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Development and Applications of Fluorescence Methods in Biosciences

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Overview and Motivation

"Development and Applications of Fluorescence Methods in Biosciences"

After the pioneering works of Kasha, Vavilov, Perrin, Jabłoński, Weber, Stokes, and Förster (including the appearance of the first book of the latter on fluorescence of organic molecules in 1951 [Foerster, T (1951) Fluoreszenz organischer Verbindungen, VandenHoech und Ruprecht, Goettingen]), fluorescence spectroscopy became a widely used scientific tool in "biosciences". Though "bioscience" is a rather broad term, it is certainly linked to the characterisation of the interaction among the major components of living cells, which are mainly proteins, membranes and DNA. Usually, those interactions are directly controlling the biochemical processes necessary for life. Blood coagulation is certainly one of those processes and is controlled by protein-protein and protein-membrane interactions. The necessity to understand the molecular mechanism of certain (blood coagulation) protein-membrane interactions gave the original motivation for **Chapter A and B** of this thesis.

Principally, the problem of protein-membrane interactions can be approached from two different sides by fluorescence spectroscopy. The first possibility is to take advantage of the intrinsic tryptophan fluorescence or of fluorescent labels attached to the protein (**Chapter B**). In tryptophan studies [publications **B2-B7**] the fluorescence properties of proteins in free and membrane bound state are compared. Fluorescence changes in the intrinsic fluorescence may be interpreted in terms of a specific conformational change occurring during membrane binding [**B4,B7**]. The use of bright covalently attached fluorescent labels allows for the application of techniques like fluorescence recovery after photobleaching [**B1**].

The second possibility is to focus on the phospholipid organisation of the membrane which might change due to protein binding. The characterisation of pyrene excimer formation as well as the determination of fluorescence anisotropy of embedded membrane probes like 1,6-diphenylhexatriene have been widely used in studies of biological and model membranes. While both methods are well suited to monitor the dynamics of the interior part of a lipid bilayer they turned out much less useful for the study of the organisation of the lipid head-group region. A fluorescence method which overcomes this limitation is the solvent relaxation method (**Chapter A**). Originally designed for the study of very fast solvation processes in non-viscous solvents the method has been successfully adapted to the study of both the hydrophilic headgroup region and the hydrophobic interior of phospholipid bilayers [**A1-A11**]. This new method in biomembrane research [**A1-A5**] was not only successfully applied in the characterisation of protein-membrane interactions [**A6,A10**], but also yielded new information on factors influencing the water organisation in bilayers [**A7-A9,A11**].

While in chapters A and B with the tryptophan and solvent relaxation approaches two "bulk" fluorescence techniques are discussed, **Chapter C** is devoted to a single molecule technique, the so-called fluorescence correlation spectroscopy. This technique is part of the new, fascinating "single molecule" field in fluorescence coming in with the recent advances in ultra-sensitive instrumentation. Driven by the necessity to develop new techniques for monitoring protein-induced changes in biomembrane organization, new approaches for the characterization of supported phospholipids bilayers were developed [**C3,C4,C6,C7**]. Moreover, FCS proofed to enable characterizing DNA-lipid [**C5,C8**] and DNA-polycation interactions [**C1,C2,C8**] and can be now considered as a valuable tool in research on Non-Viral Gene Therapy.

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Part C: New Applications of Confocal Fluorescence Correlation Spectroscopy: pages 27-38

The author selected 26 full publications out of 74 scientific contributions (including book chapters, edited books, and a patent, excluding conference abstracts) for this thesis. These 26 publications appeared between 1993 and 2005. 13 out of these 26 publications appeared within the years 2002 till 2005. The average impact factor for these 26 publications is 2.8. The amount of citations for these publications was till 22.6: 165 + (62 auto citations) = 227.

Part A

Solvent Relaxation: A New Method in Biomembrane Research

Abstract

After the understanding of solvent relaxation (SR) in isotropic solvents, scientists started about 10 years ago to characterise SR in supra- and biomolecular assemblies. It was the author's group that established the SR method for probing micro-mobilities and -polarities in biomembranes. This piece of scientific work is documented in 23 publications and four review articles. In 26 out of these 27 contribution M. Hof is the principal investigator. The two main achievments are: 1) First quantitative description of SR in all three membrane domains. 2) A series of applications of the SR technique in biomembrane sciences, including detailed binding studies of several proteins to membranes of different compositions.

1 Introduction

Fluorescence spectroscopy has considerably contributed to the present picture of the structure and function of biomembranes consisting predominately of phospholipids. Among the fluorescence techniques employed like quenching [1], energy transfer [2], lifetime (distributions) [3,4], and excimer formation [5] the determination of fluorescence anisotropy [3] has certainly been the dominating fluorescence method in studies of biological and model membranes. Fluorescence polarization studies, however, exhibit some major limitations: the experimentally determined steady-state and time-resolved anisotropies characterize the motional restrictions of the 'reporter' molecule itself and give therefore only *indirect* information about the dye environment, with the consequence that, if the probe is bound covalently to the lipid (e.g. 1-(4-trimethylamonium-phenyl)-6-phenyl-1,3,5-hexatriene; TMA-DPH), this attachment may dominate the recorded depolarization behavior. The membrane order parameters obtained from freely mobile probes like 1,6-diphenyl-1,3,5-hexatriene (DPH) result from a broad distribution of localization within the hydrophobic interior, the detailed characterization of which reveals inherent ambiguities [6]. Moreover, the anisotropy technique is limited to the characterization of the hydrophobic bilayer interior, since the anisotropy of headgroup labeled phospholipids appears to be rather insensitive to environmental changes [3]. These limitations do not restrict the application of the solvent relaxation method. It has been shown that the solvent relaxation technique in the form developed by M.Hof makes it possible to observe *directly* the viscosity and polarity changes in the vicinity of the probe molecule with well defined location in all three membrane domains.

2 Basic Principles of Solvent Relaxation (SR)

Electronic excitation from the ground state S_0 to a higher electronic excited state (such as S_1) is generally accompanied by a change of the permanent dipole moment, $\Delta\mu_c$, of the molecule ($\Delta\mu_c = \mu(S_1) - \mu(S_0)$). Since the time scale of the molecular electronic transition is much shorter than that of nuclear motion, the excitation-induced ultrafast change of the electron density happens virtually under fixed (original) positions and orientations of the surrounding solvent molecules. With the new dipole moment $\mu(S_1)$, the solute-solvent system is no longer in equilibrium. The solvation shell molecules are, thus, forced to adapt to the new situation: they start to reorient themselves into energetically more favourable positions with respect to the excited dye. This dynamic process, starting from the originally created non-equilibrium Franck-Condon state and leading gradually to a new equilibrium with the solute excited state (R) is called solvent relaxation (SR). This relaxation red-shifts the solute's fluorescence emission spectrum continuously from the emission maximum frequency corresponding to the Franck-Condon state ($\upsilon(0)$ for $t = 0$) down to the emission maximum frequency corresponding to the fully relaxed R-state ($\upsilon(\infty)$ for $t = \infty$). Since a more polar solvent typically leads to a stronger stabilization of the polar R-state, the overall shift $\Delta\upsilon$ ($\Delta\upsilon = \upsilon(0) - \upsilon(\infty)$) increases with increasing solvent polarity for a given change of the solute's dipole moment $\Delta\mu_c$. The detailed mathematical description of this relationship depends on the set of assumptions that each particular dielectric solvation theory formulates [7-14]. The fundamental "dielectric continuum solvation model" [12-14] predicts a linear proportionality between $\Delta\upsilon$ and a dielectric measure of the solvent polarity for a large variety of solvents [15]. According to this model, changes in $\Delta\upsilon$ directly reflect polarity changes in the dye environment - which can be a major desired information thus accessible through solvent relaxation studies.

The second information obtainable from the measurement of time-dependent spectral shifts is based on the fact that the SR kinetics is determined by the mobility of the dye environment. At ambient temperatures, a typical relaxation process C(t) ($C(t) = (\nu(t) - \nu(\infty)) / \Delta\nu$) in an isotropic polar solvent starts with a fast inertial motion on the 0.05 to 0.5 picoseconds (ps) time-range, followed by rotational and translational diffusion occurring on the pico- to sub-nanosecond (ns) time scale [15]. An (integral) average SR time can be defined according to

$$\langle \tau_r \rangle \equiv \int_0^\infty C(t)\,dt$$

which has been determined to about 0.3 ps in pure water [16]. If the dye is located in a viscous medium, like in solvents at deep temperatures [17], or is associated with

supramolecular assemblies like phospholipid membranes [A1,A8], micelles [18], or polymers [Egelhaaf 2000] a substantial part of the SR may be monitored on the nanosecond (ns) time scale. In vitrified solutions the solvation dynamics is much slower than ns fluorescence decay times and thus the fluorescence might origin from the Franck Condon state [A3].

3 Fluorescence Approach to Solvent Relaxation

3.1 Time-Resolved Emission Spectra and Solvent Relaxation Times

Although there have been several attempts to simplify the characterization of the SR process, the determination of the normalized spectral response function C(t) is certainly the most general and most precise way to characterize the time course of the solvent response. The C(t) function is usually determined by 'spectral reconstruction'. The primary data consist of a set of emission decays recorded at a series of wavelengths spanning the steady-state emission spectrum. The time resolved emission spectra (TRES hereafter) are obtained by relative normalization of the fitted decays to the steady state emission spectrum. Log-normal fitting of TRES yields various characteristic parameters, such as full width at half maximum (FWHM) and emission maxima (ν (t)) of TRES [15]. In order to get a complete picture on any SR process, precise knowledge about the emission spectrum of the Franck-Condon state is essential. The group of Maroncelli [15] showed that the frequency of the t = 0 peak emission, ν (t_0), of a chromophore in the system of interest can be calculated quite accurately when the absorption and fluorescence spectra in a non-polar reference solvent, as well as the absorption spectrum in the system of interest are known [15]. The combination of the latter method, which is exclusively based on steady-state data, and wavelength dependent time-resolved fluorescence decays allows the quantitative characterization of SR processes in neat solvents [15] or in biomembranes [A8].

3.2 Red-Edge Excitation Spectroscopy

Laboratories which are not equipped with a time-resolved fluorescence spectrometer, but with steady-state absorption and emission spectrometers, can still obtain qualitative information about viscosity and polarity of the probed environment by exploiting the solvent relaxation process. This application of steady-state fluorimetry is termed 'Red-Edge Excitation Spectroscopy' (REES) and it is limited to those cases where the dipolar relaxation process is comparable or slower than the fluorescence. It uses the fact that the wavelength of the

emission band maximum of polar fluorophores in motionally restricted media, such as in very viscous solutions [19,20], glass-like matrices [A3] or membranes [21], shifts to longer wavelength by shifting the excitation wavelength toward the red-edge of the absorption band [22]. Since the observed shift should be maximal if the solvent relaxation is much slower than the fluorescence, and it should be zero if SR is fast and, thus, independent of the excitation wavelength the entire fluorescence origins from the relaxed R-state, it can serve as an indicator of the mobility of the probe surrounding [19,20]. Usually, red-edge excitation shift values range from several up to 40 nanometers (nm) depending on the chosen solute and solvent system. A reported unique large shift of 76 nm for a hemicyanine dye in 'AOT'-micelles indicates an ionic rather than dielectric relaxation process [A4]. The red-edge excitation shift is especially useful when using dyes, the absorption and fluorescence maxima of which hold linear correlations with the polarity of low-viscosity solvents [A3], because then the probed polarity as well as the hypothetical emission maximum of the fully relaxed R-state can be estimated from the absorption maximum.

4 SR in phospholipid bilayers

4.1 Set of Probes for Probing SR in the External Interface, Headgroup and Backbone Region of the Bilayer: Quantitative Description of SR in Biomembranes

A major requirement for valid application or physical interpretation of SR studies in bilayers is the knowledge about the location of the used chromophore. It has been demonstrated [A1,A2,A5,A6,A8] that $\Delta\nu$ as well as τ_r are strongly dependent on the location of the chromophore within the bilayer. The SR kinetics of dyes like 6,8-difluoro-4-heptadecyl-7-hydroxycoumarin which are probing the external interface of the bilayer occurs on a rather broad time scale. About 50 % of the relaxation probed by 6,8-difluoro-4-heptadecyl-7-hydroxycoumarin in phosphatidylcholine (PC) small unilamellar vesicles at room temperature [A8] is faster than 50 ps. The second part of the solvent relaxation, on the other hand, occurs on the nanosecond time-scale. This finding can be understood when considering the following facts. Firstly, the biologically relevant fluid phase of the bilayer is characterized by the intrinsic presence of dynamic fluctuations. Thus, the appropriate description for the positions of atoms in lipids is that of broad statistical distribution functions and the membrane surface by far not be considered as “flat”. Secondly, the location of the ensemble of chromophores is characterized by a distribution along the z-axis of the membrane. Thus, at a certain time,

some of the chromophores might face an environment characterized by a large amount of "bulk" water molecules and thus monitor ps or even sub-ps relaxation kinetics. On the other hand, a significant part of the chromophores might be located several Å closer to the hydrophobic part of the membrane and thus, as explained in the next paragraph, probe solvent relaxation kinetics on the nanosecond time-scale.

SR in the headgroup region probed for example by 6-propionyl-2-dimethylaminonaphthalene (Prodan), 6-lauroyl-2-dimethylaminonaphthalene (Laurdan), 6-hexadecanoyl-2-(((2-(trimethylammonium)ethyl)methyl)amino)naphthalene chloride (Patman), or the recently designed membrane probe N-palmitoyl-3-aminobenzanthrone (ABA-C15) [A9] in PC-bilayers is purely a nanosecond process [A9,A10,A11]. The observation that an "ultrafast" component is missing indicates that there is no "bulk" water present in the environment of the chromophore of those dyes. The direct influence of the chemical composition of the headgroup on the solvent relaxation characteristics indicates that the chromophores are surrounded by hydrated functional groups of the phospholipid molecules. Changes in the solvent relaxation kinetics within the headgroup region give direct information on mobility changes of the individual hydrated functional groups. It has been for example shown [A1] that Patman is predominately probing the hydrated acyl-groups within the glycerol region, while Prodan gives information on the hydrated phosphate region of phosphatidylcholine bilayers in the liquid-crystalline phase .

SR within the hydrophobic backbone region has been monitored by a set of n-(9-anthroyloxy)stearic acids (n-AS) [A2,A8]. Again, SR is occurring on the ns time scale. Recently, we have gathered experimental and theoretical evidence [Sykora, in preparation], that similar to studies on solvation dynamics of dyes in mixtures of polar and non-polar solvent that "preferential" solvation of dyes and diffusion of water to the excited dye molecule might be a reason for the nanosecond SR responses within the hydrophobic backbone region.

In summary, we observe a slowing down of SR when starting from bulk water (τ_r in sub-ps domain), passing the external interface (τ_r in sub-ns domain) and the headgroup region (τ_r in ns domain), and finally reaching the hydrophobic backbone (τ_r is equal several ns) of the bilayer. A comparison of $\Delta\nu$ values for different membrane labels is only valid if the labels

contain the identical chromophore. Comparison of three "Prodan" like dyes and five n-AS dyes demonstrates a decreasing $\Delta\nu$ with deeper location, are in agreement with the polarity gradient within the bilayer in the liquid-crystalline phase. Changes in $\Delta\nu$ values gives direct information on the degree of hydration in the individual probed regions of the bilayer. An overview on the used dyes and their distribution relative to the molecule of phosphatidyl choline along the Z-axis in phospholipid bilayer is given in figure AI. Some representative correlation function C(t) for C_{17}DiFU, Prodan, Patman, 2-AS, and 9-AS in PC small unilamellar vesicles at ambient temperature are given in Fig. AII.

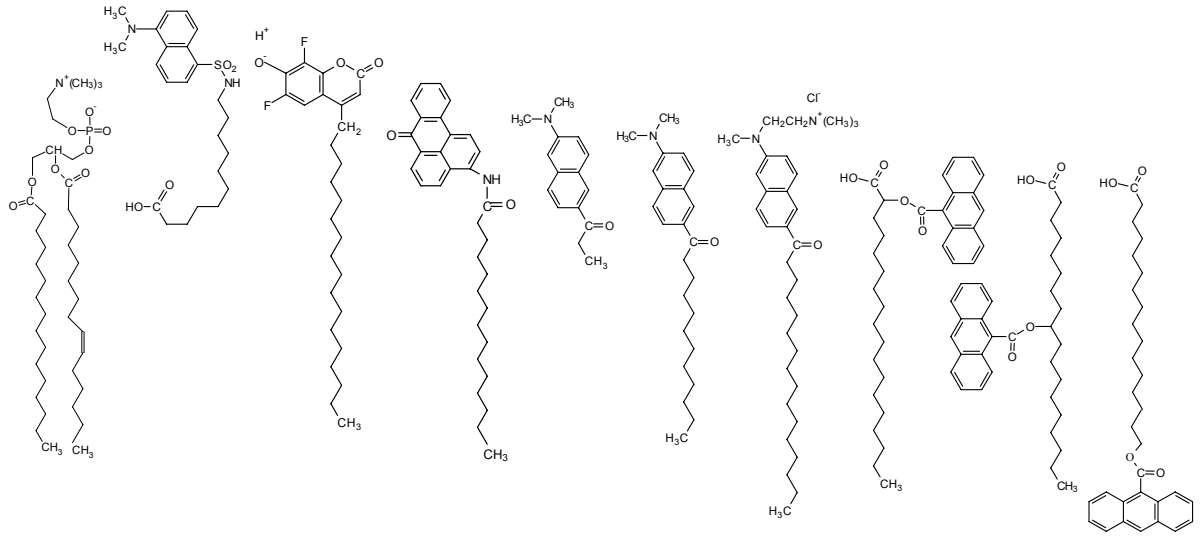

Fig.AI: Distribution of used dyes relative to a phospholipids molecule along the Z-axis in a phospholipid bilayer. From the left: palmitoyl-oleoyl-phosphatidyl choline (POPC), 11-((5-dimethyl-aminonaphthalene-1-sulfonyl)amino)undecanoic acid (Dauda) [A8], 6,8-difluoro-4-heptadecyl-7-hydroxycoumarin [A8], N-palmitoyl-3-aminobenzanthrone (ABA-C15) [A9], 6-propionyl-2-dimethylaminonaphthalene (Prodan) [A1,A5,A6,A7,A8], 6-lauroyl-2-dimethylaminonaphthalene (Laurdan) [A8,A11], 6-hexadecanoyl-2-(((2-(trimethylammonium)ethyl)methyl)amino)naphthalene chloride (Patman) [A1,A5,A6,A7,A8,A10,A11], 2-(9-anthroyloxy)stearic acid (2-AS) [A2,A8], 9-(9-anthroyloxy)stearic acid (9-AS) [A2,A8], (16-(9-anthroyloxy)palmitoic acid (16-AP) [A2].

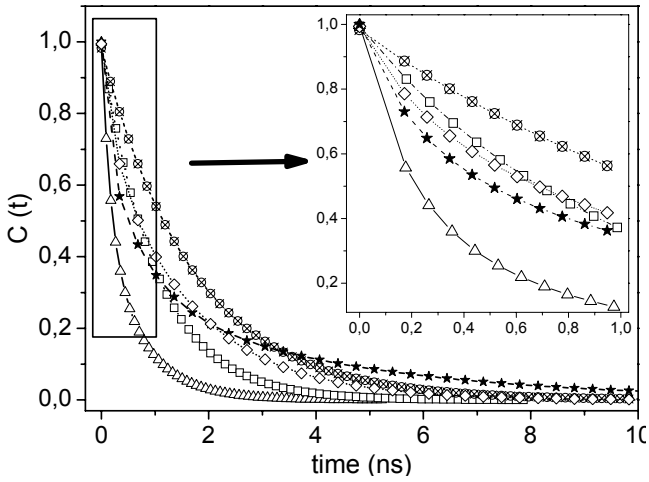

Fig.AII: Correlation functions C(t) for C17DiFU (Δ), Prodan (□), Patman (⊗), 2-AS (⬨), and 9-AS (*) in phosphatidyl choline (PC) small unilamellar vesicles at ambient temperature. Shown are the (multi)exponential fits to the experimental data, using $\nu(t_0)$ obtained by the time-zero spectrum estimation [A8].

4.2 Structural Changes in Biomembranes Monitored by the Solvent Relaxation Method (Applications)

The quantitative characterization of the SR process in different, defined depth within the membrane bilayer are the basis for the application of the SR technique in biomembrane sciences. The defined dye location provides the possibility to investigate selectively the influence of a physiologically relevant parameter on the organization of a certain membrane domain. Within the last the years the method has been applied to detect micro-"fluidity" (τ_r) and -polarity ($\Delta\nu$) changes in the bilayer due to temperature [Hutterer 1994, A1,A2,A7,A11], lipid phase transition [Hutterer 2002, A7,A9], ethanol addition [Hutterer 2002], membrane curvature [A11], and lipid composition variations [A2,A5,A6,A7, Hutterer 2002] as well as due to the binding of calcium ions [A2,A5,A6], membrane-active peptides [A10], and blood coagulation proteins [A6]. Since a detailed compilation of these results would go beyond the possibilities of this summary, the reader is referred to the individual articles. However, the validity of the SR technique will be briefly illustrated by one example, the membrane binding study of the blood coagulation protein prothrombin [A6].

This protein-membrane interaction was already investigated by two different standard fluorescence membrane techniques [23,24]. As a matter of fact both techniques, the characterisation of pyrene excimer formation [23] as well as the determination of fluorescence anisotropy of the embedded membrane DPH [24] failed in detecting the binding

of prothrombin. Within the 90th it was one of the major aims of the author of this thesis to understand the mechanism between several blood coagulation proteins and membrane surfaces. Thus, the failure of this standard fluorescence techniques gave the original motivation to develop with the solvent relaxation technique a new fluorescence technique which is in particular sensitive to structural changes within the headgroup region of biomembranes. In the publication [A6] the protein (prothrombin) induced changes in the SR kinetics monitored by Patman and Prodan were compared with changes in the DPH steady state anisotropy for the binding of prothrombin to PS-containing membranes. The SR times $\langle\tau_r\rangle$ in SUV composed of PC/PS 80:20 increase by 100% and 30% detected by Prodan and Patman, respectively, at protein concentrations leading to > 90% membrane surface coverage. The observed increase in membrane order at similar conditions reported by DPH, on the other hand, is relatively small (< 5%). It was concluded , that the binding of this peripheral protein rigidifies considerably the phospholipid headgroup region, but leads only to very small increase in the packing density of the hydrocarbon region of the bilayer. The observed deceleration of the solvent relaxation process is larger for Prodan which is bound near the surface of the membrane, than for Patman which is located closer to the hydrocarbon region. Apparently, the binding of the proteins predominantly affects the outermost region of the membrane, where the amino and the carboxyl group of the serine headgroup are supposed to be located. These observations [A6] exclude hydrophobic protein-membrane interaction and favour the formation of a coordination complex by the PS headgroup, calcium ions and the Gla residues of the proteins [25]. In further experiments [A6] the high sensitivity of the SR approach for comparative binding studies of the factors II with its N-terminal protein fragments was used for answering to the question if and by which mechanism binding sites outside the N-terminal end contribute to the binding of the entire proteins [24,26,27].

Conclusions

The SR technique can be now considered as an established technique for the characterization of the micro-polarity and -viscosity within different domains of phospholipids bilayers. The results achieved by the group of M. Hof stimulated the application of this new approach in biological membrane studies as well as an adoption of the concept of using chromophores which are defined located within the bioassembly for SR studies in proteins [28] and DNA [29].

Acknowledgments

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Publications in Journals from M. Hof used for this thesis:

Bold number indicates that all the solvent relaxation experiments in these publications were performed either by Dr. R. Hutterer, Dr. A. Haefner, and Mgr. J. Sykora in the framework of their PhD studies supervised by M. Hof or by M. Hof himself.

A1) R. Hutterer, F. W. Schneider, H. Sprinz, & M. Hof
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Impact: 1.7; Cited: 13 + (8); auto citations in parenthesis
A2) R. Hutterer, F. W. Schneider, H. Lanig, & M. Hof
‘Solvent Relaxation Behaviour of n-Anthroyloxy Fatty Acids in PC-Vesicles and Paraffin Oil: A TRES Study’ (1997), *Biochim. Biophys. Acta 1323*, 195-207.
Impact: 2.7; Cited: 7 + (11)
A3) M. Hof & P. Lianos
‘Structural Studies of Thin AOT Films by Using the Polarity Fluorescent Probe Coumarin-153’ (1997a), *Langmuir 13,* 290-94.
Impact: 3.1; Cited: 4 + (1)
A4) M. Hof, P. Lianos, & A. Laschewsky
‘An Amphiphilic Hemicyanine Dye Employed as an Extremely Sensitive Probe of Water in Reverse AOT Micelles’ (1997b), *Langmuir 13,* 2181-83.
Impact: 3.1; Cited: 13 + (0)
A5) R. Hutterer, A. Parusel, & M. Hof*****
‘Solvent Relaxation of Prodan and Patman: A Useful Tool for the Determination of Polarity and Rigidity Changes in Membranes’ (1998a) *J. Fluor. 8*, 389-393.
Impact: 1.0; Cited: 9 + (2)
A6) R. Hutterer, F.W. Schneider, W.T. Hermens, R. Wagenvoord, & M. Hof
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Impact: 2.7; Cited: 3 + (7)
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Impact: 3.1 Cited: 0 + (1)
A10) Sheynis T., Sýkora Jan, Benda Aleš, Kolusheva S., Hof Martin, Jelinek R.: Bilayer Localization of Membrane-Active Peptides Studied in Biomimetic Vesicles by Visible and Fluorescence Spectroscopies. *Eur. J. Biochem*. 270(22), 4478-4487 (2003).
Impact: 3.0; Cited: 1 + (1)

A11) J. Sýkora, Jurkiewicz P., Langner M., Kraayenhof R., Epand R & M. Hof
Does curvature influence the water structure in the headgroup region of phosholipid bilayers? (2005), *Chem. Phys. Lipids,*135(2), 212-221.
Impact: 1.7; Cited: 0

Average Impact Factor: 2.4; Citations total: 63 + (36 auto citations)

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(Bold number indicates that M. Hof is the principal investigator of the listed publication)

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A23) H.-J. Egelhaaf, B. Lehr, M. Hof, A. Häfner, H. Fritz, F.W. Schneider, E. Bayer, & D. Oelkrug ‘Solvation and Solvent Relaxation in Swellable Copolymers as studied by Time-resolved Fluorescence Spectroscopy’ (2000) , *J. Fluor.* 10(4), 383-392.

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Part B

Structure Function Relationship of Proteins involved in Blood Coagulation investigated by Intrinsic Protein Fluorescence

Abstract

The observed lipid-specificity in the prothrombinase reaction, which means that different phospholipids catalyse the prothrombin activation with very different efficiencies defines the motivation for seven here listed publications. This contributions describe the spectroscopic characterisation of the specific interactions of the substrate prothrombin and the enzyme Xa with calcium ions and with membrane surface of different lipid composition. The application of wavelength dependent picosecond tryptophan fluorescence spectroscopy led to two main findings:
1) Molecular picture of the calcium induced conformational change in the N-termini of these proteins.
2) Clear evidence for a lipid-induced conformational change in the N-termini of prothrombin. In further two publications tryptophan fluorescence was used to learn about the structure-function relationship of cytoskeletal proteins involved in platelet aggregation as well as of human blood serum orosomucoid. In 7 out of these 9 contribunions M. Hof is the principal investigator.

1 Introduction

When damage to a blood vessel occurs, the aggregation of platelets initiates the blood clotting cascade, which consists of a sequence of proteolytic enzyme activations. The protein fluorescence work presented herein is mainly focused on the last step in the blood coagulation cascade, that is, the binding of activated factors Xa and Va to membranes, assembly of these factors into the so-called prothrombinase complex, and activation of prothrombin by this complex. Mainly tryptophan fluorescence spectroscopy has been used to characterise the molecular mechanisms of the interaction of the substrate prothrombin and the enzyme Xa with calcium ions [B2,B5] and membranes [B4,B7]. Moreover, tryptophan fluorescence spectroscopy has been used to learn about the function of the vasodilator-stimulated phosphoprotein (VASP) and the mammalian drosophila enabled protein (Mena), which are cytoskeletal proteins of human platelets [B6]. VASP and Mena are important components of signal transduction pathways that regulate adhesion and aggregation of platelets.

2 Picosecond Tryptophan Fluorescence of Membrane Bound Prothrombin

The main advantages of exploiting the intrinsic tryptophan (Trp) fluorescence in protein studies are the high sensitivity of the Trp fluorescence to changes in its microenvironment and the fact that the protein has not to be modified and, thus, the spectroscopic measurements can be performed using the native protein. Since the photophysics of even a single Trp residue in proteins appears complex in most cases, the applicability of tryptophan studies is limited to proteins with a low number of Trp residues. Since prothrombin contains a rather high number of Trp residues (12 and 14 for human and bovine prothrombin, respectively [1]), tryptophan studies have been limited to its fragment 1 (F1) which contains only three Trp residues. The 1-156 N-terminal polypeptide F1 is believed to be the region predominantly responsible for the metal ion and membrane binding properties of prothrombin. Besides small but significant differences in the desorption rate, it displays basically very similar membrane binding characteristics as the entire protein [B1]. The structure of F1 is commonly divided into the N-terminal "Gla domain", characterized by 10 γ-carboxyglutamic acid residues (Gla) and a region of disulfide linkages known as the "kringle region". Calcium ions bind (almost) exclusively to the Gla domain [2] and form the native conformation required for membrane binding [3]. Figure BI depicts a sketch of the X-ray structure of Ca-Bovine (B)F1 and shows the location of its three tryptophan residues and the seven Calcium ions bound to the Gla domain. Investigating possible molecular differences in the binding of the calcium-prothrombin complex to differently composed, negatively charged membrane surfaces, one might speculate whether such differences can be found in the conformation of membrane-bound Gla domains.

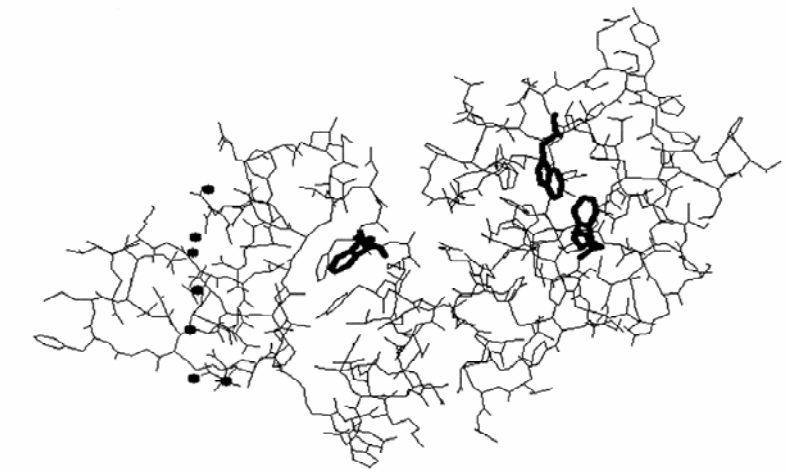

Fig. BI. A depiction of the X-ray structure of Ca-BF1. The right part of the protein is the kringle-domain, where Trp90 and Trp126 are located. The Gla-domain is the left part of the protein, containing Trp42 and seven calcium ions (dots). The coordinates where taken from the Brookhaven Protein Data Bank entries [4].

Using picosecond fluorescence time-correlated single photon counting, changes in the microenvironment of the three individual tryptophans can be separated from each other without either cleaving BF1 into the isolated Gla (containing Trp42) and kringle domains (containing Trp90 and Trp126) or modifying the protein by site-directed mutagenesis. The sensitivity of this approach to conformational changes has been demonstrated by a time-resolved study of the calcium induced conformational change in BF1 [B2]. This work [B2] comprises a detailed analysis of the wavelength dependent fluorescence decays of apo-BF1 as well as of Ca-BF1. Fluorescence lifetime distribution [B3] and conventional multiexponential analysis, as well as acrylamide quenching studies led to the identification of six distinguishable tryptophan excited states for the apo- as well as for the Ca-form of BF1. Accessibility to the quencher and the known structure have been used to associate the fluorescence decay of the tryptophan present in the Gla domain (Trp42) with two red shifted components (2.3 and 4.9 ns for apo-BF1). The two kringle domain tryptophans (Trp90 and Trp126) exhibit three decay times (0.24, 0.68 and 2.3 ns for apo-BF1) which are blue shifted. The 0.06 ns component remained unassigned due to the limited time-resolution of the experiment.

Addition of calcium ions did not change fluorescence lifetimes and intensities of those components which have been assigned exclusively to the two kringle tryptophans Trp90 and Trp126 (0.24 ns and 0.68 ns) – a result that argues against a calcium binding site in the kringle domain [2]. On the other hand, it was found that the overall fluorescence quenching is due to a "static-like" quenching of the Gla-Trp (Trp42) components D and E (2.3 ns and 5.1 ns for Ca-BF1, respectively) as a consequence of a ground state interaction between Trp42 and the Cys18-Cys23 disulfide bridge. For illustration see figure BII, comparing the decay associated spectra (DAS) of the two Gla-components in absence and presence of Calcium. It is important to note that an observed decrease in the fluorescence intensity in the “5 ns component” of 85% is by far larger than the calcium-induced changes in the parameters determined by circular dichroism [5], by antibody binding experiments [6], differential scanning calorimetric studies [7,8] and fourier transform infrared spectroscopy [9]

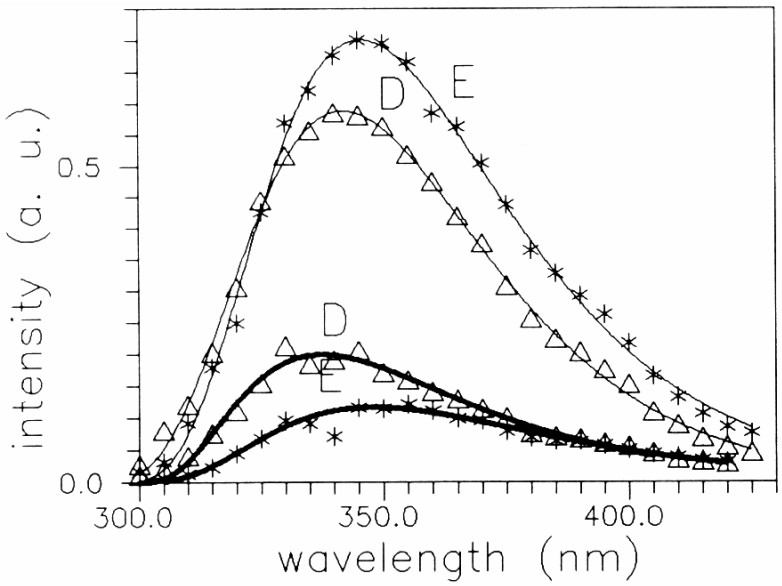

Fig. BII. Decay-associated spectra of components D (Δ) and E (*) for apo-BF1 (2.3±0.2 ns (D) and 4.9±0.3 ns (E)) and for Ca-BF1 (2.3±0.3 ns (D) and 5.1±0.4 ns (E)). Shown are the experimental points and the log-normal fits to the data. Thick lines represent the decay-associated spectra of Ca-BF1. The emission maxima are 341.9 nm (D) and 345.5 nm (E) for apo-BF1 and 337.4 nm (D) and 347.6 nm (E) for Ca-BF1.

The high sensitivity of the time-resolved Trp42 fluorescence to conformational changes in the Gla domain was one motivation to examine the hypothesis of possible lipid-induced conformational changes in the Gla-domain of prothrombin by picosecond tryptophan fluorescence spectroscopy of BF1 [B4]. Therefore, the wavelength-dependent tryptophan fluorescence decays of Ca-BF1 in presence of pure phosphatidylcholine (PC) small unilamellar vesicles (SUV) and PC-SUV containing either 25% phosphatidylserine (PS) or 40% phosphatidylglycerol (PG) were characterized. Based on the determined apparent membrane dissociation constant K_d (The K_d -values are 0.9±0.1 μM and 0.8±0.1 μM for 25 mol % PS, and 40 mol % PG, respectively), Ca-BF1 (4 μM) should have been > 90% bound to the membrane surface at a lipid concentration of 1.3 mM in both investigated lipid systems. In both cases the lifetime analysis identified the existence of five wavelength independent lifetimes. Specific binding to PS-containing membranes did neither change the fluorescence lifetimes nor the corresponding wavelength dependent amplitudes. Apparently, the membrane binding part of calcium-prothrombin remains in its native conformation when bound to the highly procoagulant PS-containing membrane surface. In contrary to the PS-results, the tryptophan studies of the PG-bound BF1 yielded an interesting new result, i.e. a lipid induced conformational change in the Gla-domain, observed by a significant prolongation of the lifetime of component E. At protein/lipid concentration ratios ensuring that the majority of the

protein is bound to the 40% PG / 60% PC surface, the lifetime of component E shifts from 5.1 to 7.5 ns, when compared with Ca-BF1 in solution. The prolongation of the Trp42 fluorescence lifetime can be observed as well by an apparent shift of the component D from 2.2 ns to 2.8 ns. On the other hand, as in the case of the binding to PS containing surfaces, the kringle components B and C remain unchanged. Since component D is due to the fluorescence of Gla and kringle tryptophans, the constant kringle tryptophan fluorescence portion in D might mask the entire magnitude of the lifetime shift in the Gla-portion of component D.

For the N-terminal membrane binding part the comparison of the PS- with the PG-results leads to the conclusion that Ca-BF1 exhibits already the "perfect" conformation for binding and proteolysis and, thus, retains its conformation when bound to PS surfaces. The PG-induced conformational disruption of the Gla domain possibly might affect the protein conformation in the non-fragment 1 part of the protein and/or the lateral diffusion on the membrane surface. Both scenarios could yield a possible explanation for the lower procoagulant activity of PG when compared to PS.

3 Picosecond Tryptophan Fluorescence of Membrane Bound Factor Xa

The described investigations were extended to the enzyme Xa, by using its 86 aminoacids containing N-terminus (FX-GE). It consists of two parts, a Gla-domain and in contrast to the kringle of BF1, a EGF-(Epidermal Growth Factor)-like part [10,11]. FX-GE binds calcium ions in a similar way as the entire Xa [11] and reveals only one Trp (Trp41), which is localised in the Gla-domain. In comparison to the 3 Trp-BF1, the characterisation of a calcium-induced Trp-disulphide adduct formation could be characterized more precisely [B5]. Similar to BF1 the addition of calcium ions led to a conformational change in the Gla domain resulting in a “static-like” quenching of the fluorescence of the Trp41. A comparison of the fluorescence lifetimes and the calcium-induced quenching pattern between BF1 and FX-GE give strong evidence for a structural and functional homology between the Gla-domains in prothrombin and factor Xa. The next step was to investigate whether the Gla domain of FX-GE shows a similar membrane binding behavior as BF1 and whether the fluorescence characteristics are altered again in a lipid specific manner. Therefore fluorescence decays of FX F1-86 bound to large unilamellar vesicles (LUV) of different phospholipid compositions

were characterized [B7]. In contrast to BF1 it was found that neither PG- nor PS-binding is influencing the microenvironent of the Gla tryptophan residue (Trp41) of FX F1-86. Considering the high sensitivity of the fluorescence of the Gla tryptophan residue to changes in the microenvironment as a consequence of a calcium-induced conformational change [Hof 1996a, Haefner 2000] and the fact that we did find a significant PG-induced change in the fluorescence of the Gla tryptophan of prothrombin [B4], we gathered reasons to state that PG alters the conformation of the Gla domain in prothrombin, but possibly not in factor Xa. It should be point out that the structures of the Gla domains of BF1 and FX F1-86 differ regarding the influence of calcium binding on them, which might be the reason for the observed molecular differences [B7]. Concerning the catalytic activity of the prothrombinase, this results suggest that the Gla domains of factor Xa and prothrombin might play a role in regulating the lipid specifity observed in the prothrombinase reaction.

4 Tryptophan Fluorescence Spectroscopy of the N-terminal EVH1 Domain of Vasodilator-stimulated Phosphoprotein (VASP) and the Mammalian Drosophila Enabled Protein (Mena)

Drosophila enabled/vasodilator-stimulated phosphoprotein homology I (EVH1) domains are 115 residue protein-protein interaction modules which provide essential links for their host proteins to various signal transduction pathways. Many EVH1-containing proteins are associated closely with actin-based structures and are involved in re-organization of the actin cytoskeleton and associated processes such as cell-cell adhesion or platelet function. It could be shown by steady-state tryptophan fluorescence spectroscopy that EVH1 domains recognize and bind specific proline-rich sequences [B6]. The binding is of low affinity, but tightly regulated by the high specificity encoded into residues in the protein:peptide interface. Moreover, it was shown that a small 'core' proline-rich sequences in the target protein binds a 'recognition pocket' on the domain surface. Further affinity- and specificity-increasing interactions are then formed between additional domain epitopes and peptide 'core-flanking' residues [B6].

Conclusions

By using multiwavelength picosecond tryptophan spectroscopy in combination with modern data analysis methods, it was possible to investigate conformational changes in two protein domains simultaneously and separately. In the case of blood coagulation proteins conformational changes in the Gla-domain can be characterized by tryptophan fluorescence with a very high sensitivity. The results on prothrombin and factor Xa indicate that the Gla domain plays a role in the lipid specificity of the prothrombinase reaction.

Acknowledgments

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Publications in Journals from M. Hof used for this thesis:
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Impact: 6.5; Cited: 12 + (3)

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Impact: 2.7; Cited: 3 +(2)
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'Dual Epitope Recognition by the VASP EVH1 Domain Modulates Polyproline Ligand Specificity and Binding Affinity' (2000), *EMBO J. 19*, 4903-4914.
Impact: 10.5; Cited: 37 + (0)
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Impact: 1.0; Cited: 0 +(0)

Average Impact Factor: 4.1; Citations total: 68 + (11)

Contributions on this topic by M. Hof not used in this thesis:
(Bold number indicates that M. Hof is the principal investigator of the listed publication)

Reviews in books:

B8) R. Hutterer & M. Hof (invited review)
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Part C

New Applications of Confocal Fluorescence Correlation Spectroscopy

Abstract

After the introduction of the first commercial Fluorescence Correlation Microscope (Carl Zeiss Confocor 1) in 1996, a fast growing community of scientists tried to a) demonstrate applications of this "new" single molecule technique and b) to further improve FCS technique in terms of precision, validity, and applicability. The authors group contributed significantly to both aims by presenting pioneering contributions in the fields of a) DNA condensation, b) production and characterization of solid supported phospholipids bilayers, as well as c) time-resolved fluorescence correlation spectroscopy. The scientific contribution is documented in 20 publications and one patent. All FCS experiments described in these publications were performed in the laboratory under the supervision of M. Hof.

1 Introduction

Fluorescence correlation spectroscopy is a relatively old technique, originally introduced by Elson, Magde and Webb in the early seventies [1-3]. However, it took more than two decades until the development of new lasers with high beam quality and temporal stability, low-noise single-photon detectors, and high-quality microscope objectives with nearly perfect imaging quality at high numerical aperture that the technique became a fluorescence techniques valuable for a larger scientific community. The group of M. Hof acquired a FCS microscope in December 1999 and succeeded to present a) the first application of FCS in DNA condensation [C1,C2, Kral 2002c], b) the first confocal FCS measurement on supported phospholipids bilayers [C3], c) a protocol for precisely measuring 2-dimensional diffusion [C4], and d) the first experimental realisation of time-resolved FCS [C7].

2 Principles of Fluorescence Correlation Spectroscopy (FCS)

FCS is based on a statistical analysis of temporal behavior of detected spontaneous fluorescence intensity fluctuations. In other words, by means of normalized autocorrelation function $G(\tau)$ it studies the relation between the detected intensity in certain time t and time τ later.

$$G(\tau) = \frac{\langle I(t) * I(t+\tau) \rangle}{\langle I(t) \rangle^2}$$

In order to be able to subtract any information from the fluctuations, the magnitude of the fluctuations compared to the averaged intensity must be as high as possible. In reality it means to detect signal from single molecules, as only than any change of state of the measured molecule highly influences the overall fluorescence intensity. The first reasonable realization of favorable signal-to-noise setup came only in 1993 [4]. It makes use of stable lasers, aberrations free epifluorescence confocal microscopes with high numerical aperture, interference filters, sensitive single-photon detectors and fast hardware correlators. This setup is still one of the most widely used. Further technical development later brought in a pulsed two photon excitation, pulsed diode lasers, a combination of several lasers, polarizators and detectors in one setup, and fast computers enabling online software correlation with saving all information about every detected photon, thus enabling later more elaborate analysis.

The highly focused laser beam creates diffraction limited Gaussian-Lorentzian intensity profile with minimum lateral radii of order 200 – 300 nm. The detection in axial dimension is limited by pinhole in image plane of microscope to value between 2 and 4 μm. The size of the detection volume is than 0.3 to 1 fl. If such a volume is combined with nM concentration of fluorescent molecules, one comes to a single-molecule level. There are different sources of fluctuations in the studied system. The decrease of autocorrelation towards shorter times on the nanosecond time scale (antibunching) is caused by finite fluorescence lifetime, but is not observable with an usual 50 - 200 ns time resolution. On a microsecond time scale, the shape of $G(\tau)$ is governed by the triplet state, possible photo-isomerization and blinking dynamics, or a chemical equilibrium among different fluorescent species. Finally, and actually most important for the applications of FCS, the longest observable auto-correlation decay is caused by translational diffusion, flow or photobleaching.

To obtain real physical parameters from the autocorrelation function, one needs to apply a proper physical model. At this step first approximations take place. The detection volume is for bulk measurements approximated as a 3D Gaussian. Assuming small point-like non-interacting molecules freely diffusing in a space much larger than the detection volume, showing up only triplet state dynamics, the *G(τ)* takes form,

$$G(\tau) = 1 + \left(1 - T + Te^{-\tau/\tau_{tr}}\right)\left(\frac{1}{PN[1-T]}\right)\cdot\frac{1}{1+(\tau/\tau_D)}\left(\frac{1}{1+(\tau/\tau_D)(\omega_0/\omega_Z)^2}\right)^{1/2}$$

where T is a triplet fraction, τ_{tr} is a triplet decay time, PN is the apparent particle number, τ_D is a diffusion time and ω_0 and ω_Z are lateral and axial radii of detection volume, respectively. The derivation of equations for the applied models makes use of the natural laws applied in classical methods of perturbation kinetics as the only difference is in the source of fluctuations.

The parameters PN and τ_D are related with macroscopic values of concentration c and diffusion coefficient D via:

$$\tau_D = \frac{w_0^2}{4D} \quad \text{and} \quad PN = \pi c w_0^2$$

The diffusion coefficient for spherically symmetric molecules is related to hydrodynamic radius r_h via Einstein-Stokes equation:

$$D = \frac{k_B T}{6 \pi \eta r_h}$$

where k_B is Boltzmann constant, T is thermodynamic temperature, η is dynamic viscosity and r_h is hydrodynamic radius.

3 Characterisation of Supported Phospholipid Bilayers by FCS

Biological membranes constitute one of the most essential functions for life. Cellular membranes are protein-loaded phospholipid bilayers, separating two electrolyte solutions. The phospholipid molecules in these membranes contain two highly hydrophobic acyl-chains and relatively hydrophilic headgroups. These specific features cause self-assembly of these molecules into so-called bilayers, consisting of two adjacent sheets of molecules with the headgroups exposed to water, and the chain regions buried into the hydrophobic membrane interior. Several model systems, mimicking cellular membranes, have been produced. An important step forward was the production of so-called supported phospholipid bilayers (SPBs) by exposure of a solid support to phospholipid vesicle suspensions [5]. Vesicles adsorbing onto such supports often fuse spontaneously into bilayers, and this procedure is much easier than the earlier-used laborious stacking technique. SPBs have now been applied in biosensors, micro- and nano-structures, blood-compatible surfaces, medical implant

devices, and production of catalytic interfaces [6]. Although many additional applications were proposed, the study and application of these systems has long been hampered by the lack of techniques suitable for membrane research in electrolyte solutions.

In the last two decades this situation has changed with the development of sophisticated techniques, capable of measuring physicochemical surface parameters of membranes at molecular resolution in buffers. These techniques include ellipsometry [C6], quartz crystal microbalance (QCM), impedance spectroscopy, atomic force microscopy (AFM), and surface plasmon resonance (SPR). These techniques, however, do not give information of the diffusion properties (D) of single lipid molecules within the bilayers. Since it is obvious that confocal FCS would be the method of choice for diffusion properties of single lipid molecules within model membranes, a series of experiments on black lipid membranes was published immediately after the introduction of the FCS method [7-9]. However, following these first FCS experiments a different technique, fluorescence photobleaching recovery was used preferentially to determine the diffusion of various membrane components both in artificial and in biological membranes. In 1999 a confocal FCS characterisation of lipid bilayers was performed on giant unilamellar vesicles and cell membranes [10,11]. However, the relative error in the determined diffusion coefficients in these publication was up to 100 %, demonstrating the need for the elaboration of a more systematic confocal FCS approach.

This facts gave the motivation a) to perform the first confocal FCS studies on SPB's [C3], and b) to elobarate a protocol for the precise determination of diffusion coefficients within bilayers [C4], and c) to combine these FCS experiments with complementary techniques, like ellipsometry [C3,C6]. The key step for the first valid (and thus publishable) application of confocal FCS for the characterization of SPB's was a systematic variation of solid supports as well as labeled phospholipids. It was shown that FCS experiments on Bodipy-labelled lipids in SPB's adsorbed on muscovite (Mica) yielded diffusion coefficients and surface concentrations with an experimental error of about 20 %. The main reason for the rather low precision was the way how the sample plane was positioned relative to the laser focus. In the above cited contributions ([10,11,C3]) the vertical (z-) position of the phospholipid membrane was localized by focusing to the point of maximum fluorescence intensity when scanning the z-axis in 1 μm steps and the illuminated surface area was determined by an external measurement of a standard system with a known diffusion coefficient. In [C4] a

modified method for the determination of diffusion coefficients and phospholipid surface densities of planar phospholipid systems was presented. The so-called “Z-scan” involves the determination of diffusion times and particle numbers in 0.1-μm steps along the z-axis. From the dependence of those parameters on the position of the focus, diffusion coefficient and surface density are directly determined without the need of an external calibration measurement. The comparison of results on mica- and borosilicate glass-supported SPB’s obtained by this approach with those obtained by the traditional method applied in these above cited contributions [10,11,C3] not only demonstrated significant higher precision of the “Z-scan” (standard deviation in D < 10 %), but also explains the origin of apparent anomalous slow diffusion patterns contributions [10,11,C3]. This approach was applied for characterizing the effect of cholesterol content on the diffusion coefficients in SPB's composed of dioleoyl-phosphatidylcholine (DOPC). Moreover, the so far first determinations of diffusion coefficients in phospholipid monolayers at oil/water interface were presented. In addition to these FCS experiments, ellipsometry was applied in order to elucidate the role of the support on mechanism of formation of SPB’s [C6]. It was shown that either vesicle-vesicle or vesicle-surface interactions are dominating the mechanisms the bilayer formation. By what mechanism the SPB’s are formed depends on the chemical composition of the support, presence of ions, as well as lipid composition [C6].

4 DNA condensation characterized by FCS

The condensation of DNA involves a dramatic decrease in the volume occupied by a DNA molecule and is of immense biological importance. Recently, such structural, physico–chemical and energetic aspects of the condensation continue to receive much attention and have lead to develop non-viral gene therapy (NVGT) delivery vectors [12]. NVGT protocols require reproducible and efficient delivery systems especially for the introduction of plasmid DNA into the cancer cells [13]. The NVGT vectors normally carry positive charges which interact with DNA phosphate, leading to DNA condensation into nanoscale complexes. This DNA condensation gives protection from nuclease and right size to enter cells [14].

Since FCS is a method which is sensitive to volume changes of macromolecules, it has been the aim of several research groups to establish this single molecule technique for direct monitoring of the condensation process and characterisation of the resulting condensing

agent-DNA nanoparticle. In 2002 it has been shown for the first time that FCS is indeed able to visualize DNA condensation induced by the natural DNA condensing agent spermine as well as by the positively charged amphiphilic compound HTAB [C1,C2, Kral 2002c]. Plasmides of different sizes have been stained by the DNA intercalating agent Propidium Iodide (PrIo) and Ethidium bromide (EtBr). Since the condensation processes were characterised by apparent increases in the diffusion coefficients up to a factor 10, this single molecule technique proofed to be a very sensitive tool for studying DNA conformational changes resulting from polycations-mediated condensation. However, it was found that both stains have large effects on the diffusion coefficients of DNA as well as on the critical condensing agent concentration. Additionally the condensation process leads to PrIo and EtBr release from DNA and this to a significant reduction in fluorescence, making the interpretation of the FCS analysis more complicated [C1,C2].

In recent contributions [C8, Kral 2005b] we have shown that increasing amount of labelling by PicoGreen® (PG; [2-[N-bis-(3-dimethylaminopropyl)-amino]-4-[2,3-dihydro-3-methyl-(benzo-1,3-thaizol-2-yl)-methylidene]-1-phenyl-quinolinium]) do not influence the determined diffusion coefficients. Due to the high quantum yield and absorption coefficient of this dye, 10 times lower dye loading is need for FCS studies when compared to PrIo and EtBr. Moreover, count rate is much less sensitive to the condensation process, indicating that dye release is -if at all- only to a minor extent interfering with the condensation process. The latter advantage of PG staining led to a better understanding of the PN/condensing agent titration curves. In case of the uncondensed DNA the concentration calculated form the apparent PN is up to 20-fold larger as the expected one. When the condensation process is completed, the PN reaches for some condensers its expected value. In the uncondensed form the size of the DNA molecule is considerably bigger than the confocal element and segmental motions of the multiple labeled DNA molecule as a possible additional cause for fluorescence fluctuations have to be considered [C8, Kral 2005b]. On the other hand, the PN, which is the most accurate read-out parameter of a FCS experiment, gives quantitative information on the packing density of DNA-condensing agent aggregates. Thus, direct information on the quality of condensing molecules can be derived by determining PN [Hof 2005, Adjimatera 2005]. Figure CI is illustrating the effect of particle size on the number of detected fluorescence fluctuations.

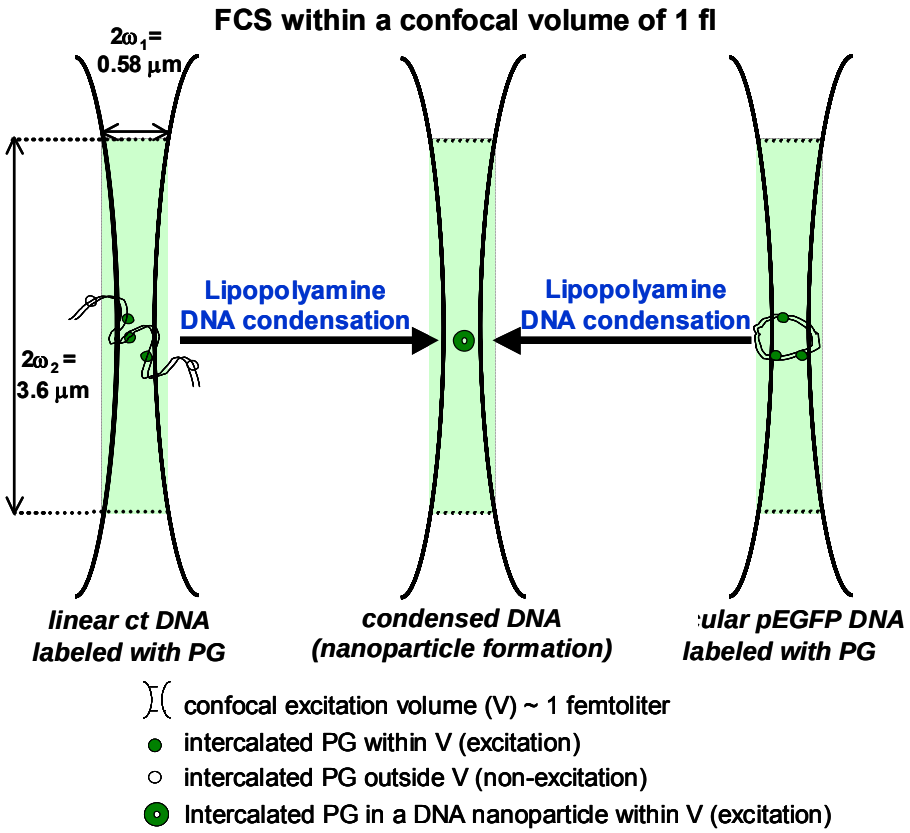

Fig. CI: Illustration of DNA molecules in the FCS confocal excitation volume (V); V is an approximate cylinder of 1 fl.

Left: a free (no condensing agent (lipopolyamine)) linear DNA molecule (ribbon) labeled by intercalated PicoGreen (PG; both open- and filled-circles), but only a fraction of fluorophores (filled-circles) lie within the confocal volume, and are therefore excited.

Right: a free circular molecule labeled by intercalated PG which are excited (filled-circles).

Center: a lipopolyamine-condensed DNA where all the PG reporter molecules now lie within the confocal volume, and all are therefore excited; this nanometer-sized complex acts as a point-like molecule (a single nanoparticle).

In recent applications this methodology has been used to study the interactions of different sized DNA molecules (4.7, 10 and 13 kilobase pairs) with liposomes formulated from cationic lipids [Kral 2005b] as well as with two newly synthesized lipopolyamines were studied [Adjimatera, 2005]. In particular the FCS data shows that N^4,N^9-Dioleoylspermine efficiently condenses DNA molecules by forming DNA-lipololyamine nanoparticles [Adjimatera 2005]. Moreover, the group of M. Hof presented the first FCS study on the association of oligonucleotides with liposomes [C5].

5 Time-Resolved FCS

As demonstrated in the previous chapters FCS allows the straight forward determination of diffusion coefficients of a single population of fluorescent molecules. If the particle motion is governed by two particle species of different mobility the precise determination of the individual diffusion coefficients from a single experiments measurements becomes difficult, in many cases even impossible. One way to overcome this limitation is the so-called dual (or multi) colour fluorescence cross correlation spectroscopy [15]. In dual color (or two channel) FCS the two species are labeled by two dyes with well separated emission spectra (e.g., green and red), which requires two different laser beams for excitation. These laser beams have to be focused on the same spot and the fluorescence arising from the two dyes have to be detected by two different detection devices. Such an experimental setup allows in a single experiment the simultaneous determination of both diffusion coefficients. Moreover, the cross correlation (also called coincidence) analysis enables the direct monitoring of the aggregation (reaction) product of both (differently labeled) species. The technical challenge of multi-channel excitation/detection is to assure perfect overlap of the detection regions at different wavelengths, which is not a trivial task due to chromatic aberrations of most optical components. An alternative is to use the fluorescence lifetime instead of the fluorescence spectrum to distinguish between different molecules. This so-called time-resolved FCS has been suggested in 2002 by the group of J. Enderlein [16] and first time experimentally realised in the laboratory of M. Hof [C7]. By applying a sophisticated analysis that combines autocorrelation with fluorescence-lifetime measurements, a quasi-two channel FCS without the need of multiple excitation and/or detection channels can be realized [C7]. Similar to two colour FCS the time-resolved FCS allows simultaneous and independent monitoring of diffusion of two species in one sample as well as the quantitative characterization of the interaction among those species by cross-correlation analysis. In addition, TR-FCS measurements yields the fluorescence lifetime of the single fluorescent particle, which is another useful parameter sensitive to changes in the microenvironemt of the probe.

Conclusions

Confocal fluorescence correlation spectroscopy enables to directly monitor DNA condensation. The high sensitivity of this approach is given by large changes in the read out parameters diffusion time and particle number as well as low used concentrations of fluorescent label. This approach has its large potential in future "in vivo" experiments. The application of the so-called "z-scan" allows for a precise determination of diffusion coefficients in 2D systems without the need of external calibration. Since FCS was shown to be compatible with ellipsometry, the combination of both techniques appear to be useful in studies on supported phospholipids bilayers. Time-resolved FCS allows to simultaneously and independently monitor the diffusion of molecules. Considering that the fluorescence lifetime of certain fluorophores strongly depends on its microenvironment, time-resolved FCS appears to have a larger potential for "in vivo" experiments than two-color experiments.

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Publications in Journals from M. Hof used for this thesis:

(Bold number indicates that all the FCS experiments described in these publication have been performed in the laboratory of M. Hof. The FCS experiments on lipid systems were performed by A. Benda and M. Beneš within the framework of their PhD studies supervised by M. Hof. The FCS experiments on DNA condensation were performed by Dr. Teresa Kral under the supervision of M. Hof.)

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