the surface of a germanium ATR plate [67][74]. Aminopropyltriethoxysilane has turned out to be optimum as silanizing agent.

**4.4. Interaction of Drugs with Model Membranes**

Local anesthetics (LA) of the tertiary amine type (procaine (I), oxybuprocaine (II), falicaine (III), and dibucaine (IV)) were used as model compounds to interact with lipid bilayers of the type described in Sect. 4.1 (Formulas of LA).

All LAs were found to adsorb to the membrane. The higher the physiological activity of a given LA, the higher its affinity to condense to the DPPA/POPC bilayer, as well as to already adsorbed LA. Equilibrium surface concentrations have been determined and fitted by the Brunauer-Emmett-Teller (BET) isotherm (Fig. 11) [75].

From molecular-model considerations, one may estimate the maximum and minimum monolayer concentration of oxybuprocaine to be $\Gamma_{\text{max}} = 4.15 \cdot 10^{-6}$ mol/m$^2$ and $\Gamma_{\text{min}} = 1.38 \cdot 10^{-6}$ mol/m$^2$, respectively. Therefore, one has to conclude LA-multilayer condensation even at clinical application concentrations (5-100 mm).

More recent interaction studies of dibucaine (DIBU) with DPPA/POPC and DPPA/DMPC bilayers [61] have confirmed this finding. For these studies DIBU was dissolved (0.5-10 mm) in aqueous solutions of 100 mm NaCl at pH 5.5 (borate-phosphate-citrate buffer, 20 mM) for DPPA/POPC membranes, and at pH 6.7 (phosphate buffer, 20 mM) with DPPA/DMPC-d_{54} membranes. The volume of the ATR cell was 250 μl. To establish constant bulk concentration in the sample ATR cell, 5-10 ml of DIBU solution was permanently circulated in a closed cycle, (Fig. 10). Fig. 12 shows typical polarized spectra of 10 mm DIBU in contact with a DPPA/POPC membrane (trace a). A corresponding membrane buffer solution was in the reference (R) ATR cell, (see Fig. 6).

Trace a of Fig. 12 reveals, therefore, membrane-bound and bulk DIBU, as well as DIBU-induced structural changes of the membrane. For comparison, corresponding spectra of 100-mm bulk DIBU were scaled (x0.37) to about the same intensity (trace b). Both experiments were performed at rather non-physiological pH 5.5 in order to enable direct comparison with published NMR data [76-79]. The fact that the absorbance spectrum of 100-mm bulk DIBU has to be scaled by 0.37 instead of 0.10 in order to match the absorbance of 10-mm DIBU interacting with the membrane, reveals unambiguously that about 2/3 of the absorbance of DIBU results from membrane bound LA. Quantitative analysis by Eqs. 8, 9, and 25-30 results in a surface concentration of total DIBU (LA_{tot}) of $7.8 \times 10^{-6} \pm 0.4 \times 10^{-6}$ mol/m$^2$. 

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**Fig. 9.** a) Film balance. T, trough filled with aqueous subphase; S, sweep for compression of lipid monolayer (L) spread at the air-water interface; F, float connected to torsion wire (T') for film pressure measurement. b) Transfer of a compressed lipid monolayer from the air-water interface to a solid substrate (MIRE) by the Langmuir-Blodgett technique, see [69-70].
Fig. 10. Schematic setup for IR-ATR in situ experiments. 1: Multiple internal reflection element (MIRE, ATR plate), e.g., with immobilized membrane; 2: liquid sample cell for flow-through and stationary experiments; 3: thermostat; 4: pump; 5: thermostat for substrate reservoir; 6: substrate reservoir; 7: MIRE thermostat. From [71].

Fig. 11. Adsorption isotherms of procaine (+), lower trace, oxybuprocaine (Δ), falcaine (*), and dibucaine (○), upper trace. 20 mM phosphate buffer, pH 7.0, 100 mM NaCl. Note, that increasing physiological LA activity is reflected by the increasing adsorption tendency. From [75].

Assuming a cross-section of 45–67 Å² per molecule [77], one obtains 2–3 DIBU monolayers bound to the DPPA/POPC membrane at 10-mM bulk concentration. In contrast, Seelig et al. [77], reported still sub-monolayer coverage at the same bulk concentration.

Fig. 12. Polarized IR-ATR absorbance spectra of a 10-mM dibucaine (DIBU) solution (100 mM NaCl, 20 mM borate-phosphate-citrate (BPC) buffer, pH 5.5) interacting with a DPPA/POPC bilayer membrane (trace a) and for comparison, 100-mM DIBU solution in contact with the pure Ge ATR plate (trace b), scaled with 0.37 in order to get approximately the same band intensities (same buffer). Insert: Compressed wavenumber scale of the latter in the u(NH⁺) region (same absorbance scale). Angle of incidence: θ = 45°, number of active internal reflections: N = 19, temperature: T = 25°. Reference: DPPA/POPC bilayer (see Sect. 4.1), same buffer. Wavenumber range: 3000–2200 cm⁻¹ (A), 1800–1000 cm⁻¹ (B). Note. i) Only ca. 1/3 of the DIBU absorbance (trace a) results from dissolved DIBU; 2/3 is bound DIBU as a consequence of the scaling factor 0.37 for the trace b spectrum. Calculated surface concentration: r = (7.8 ± 0.4) × 10¹⁶ mol m⁻². ii) Dissolved DIBU is protonated (pKₐ = 8.83). Membrane-bound DIBU appears partly as base, as concluded from corresponding v(NH⁺) absorbance near 2700 cm⁻¹. iii) The unusual behavior of typical lipid bands (v(CH), ν(C=O), ν(C–O), ν(PO₂⁻)) is reversible and does not reflect lipid loss, see text.
DIBU concentration and pH. One reason for the discrepancy results probably from different bilayers. Symmetric POPC multilayers were used in [77]. In our case, also POPC was facing the aqueous environment (DIBU), however, the counter monolayer, facing the Ge-ATR plate, was Ca**- DPPA. Although the DPPA polar region is not directly accessible by DIBU (Fig. 8), the resulting negative trans-membrane electric field could facilitate DIBU binding and incorporation into the membrane. A second reason could be of systematic nature, since the experimental approaches are quite different (see Sect. 4.3).

Concerning the lipid response to DIBU binding, there is evidence for a reorientation of the choline head group (negative band at 1491 cm\(^{-1}\) [45]) in agreement with [77][80]. More significant lipid responses, however, result from \(\nu(CH_2): 2800-3000 \text{ cm}^{-1}\), \(\nu(C=O): 2000-2300 \text{ cm}^{-1}\), \(\nu(C=O): 1740 \text{ cm}^{-1}\), \(\nu(CH_2): 1470 \text{ cm}^{-1}\), \(\nu(C=O): 1180 \text{ cm}^{-1}\), \(\nu(PO_2)\) and for \(\nu(CH_2): 1100 \text{ cm}^{-1}\), \(\nu(PO_2)\) : 1000 cm\(^{-1}\).

Since the reference to the spectra shown in Figs. 12 and 13 are the corresponding bilayer membranes and buffer solutions the negative responses of lipid bands could indicate material loss upon LA interaction. This, however, is not the case, because the effects are reversible after LA replacement by buffer [61].

A similar, also less pronounced behaviour was also observed earlier with the LA oxybuprocaine [62]. LA-induced conformational changes in the hydrocarbon chain, fatty-acid ester, phosphate, and choline regions have to be concluded. Hydrocarbon chain disordering is also indicated by broadening and upwards shifts of \(\nu(CH_2)\) and \(\nu(CD_2)\) [61]. Both phenomena are qualitative features for the formation of gauche defects [81]. Quantitative analysis is possible via the dichroic ratio dependence of \(\nu(CH_2)\) on bulk DIBU concentration (Fig. 14), or by dichroic analysis of the difference bands (Figs. 12 and 13). Concerning the former, Fig. 14 shows the dependence of the mean dichroic ratio of \(\nu(CH_2)\) and \(\nu(CH_3)\) of a DPPA/POPC membrane interacting with DIBU (S) at pH 5.5 and with buffer (R).

Since data were collected in the SBSR mode (see Fig. 6) the age of both membranes, \(S\) and \(R\), was exactly the same. The \(\nu(CH_2)\) dichroic ratio of the R membrane remained constant over the whole duration of the experiment. Maximum dichroic ratio (1.37 ± 0.04) is reached at ca. 3-mm bulk concentration, indicating saturation of the effect. This could mean that maximum chain disordering is reached. Since 3-mm bulk DIBU correspond to more than a monolayer adsorbed DIBU [61], and since an isotropic layer would have a dichroic ratio of \(R_{iso} = 1.75 ± 0.05\), it rather tends to the interpretation, that DIBU coats the membrane, thus, preventing further disordering. On removing the 10-mm DIBU solution, a certain hydrocarbon chain disordering is restored, although no remaining DIBU could be detected in the membrane. These data, and the directly measured difference spectra (Figs. 12 and 13) enable the determination \(\sum \sigma_i^{CH_2} - \sum \sigma_i^{CH_3}\) and \(\sum \sigma_i^{CH_2} - \sum \sigma_i^{CD_2}\) respectively. \(\sigma_i\) denotes the orientation function (Eqn. 2) of the k-th CH_2 group in the i-th chain (i = 2) and \(\sigma_i\) of the i-th CH_2 group in the k-th CH_3 group (to be published).

5.4. Drug-Membrane Partition Coefficient

Since the volume of a bilayer membrane is small and in some cases may become comparable to that of bound drug, it is reasonable to define the apparent partition coefficient by

\[
P_{app} = \lim_{\text{conc} \to 0} \frac{c_m}{c_b}
\]
Fig. 15. Stationary and temperature (T) modulated FTIR ATR spectra of hydrated poly(L-lysine) multilayers on a Ge ATR plate (MIRE). Environment: 90% rel. humidity H₂O (A–D) and D₂O (E–H). Mean temperatures: T = 26°C (A,B,E,F) and T = 35°C (C,D,G,H). Stationary spectra: Predominant antiparallel β structure (pleated sheet) at 35°C (1625/1695 cm⁻¹, C, and 1618/1692 cm⁻¹, G). More complex conformational equilibrium at 26°C, α-helix and/or random (1655 cm⁻¹, A, 1643 cm⁻¹, E) in equilibrium with antiparallel β structure. Amide I band in H₂O environment overlapped by 8H₂O (-1640 cm⁻¹, A,C). Complete H/D-exchange in D₂O environment (E,G, α(NH) ~ 3300 cm⁻¹, α(ND) ~ 2400 cm⁻¹, amide II: ~ 1540 cm⁻¹, amide II: ~ 1450 cm⁻¹). T-modulation spectra: amplitude: ΔT = ±1.5°C (B,F), ΔT = ±1.0°C (D,H). Period: t = 11 min (i.e. 5.5 min T–ΔT and 5.5 min T+ΔT). Phase shift with respect to stimulation: 0° (lower trace), 45°, 90°, 135° (upper trace). Modulation bands of H₂O (-3400 cm⁻¹, -1640 cm⁻¹) and D₂O (-2500 cm⁻¹, -1200 cm⁻¹) result from temperature- and conformation-induced hydration changes, therefore, amide I (-1650 cm⁻¹) analysis is more relevant in D₂O environment (F,H). Modulation at T = 35°C stimulates a transition (1625/1619 cm⁻¹, D, 1620/1616 cm⁻¹, H) probably between two β structures. This transition occurs at T = 26°C, too (90° phase shift, 3rd trace from bottom, B,F). However, α-helices and/or random structures are also involved (~ 1655 cm⁻¹) The complexity of the transition is reflected by the phase dependent shapes of the amide I, II bands, which is typical for multistep reactions. Angle of incidence: 45°, number of active internal reflections: N = 26. Reference for stationary spectra: Clean MIRE.

[76–79][83–86]. Other methods are based on dialization [87], freezing-point depression [88][89], and electrophoretic mobility (ζ-potential) measurements [83][90]. Since many drugs may assume a charged and uncharged state (amines, carboxylic acids) depending on the environment, it is reasonable to take electrostatic effects into account for the discussion of membrane partitioning. Most of these approaches are based on the Gouy-Chapman model [77][83][88][91–94]. There is a general agreement that tertiary amines (e.g. LAs) undergo an apparent pKₐ decrease of ΔpKₐ = 1.5 upon membrane binding. Membrane bound LAs with a bulk pKₐ between 8 and 9 may, therefore, be expected predominantly in the protonated state at bulk pH 5.5 [76–79], which is in contrast to our finding, see below.

Although these methods are generally applied, there is still room for some critical remarks. Firstly, concerning the centrifugation approach: to get a supernatant free of lipids, up to 300 000 g are applied for 1–3 h. This leads probably to an equilibrium between drug in the supernatant, in the interlamellar water phase (the pellet consists of multilayers), and in the membrane. One may ask, whether this system at the moment of separation of supernatant and pellet still represents the equilibrium state before centrifugation? From the thermodynamic point of view, it is not only a question of high-pressure application, but also of activity alteration in the interbilayer waterphase. Secondly, many questions arise with respect to the electrostatic approach: is the Gouy-Chapman model adequate to describe the inhomogeneous surface potential profile resulting from single adsorbed charges? In this respect, an alternative approach has been given by Ninham and Parsegian [95] taking account of the local potential of membrane-bound charged species. Furthermore, can-counter-ion binding be neglected as in most applications cited above? Why is the interfacial and bulk permittivity set to be equal, although considerable differences have to be expected [96]? This has influence on the Boltzmann distribution of ions between bulk and interface and results in a Born energy term. Concerning ζ-potential measurements, there are also uncertainties with respect to non-uniform vesicle population, distance of the plane of shear from the vesicle surface (generally assumed to dₛ = 2 Å). Alteration of surface structure by the adsorption of charged molecules may also influence dₛ.

Finally, desorption of charged particles leads to a surface potential, which will induce compensating reactions such as counter-ion adsorption and dissociation or association of H⁺ (pKₐ shift). Structural alterations may occur, too.
One should be aware, that in cited applications many 'hidden' parameters had to be estimated in order to enable a quantitative analysis of experimental data. The need for complementary experimental techniques with direct access to the quantities of interest is obvious. In this respect, IR ATR spectroscopy is a promising approach. Typically, the drug solution is circulated through the small cell (see Figs. 6 and 10). Therefore, there is no ambiguity with respect to the bulk concentration. If necessary, it can be monitored on line. On the other hand, drug binding is detected simultaneously in situ via ATR. Quantitative analysis is straightforward as described in Sect. 2.

Concerning DIBU binding to a DPPA/POPC membrane, we have measured the adsorption isotherms for both, total DIBU (LA\textsubscript{tot}) using the aromatic ring vibration at 1407 cm\(^{-1}\), and protonated species (LA\textsuperscript{+}) by means of the N-H\(^+\) stretching vibration at 2700 cm\(^{-1}\) [61]. It follows that, already at bulk DIBU concentration of ca. 1 mM, the bound total amount of DIBU is sufficient to form a compact monolayer. However, the bound protonated species LA\textsuperscript{+} reaches this limit only at 2-6 m\textsuperscript{M} bulk concentration, although there is 99.96\% LAH\textsuperscript{+} and only 0.04\% LA at pH 5.5 in the bulk environment (pK\textsubscript{a} = 8.83). Furthermore, about one half of bound DIBU is deprotonated and exists as base (LA) even at bulk pH 5.5. This ratio of charged to uncharged DIBU is also maintained in the DIBU multilayer region at \(\geq 5\ \text{mM}\) bulk concentration. Note that Fig. 12 gives already evidence for significant DIBU deprotonation upon binding to the membrane. It should be mentioned that the bulk concentration of LA and LA\textsuperscript{+} are known and maintained constant during the adsorption process by using a closed cycle flow-through system (Fig. 10) with a large buffered substance reservoir. The adsorption isotherms of LAH\textsuperscript{+} and (LA\textsubscript{tot}) fit well to the simple Langmuir isotherm \(\Gamma = c_0 B/(a + c_0)\), \(B\) and \(a\) denote the monolayer surface concentration, and the ratio of desorption/adsorption rate constants. The concentration of a compound in the monolayer \(c_0\) is related to the experimentally accessible surface concentration \(\Gamma\) by \(c_0 = \Gamma/a d_e\), where \(d_e\) denotes the membrane thickness (\(d_e = 5\ \text{nm}\)). According to Eqn. 35, the apparent partition coefficient may be expressed by the Langmuir parameters and the membrane thickness

\[
P_{\text{app}} = \frac{B}{a d_e} \tag{36}
\]

The resulting partition coefficients are found to be \(P_{\text{app}}(\text{LA\textsubscript{tot}}) = 1060 \pm 86, P_{\text{app}}(\text{LA\textsuperscript{+}}) = 475 \pm 93, \text{and } P_{\text{app}}(\text{LA}) = 9.99 \cdot 10^4 \pm 1.87 \cdot 10^5\), all data with 95\% confidence limits [61]. The values for the total amount LA\textsubscript{tot} and for LA\textsuperscript{+} are experimental, whereas values for the base LA are calculated from the difference of the surface concentrations of LA\textsubscript{tot} and LA\textsuperscript{+}. The Langmuir parameters \(B\) and \(a\) lose their original meaning and have to be considered as fitting parameters for an experimental isotherm, since electrostatic interactions as well as surface reactions (LA\textsuperscript{+} \rightarrow LA + H\textsuperscript{+}) and multilayer adsorption occur. In view of the fact that the pH is known and maintained constant during the adsorption process, as well as on surface roughness and optical constants.

\[P_{\text{app}}(\text{LA\textsubscript{tot}}) \approx 1060 \pm 86, \ P_{\text{app}}(\text{LA\textsuperscript{+}}) \approx 475 \pm 93, \text{and } P_{\text{app}}(\text{LA}) \approx 9.99 \cdot 10^4 \pm 1.87 \cdot 10^5\]
5. Prospects

This review article has featured general and special aspects of IR ATR spectroscopy. Results justify the hopes that this technique may become a serious complement in drug design and drug delivery. Of course, there is need for further refinements of experimental methods and theory. In this context, it is planned to introduce more general ultrastructural models adequate for dichroic analysis of oriented poly- and oligopeptides, as well as of hydrocarbon chains. For that purpose, it is essential to get information on energetically reasonable local conformations, i.e., on local distribution functions. Such data are available from molecular modeling. On the other hand, data on molecular structure derived from IR ATR can be used to filter the variety of possible structures derived from molecular modeling. Such concerted investigations are not limited to immobilized oriented samples. Molecular structures can be studied in solution, too, provided the concentration of the solute is greater than 1–10 mm, depending on the molar absorption coefficients. Especially external parameter modulation techniques will be applied in a variety of different forms enabling the detection and analysis of conformational equilibria in immobilized and dissolved samples.

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