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Fluorescently Labeled Phospholipids – New Class of Materials for Chemical Sensors for Environmental Monitoring

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1. Introduction

Reliable environmental monitoring strongly depends on the quality of chemical and biochemical sensors. There are still some unsolved problems especially when higher selectivity is required. In this chapter we propose a new class of materials – fluorescently labeled phospholipids, which can be used as chemical and biochemical sensors. We focus our attention on the most promising compound - head labeled with nitrobenzoxadiazole (NBD) phosphatidyl ethanolamines. We were the first to study these compounds in one component layers. Three new phenomena were discovered for this material that can be used for successful sensor applications. In our research we use the Langmuir and Blodgett method for investigation of organic monolayers at the air-water interface and for thin film deposition. It can also independently be used for environmental monitoring, e.g. water purity monitoring.

2. The Langmuir and Blodgett method - Use of the Langmuir film method for measuring the quality of water in natural basins

Probably the most promising method for the creation of supramolecular architectures in a well controlled manner is the method of Langmuir and Blodgett. This method is schematically described on Fig. 1. A trough, usually manufactured from well cleanable and inert material Teflon® (polytetrafluorethylene) is filled with ultra pure water. The organic substance to be investigated and deposited is spread from a solution. Molecules of the substance should be with the proper hydrophilic–hydrophobic balance so they remain at the air-water interface and do not penetrate the water. These molecules consist of a hydrophilic head group, which is attracted to the water and a hydrophobic tail (most often – hydrocarbon groups) which is repelled by the water. Some time is allowed for the solvent to evaporate until something like a 2D gas of the investigated molecules remains at the air-water interface. This is called Langmuir film. After this a compression of the organic monolayer with a barrier is started.

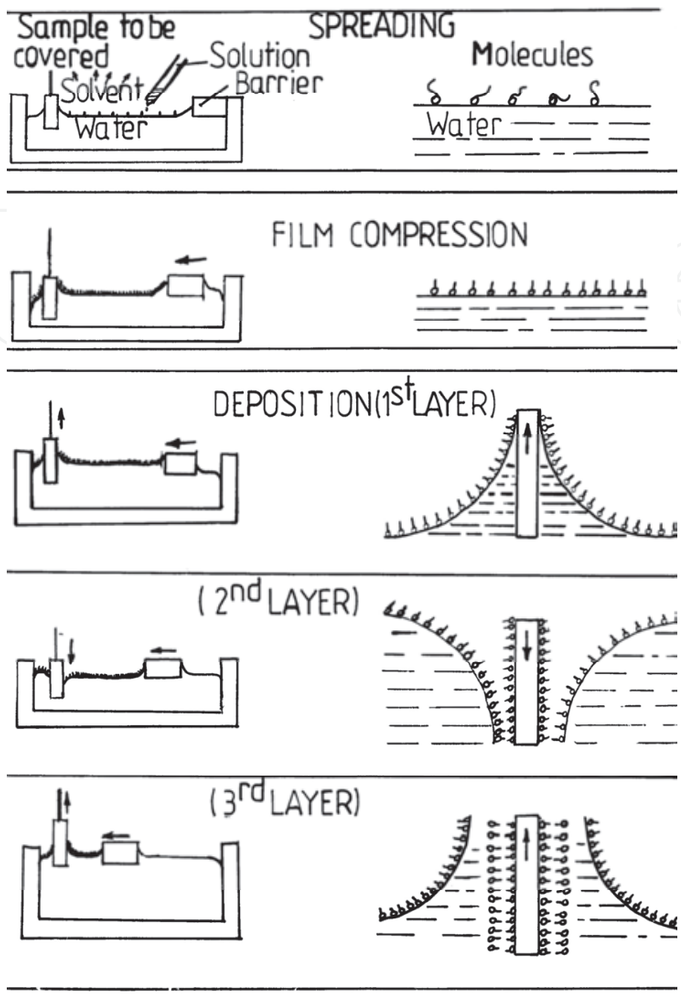

Fig. 1. The Langmuir and Blodgett method for investigation of organic monolayers at the air-water interface (Langmuir films) and for thin film deposition.

The surface pressure is constantly measured by a surface tensiometer (not shown on Fig. 1). A surface pressure – mean area per molecule isotherm can be measured. Additionally, the trough can be integrated with another instrument, e.g. fluorescence microscope and additional data can be gathered. At any point the compression of the monolayer can be stopped, the regime of constant surface pressure maintenance can be switched on, and a deposition on a solid substrate can be started. If we have a hydrophilic substrate which attracts the molecules' heads and it is immersed in the water before the monolayer spread then on the first movement up the first monolayer is deposited. Now the substrate becomes hydrophobic because the hydrophobic molecular tails are on the surface and on a downward movement of the substrate a second layer is deposited. Again the substrate surface becomes hydrophilic and on a subsequent movement upwards a 3rd layer is deposited. And the layer by layer deposition can continue. This deposition method has the following advantages compared to alternative methods of thin film deposition like spin coating, vacuum evaporation and self-assembling:

- This is a discreet method of deposition, a complete layer after complete layer are deposited. This gives the possibility for a very precise control of the film thickness. Phospholipid molecules are with height of around 3 nm but also the tail chain length can be varied so a thickness control to 0,1 nm accuracy can be achieved.
- The molecules are well oriented. This is very important for some applications, e.g. non-linear optics.
- The molecules are prearranged on the water surface before the deposition process. Thus the surface density of defects is much smaller.
- This is the most suitable method for molecular architecture. Different layers can be from different molecules. Inside the layer mixture of different molecules can be used. There is a possibility for interface reactions (e.g. CdSe nanoparticles incorporated in the lipid matrix can be prepared in this way). Also absorption from the water subphase of e.g. proteins is possible.

The development of rapid, economical and sensitive techniques for characterization of the purity of natural and drinking water represents leading ecological problem. The surface properties of natural waters (sampled from rivers, lakes and gulfs) are already successfully used for evaluation of the ecology standard and purity of water basins. Recently Pogorzelski et al. (e.g. Pogorzelski and Kogut, 2003 and references there in) proposed a Langmuir monolayer based technique which by measuring the surface pressure-area isotherm of the samples collected from a range of natural water basins, yields the so called "structural signatures" of water, which adequately predicted the quality and the purity of the basin. Major advantage of the Langmuir monolayer technique is that it combines ease of use, high sensitivity and possibility for rapid application with much lower price in comparison with the most commonly used chromatography techniques.

The "structural signatures" of samples of natural water result from the generalized scaling procedures applied to the surface pressure-area isotherms of the natural films. They appear to reflect in a quantitative and sensitive way the film composition, film solubility and the miscibility of its components, the kinetic mobility of surfactant molecules, and the compound's surface concentration. It is suggested that certain classes of film-forming components or "end-members" may dominate the static and dynamic surface properties. Variation in the surface rheological parameters of source-specific surfactants is postulated to reflect organic matter dynamics in natural waters. The reported results demonstrate that natural films are complex mixtures of biopolymeric molecules covering a wide range of solubilities, surface activities and molecular masses with a complex interfacial architecture.

The natural water's (sea, lakes, rivers) surface microlayer plays an important role in air-water interactions. A certain fraction of dissolved organic matter in the water basins has surface-active properties and makes up a very reactive part of the organic matter (Druffel and Bauer, 2000). According to their surface-active properties, these substances accumulate at water interfaces thereby influencing gas, mass, momentum and energy transfer between the so modified interfaces. The intensity of the film-effect depends strongly on film surface concentration, composition, and viscoelastic properties of the surface microlayer films. Processes taking place in the water body bulk (biological event, organic matter transformation or degradation, anthropogenic effluents, etc.) are sources of surface-active substances. Surfactants are concentrated at the air–water interface by numerous physical processes including diffusion, turbulent mixing, bubble and particle transport, and convergent circulations driven by wind, tidal forces, and internal waves.

The composition of the natural water's surface films is largely undefined, although significant enrichments of many specific classes of compounds in the surface microlayer have been demonstrated (for review, see Hunter and Liss, 1981). Natural sea/river/lake films mostly resemble layers composed of proteins, polysaccharides, humic-type materials and long chain alkanoic acid esters (Van Vleet and Williams, 1983). The generally accepted view is that the ubiquitous background of degraded biopolymeric and heterogeopolymeric material in the bulk waters has the potential to generate measurable surface films even in oligotrophic waters. Specific inputs of fresh bioexudates and biopolymeric material from local events are superimposed on this background signal.

The emphasis in the published studies (Pogorzelski, 2001; Pogorzelski and Kogut, 2001 a, b; 2003 a, b) has been on the multicomponent character of natural surfactant films and the consequent complexities involved in any attempt to predict the interfacial viscoelastic properties (playing a crucial role in modeling of physical systems with surface film-mediated interfaces) due to the diverse chemical composition of such films.

A complete compositional or structural description of naturally occurring surfactants is not currently feasible.

Instead of analyzing the chemical composition, it should be possible to scale microlayer film surface pressure–area isotherms in terms of the structural parameters, reflecting the natural film morphology, and resulting from the generalized physical formalisms adopted to multicomponent surfactant films. Particularly efficient approach to scale the surface pressure (π) - area (A) isotherms of the microlayer films adsorbed on the surface of natural waters proved to be the fitting of the isotherms by the virial equation of state as proposed by Barger and Means (1985):

$$\pi A = C_0 + C_1\pi + C_2\pi^2 \tag{1}$$

where C_0, C_1, C_2 are virial coefficients, and A is the film area (in cm^2).

As demonstrated by Pogorzelski, 2001; Pogorzelski and Kogut, 2001 a, b, 2003 a, b C_1 can be interpreted as the limiting specific area occupied by the molecules in the film, and C_o can be assumed equal to XnkT in the limiting case when π approaches zero:

$$C_0 = XnkT \tag{2}$$

where the parameter X is related to the interaction forces between molecules in the monolayer, n is the number of molecules in the unknown film, k is the Boltzmann constant, T is the temperature in degrees Kelvin.

The limiting specific molecular area A_{lim} (in nm^2) can be expressed as (Frew and Nelson, 1992):

$$A_{lim} = C_1 n^{-1} \times 10^{14} \tag{3}$$

Since the area covered with a film of a pure substance at a constant value of k is directly proportional to the mass m on the surface, it is possible to extend this computation to all the natural films (Barger and Means, 1985).

Similarly, fitting procedures can be applied to quantitatively analyze the hysteresis of natural water's surface films when subjected to cyclic area compression/expansion, and also to describe the sample's surface pressure–temperature isochores.

Thus it is possible to avoid the expensive, time consuming and cumbersome analysis of the chemical composition of natural waters, and instead to characterize the sample's quality by the introduction of sensitive and much easier to obtain physicochemical "structural signatures" of the natural microlayer films. The structural state of natural water films, which can be incorporated with such source-specific markers of both biogenic and anthropogenic origin, can be assessed through the quantification of the parameters variability. They can be useful for tracking organic matter dynamics, as already established factors like the carbon to nitrogen C/N ratio (Bock and Frew, 1993), used in microlayer film studies, for instance. The main expectation of such studies is that variation in the surface rheological parameters of natural biosurfactant films manifested at the air–water interface could be followed to trace and map surface-active source-specific compounds spatial-seasonal-temporal evolutions.
Compared to the evaluation of the ecological quality of natural waters, the characterization of the purity of drinking water poses higher challenges as it requires precise identification of even trace amount of detrimental ingredients. Some compounds, like the ions of heavy metals or membrane-active molecules, can have profound detrimental effect on the consumers' health even in very low doses that can not be detected by direct measurement of the sample's surface pressure. More precision quantitative measuring techniques are needed. For the purpose of such demanding measurements we further advanced the monolayer technique by introducing the use of fluorescently labeled LB solid supported phospholipid films. This is because fluorescence in some molecules is highly sensitive to even most delicate environmental changes (like slight changes in ionic strength, presence of quenchers in trace concentrations, etc.) LB films from these materials have high potential to be used as sensitive, selective and fast chemical sensors. In this new class of compounds for sensor applications - fluorescently labeled lipids, fluorescence intensity and lifetime are strongly influenced by minimal amounts of tested substances. In the following chapter we look in greater detail to the most promising fluorescence label in this class of compounds – the NitroBenzoxaDiazole (NBD) label. Then, results from our research of NBD labeled phospholipids at the air-water interface, as LB film on solid support, and molecular modeling are presented and discussed in view of sensor applications.

3. Previous research of NBD fluorescently labeled lipids

Synthesis, where to the polar head (to the amino group) of egg phosphatidylethanolamine (PE) covalently is bound the NBD chromophore was first described by Monti et al. (1978). Due to the use of egg phosphatidylethanolamine (PE) tail length varies. Solution of NBD-PE in ethanol shows absorption maxima at about 330 nm and 460 nm, and the fluorescence maximum is at 525 nm. Fluorescent intensity in ethanol is proportional to the concentration in the range of 1 ng/ml to about 3 μg/ml. This article studied the dependence of the intensity of absorption and fluorescence of NBD-PE to the change in dielectric constant of the solvent used. The observed strong sensitivity of the spectral characteristics of NBD-PE to the polarity of its surrounding makes this molecule an excellent indicator of conformational changes in the membrane. This article notes that small amounts of non-ionic detergent can lead to increase in fluorescence intensity and peak position change. Without problems is the incorporation of NBD phospholipid molecules in liposomes and biological membranes. For

the NBD chromophores the angle between absorption and emission dipole is about 25 ° (Thompson et al., 1984) and therefore the real environment of the chromophores may be different for absorption and emission. Overview of the spectral characteristics of NBD was made by Suzuki and Hiratsuka (1988).

There are a large number of papers in which NBD labeled lipids are used especially as a small percentage additive in the biomembrane studies. Here we will review only the work related to the chemical sensor applications of these molecules. The presence of large paramagnetic metal ions can be monitored by the fluorescence quenching of the NBD chromophore. Morris et al., 1985 used cobalt ions to quench the fluorescence of NBD-PE incorporated in phospholipid liposomes. Large paramagnetic ions such as Co^{2+} efficiently quench the fluorescence. The mechanism that is suggested is of lateral diffusion of Co-lipid complex followed by collisional quenching with NBD-PE. The addition of the chelator EDTA restores the initial fluorescence to 90%. EDTA quenches itself about 10% of the fluorescence. Fluorescence is quenched in the outer layer of the liposomes within milliseconds after the addition of cobalt ions, then, if possible, it penetrates the inner layer. For small monolayer liposomes the process is 10-20 times slower, but in all cases completed in the first few seconds. This technique is used also for measuring the surface potential of the membrane. Another paramagnetic ion copper Cu^{2+} is also used for NBD fluorescence quenching (Rajarathnam et al., 1989). Chattopadhyay and London (1987) proposed a method for measuring the position of NBD chromophores in the biomembrane by quenching its fluorescence by spin-labeled in a different position phospholipids. A comparison of the fluorescence intensity is made when two located in different depths quenchers are used. Results show that the greatest distance from the center of the bilayer is for NBD chromophores in the molecules of the Dipalmitoyl-NBD-PE – 1,42 nm. This means that due to its strong hydrophilicity the NBD chromophore is folded to the hydrocarbon tails and is positioned on the border tail - head, which is 1,5 nm from the center of the bilayer. For 6-NBD-PC this distance is 1,22 nm, for 12-NBD-PC, this distance is 1,26 nm, i.e. the tail in which is the NBD chromophore is folded and goes to the water surface. In this paper is calculated the critical distance R_c, below which the fluorescence of NBD is effectively quenched by the spin-label – 1,2 nm. Calculations show that if fluorescence is quenched due to presence of acceptor this distance is 10% larger.

Another important characteristic of the NBD chromophore that can be used in sensor applications is the dependence of its fluorescence lifetime on the polarity of the surrounding media. In general, reducing the polarity of the environment increases the lifetime. Lifetime of dilauroyl and dimiristoyl-NBD-PE in liposomes of egg lecithin is 6-8 ns (Arvinte et al., 1986). Detailed analysis of the fluorescence lifetime characteristics of NBD-aminohexane acid (NBD-$NH(CH_2)_5C0_2H$) at low concentrations in solvents of different polarity and donor hydrogen connection strengths was conducted by Lin and Struve, 1991. This substance has aminoalkane side chain similar to chains in which NBD chromophore is conected to phospholipids and the results are comparable. The conclusions are that the line shift of absorption and luminescence is due to the polarity of the solvent, while the drop in luminescence intensity due to non radiation transitions is much more affected by the hydrogen connection strengths. Fluorescence lifetimes in aprotic solvents is from 7,37 ns in DMSO to 10,6 ns in ethyl acetate, but are shorter in alcohols (5,65 ns in methanol). Extremely fast is the NBD luminescence in water - 0,933 ns. Low quantum yield in water is explained

by anomalously short lifetime of non radiation transitions combined with radiation transitions which are with 3 times longer lifetime than those in other solvents. Oida et al., 1993 developed the so-called Fluorescence Lifetime Imaging Microscopy (flimscopy) which uses DP-NBD-PE and rhodamine labeled lipids.

Fluorescent transduction of changes in the structure of the lipid membranes shows properties necessary for biosensor applications. When connected with the substrate a single membrane associated "receptor" protein may affect a significant number of surrounding molecules via electrostatic interactions, spatial interactions, interface changes in ionic strength or pH. The result is that: 1) perturbation of the lipid layer that is caused by the interaction receptor - ligand can be qualitatively related to the degree of connectivity, and 2) have amplified the original signal after the interaction of biomolecules. Placing a "receptor" protein in the phospholipids layer, which simulate the biological membrane, and provides improved stability against denaturation of the protein, gives biosensors with improved operational life span. Mixed lipid monolayers containing small amounts of DP-NBD-PE were shown to be able to convert changes in pH due to the hydrolytic enzyme activity at the membrane interface. This conversion scheme is used to determine the acetylcholine by acetylcholinesteraze (Brennan et al., 1990) and urea by ureaze (Brennan et al., 1992, 1993). In these studies a small concentration (about 1 mol %) of DP-NBD-PE and the respective enzyme are added in the phospholipid membrane. Changes in interface pH caused by hydrolytic enzyme reaction, lead to a change in the ionization of acidic phospholipid heads. This causes a change in the forces of electrostatic repulsion between neighboring heads. Structural changes in the membrane lead to an analytical signal in the form of change of fluorescence intensity due to fluorescence selfquenching of NBD-group caused by local increase in concentration. A comparison of different fluorophores connected to the same position of a protein showed that NBD-group gives the highest sensitivity (typically 4 times better than the next fluorophore (see Brennan et al., 2000 and references therein).

From the viewpoint of sensor applications of DP-NBD-PE important is the optimization of: a) the concentration of DP-NBD-PE molecules in the membrane, and b) the composition and structure of the phospholipids membrane. This is done by the Krull's group in Toronto (Brown et al., 1994 and Shrive et al., 1995). The results are applicable to both LB film layers and liposomes. Fluorescent measurements were performed on liposomes because the fluorescence signal from LB monolayers is weak and leads to significant errors. For the optimization process a model was developed for the fluorescence selfquenching of DP-NBD-PE. It considers the probability for static quenching by the formation of emissionless traps consisting of pairs of statistical DP-NBD-PE molecules which are at critical distance R_c. The model also considers the dynamic quenching due to Förster transfer of energy from DP-NBD-PE monomers to the traps. Assumptions in this model are: 1) statistical traps are formed according to two-dimensional equation of Perrin; 2) all DP-NBD-PE molecules that do not participate in the traps are uniformly distributed throughout the monolayer; 3) there is no diffusion during the lifetime of the excited state, 4) energy can move between and among fluorophores and traps, but once traps are reached energy immediately and without emission decreases; 5) passing of energy in more than one DP-NBD-PE molecule before reaching the trap is negligible. It is estimated that the distance at which the efficiency of Förster transfer of energy becomes 50% R_0 = 2,55 nm and that R_c = 0,94 nm. The optimum

concentration of DP-NBD-PE molecules is one in which the theoretical expression undergoes a maximum change, i.e. the second derivative of the expression to the change in concentration is calculated. According to theoretical calculations, the optimal concentrations were 0,027 and 0,073 DP-NBD-PE molecules per nm^2. These values were the same within the experimental error when comparing results of three different types of liposome compositions.

Optimization of composition and structure of membrane phospholipids showed the need for structural heterogeneity in the membrane at microscopic and not at molecular level in order to produce significant changes in fluorescence intensity. In membranes without heterogeneity the signal change is only 5-6%. Heterogeneity is achieved by the mixing of dipalmitoyl phosphatidyl choline with dipalmitoyl phosphatidic acid at a ratio of 7:3. At surface pressure of 30 mN/m, which is considered the liposome pressure, this mixture gives domain structure as observed in Langmuir films by fluorescence microscopy. The resulting changes in the average fluorescence intensity on pH change in this case reaches 60%. The mechanism of response of the membrane is shown to depend on the surface potential (Nikolelis et al., 1992) and is the result of changes in the ionic double layer and the rearrangement of the lipid heads and tails. This indicates that the mechanism of response in these biosensors is much more complicated than changing the distance between the heads. Moreover, the choice of phospholipid for these biosensors must be based on constraints coming from the ionic strength and pH, imposed on the activity of immobilized, chemically selective protein as enzyme activity is highly dependent on pH (Brennan et al., 1994).

4. Investigations of monolayers at the air-water interface

On Fig. 2 are shown the isotherms of head labeled Dipalmitoyl-NBD-PE (DP-NBD-PE, chemical formula is in Fig. 14) at three different temperatures and in the presence of cobalt ions in the water at 20° C. Along with these measurements the monolayer was studied with fluorescence microscopy. The results for 20° C are published and discussed in detail elsewhere (Ivanov, G.R. (1992)). This was the first time that fluorescence self quenching in organic monolayers at the air-water interface (Langmuir films) was described. Here on Fig. 3 for the first time we publish the fluorescence microscopy data at 5° C. At room temperature the average area per molecule in the liquid phase is 1,4 nm^2, and in the solid phase - 0,45 nm^2. Adding $CoCl_2$ in the water increases the surface area of the molecule in the solid phase to 0,67 nm^2. The addition of $CaCl_2$ (not shown) leads to a smaller increase in the area in the solid phase - 0,56 nm^2.

The shape of the solid domains is due to an interplay of several forces: the growth kinetics which at these compression speeds is negligible; the edge energy at liquid phase – solid phase interface which is minimal for circular domains; and the electrostatic repulsion between the similarly oriented dipoles of the molecules which is minimized when the molecules are further apart. Due to the last force the domains repulse each other at low surface pressures and when the area of the solid domain is increased at higher pressures the domains obtain the dendridic shape which increases the distance between molecules. The fluorescence microscopy data at 5° C reveals also something that is not well observed at higher temperatures. The solid domains grow in size largely due to the attachment of smaller solid domains from the second population of solid domains.

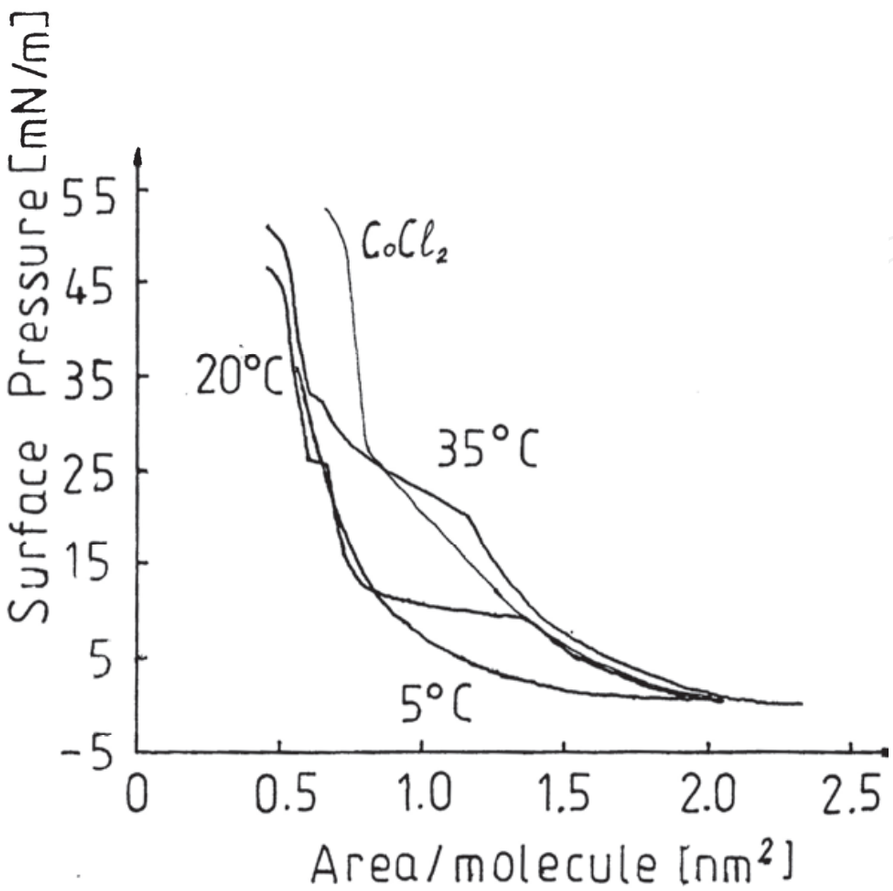

Fig. 2. Isotherms of monolayers from DP-NBD-PE at 5° C, 20° C, 35° C and at 20° C with the presence $CoCl_2$ in the water.

The presented data here is for single component monolayers composed only from the fluorescently labeled in the head phospholipid DP-NBD-PE. The fact that we are able to observe the picture of phase coexistence with an excellent contrast is due to the fluorescence self quenching of this molecule in the solid phase when the distance between the molecules becomes much smaller and this allows for non radiation transfer of energy between them. This new phenomenon can be used with great success in sensor applications. If due to interactions of the sensor with the substance to be detected some conformational changes in the DP-NBD-PE molecules arise, this will lead to a strong measurable change in the fluorescence intensity. So this provides a second mechanism for component detection apart from the already discussed influence of the fluorescence peak maximum, intensity and lifetime on the polarity of the surrounding medium.

On Fig. 4. is shown the equilibrium spreading pressure measurement of DP-NBD-PE at 20° C. In the first few seconds after placing some crystals from the material on the water surface the surface pressure increases insignificantly, then within a few seconds it increases by more than 15 mN/m. Then within a minute it reaches its equilibrium value of 19,6 mN/m. This value is quite high and indicates that at room temperature the majority of studies described

in this work were conducted under equilibrium conditions. This is not quite so with much of the work conducted with LB films. For example the most widely studied arachidic acid has an ESP of 0 at room temperature indicating that the molecules are in metastable state when deposited.

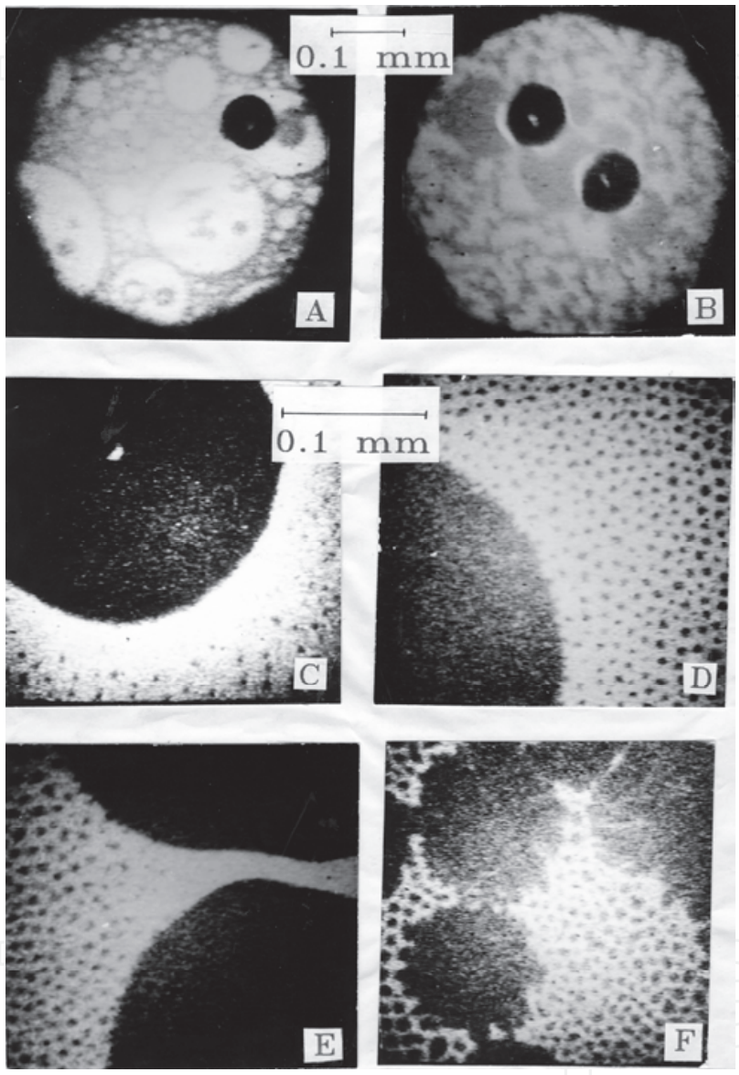

Fig. 3. Fluorescence microscopy of monolayers at the air-water interface from DP-NBD-PE at 5° C at different surface pressures Π: (A) Coexistence of liquid and solid phase at zero pressure; (B) the same at Π = 0,2 mN/m; (C) the occurrence of a second population of solid phase (the small black dots), which is repelled from the large solid domains Π = 6 mN/m; (D) the small solid phase domains overcome the repulsion of the big domains and begin to attach to them at Π = 9 mN/m; (E) large domains close the distance between them at Π = 12 mN/m; (F) domains of the solid phase obtain the dendridic shape and begin to merge with each other at Π = 15 mN/m.

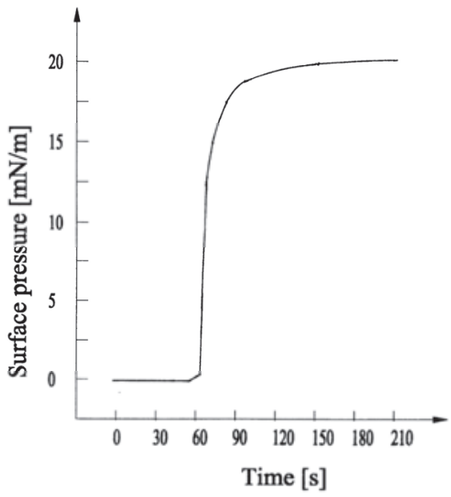

Fig. 4. Measurement of the equilibrium spreading pressure (ESP) of DP-NBD-PE at 20° C.

5. Investigations of deposited on solid support thin films using the LB method

For the possible sensor applications of DP-NBD-PE molecule it is important to obtain quality deposition on solid support. Deposition of multilayer structures of phospholipids by the LB method is complicated. Usually when immersed for the second layer deposition the first one is thrown off back into the water probably due to the presence of residual water between the layer and the substrate. A similar phenomenon was observed in the case of DP-NBD-PE. A typical way to overcome this problem is to use extremely low deposition speed. So no water is entrapped and relatively high quality multilayer structures are obtained. The problem is that very few commercial instruments have such low deposition speeds and these speeds are not suitable for industrial applications. Therefore we proposed a new method of obtaining multilayer structures. In it, after each immersion the bilayer was blown with heated air for several minutes at 55°C, which is below the melting temperature of the monolayer. The deposition results are compared in Table. 1. The quality of the film, or more precisely the amount of transferred substance, is judged by two criteria. On one hand this is the transfer ratio (Tr), which ideally is 1. But its determination has large errors due to difficulties in maintaining a constant surface pressure, causing the barrier to move back and forth without much correlation with the deposited layer. Far more accurate method is the measurement of the optical absorption of the film. In it the area of the line of maximum absorption of DP-NBD-PE at 465 nm is integrated.

Usually, in order to improve the LB film quality metal ions are added in the water subphase. With fatty acids good results are obtained when divalent ions of heavy metals such as Cd^{2+} are added but in our case this did not lead to good results. For phospholipids it is usually recommended the use of univalent metal ions. They bind to the negatively charged phosphate head of the phospholipid and neutralize the electrostatic repulsion between neighboring layers. With our molecules best results were obtained with the use of NaCl.

Table. 1 shows that the use of thermal treatment of the film increases the transfer rate which means that more substance is deposited. Peeling off the film on the down substrate movement is greatly reduced, although almost always the transfer ratio on the down

movement is less than the coefficient in the upward movement of the substrate. The results of optical absorption also confirm that more substance is deposited when heat treatment is used.

Deposition conditions			Transfer ratios (Tr) for the corresponding layer												Integral Absorp-tion
No	Π (mN/m)	Subphase	Tr_1	Tr_2	Tr_3	Tr_4	Tr_5	Tr_6	Tr_7	Tr_8	Tr_9	Tr_{10}	Tr_{11}	Tr average	(a. u.)
Gl	20	D.W./H.	3,2	0,4	2,1	-0,3	1,9	-0,5	1,7	-0,5	1,5	-0,6	1,5	1,40	0,45
G2	31	D.W./H.	2,7	0,8	1,3	0,7	1,3	1,0	1,1	1,0	0,6	1,1	0,3	1,21	3,16
G3	40	D.W./H.	2,1	0,1	0,7	0,4	1,1	0,7	1,0	0,6	0,7	0,7	0,7	0,70	2,56
G4	43	D.W./H.	1,1	0,5	0,5	0,3	0,6	0,4	0,7	0,6	0,7	0,4	0,7	0,80	2,02
G5	47	D.W.	1,3	1,4	1,1	0,9	1,1	0,9	1,9	0,9	1,3	0,9	0,9	1,67	-
G6	31	$CdCl_2$/H.	3,2	-4,8	4,8	-11,8	11,8	-11,0	11,7	-13,2	10,6	-10,0	9,2	0,35	0,74
G7	31	KCl/H.	4,0	1,9	2,1	1,1	2,3	1,4	3,1	1,3	2,1	1,0	2,3	1,80	0,82
G8	35	NaCl	2,3	-5,7	5,8	-5,6	5,4	-4,4	5,5	-3,7	4,0	-3,7	4,1	0,36	0,76
G9	35	NaCl/H.	2,7	0,4	3,4	0,4	3,1	0,6	2,5	0,5	2,7	1,3	3,4	2,29	2,59

Table 1. Transfer ratios (Tr) and integral absorption of LB films from DP-NBD-PE. H means heat treated, DW means distilled water.

To examine the effect of thermal heat treatment on the morphology of the resulting LB films comparative optical microscopy studies of samples G8 and G9 were performed. They are deposited at the optimum surface pressure of 35 mN/m and in the presence of NaCl in the water subphase. The only difference is that in G9 each bilayer was heat treated. Results from the dark field and phase-contrast microscopies clearly showed that considerably more substance is deposited when heat treatment is used and the density of defects is significantly lower. However, a significant number of defects and distinct domain structures with dimensions of tens microns can be seen.

Another way of assessing the quality of the deposition is to measure the mass of the deposited substance. This is done when the LB film is deposited directly on a quartz crystal resonator. We used a resonator operating at 10 MHz frequency. It should be noted that sensitivity depends on the square of the frequency of the resonator. This is one of the most promising methods for creating gas and biochemical sensors which directly measures the mass of the substance to be detected. The accuracy of the measured frequency shift is below 0,1 Hz and the sensitivity of the method can be seen. It was possible to observe the water evaporation from the layer. In the middle of the resonator is evaporated a gold heater which can be used in chemical sensor applications for desorption of the absorbed studied substance. On the same resonator were sequentially deposited 21 layers, then frequency was measured, then 12 more layers were deposited and the frequency was measured, and finally 6 more layers were deposited and frequency measured (Fig. 5). In the case of an ideal deposition the mass (evaluated from the frequency change) should lie on a straight line. In our case this occurs with a deviation of around 10% indicating a high quality deposition. During this deposition every bilayer was heat treated.

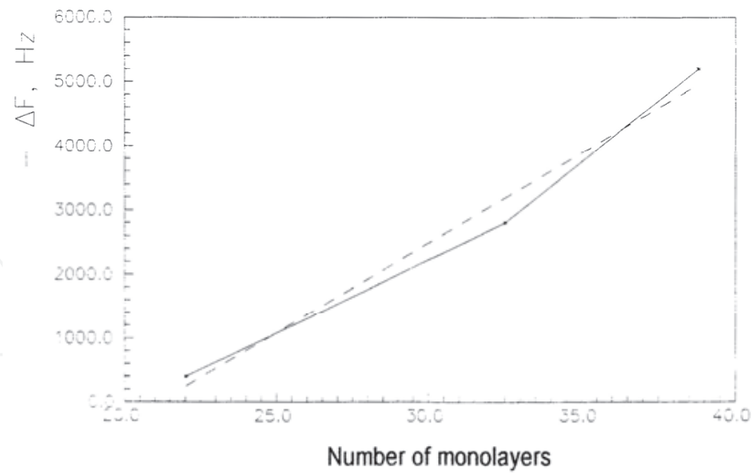

Fig. 5. Increase of the mass of 21, 33 and 39 LB monolayers from DP-NBD-PE measured with quartz resonator.

To get an idea of the effect of heat treatment at molecular level polarization Fourier transformed infrared spectroscopy in attenuated total reflection mode of multilayer LB films deposited at 35 mN/m was conducted. The results are shown in Fig. 6. The bottom curve shows the spectrum of film obtained at very low deposition speed. The middle curve shows the same film heated for several minutes at 55 ° C. The upper curve shows the spectrum of

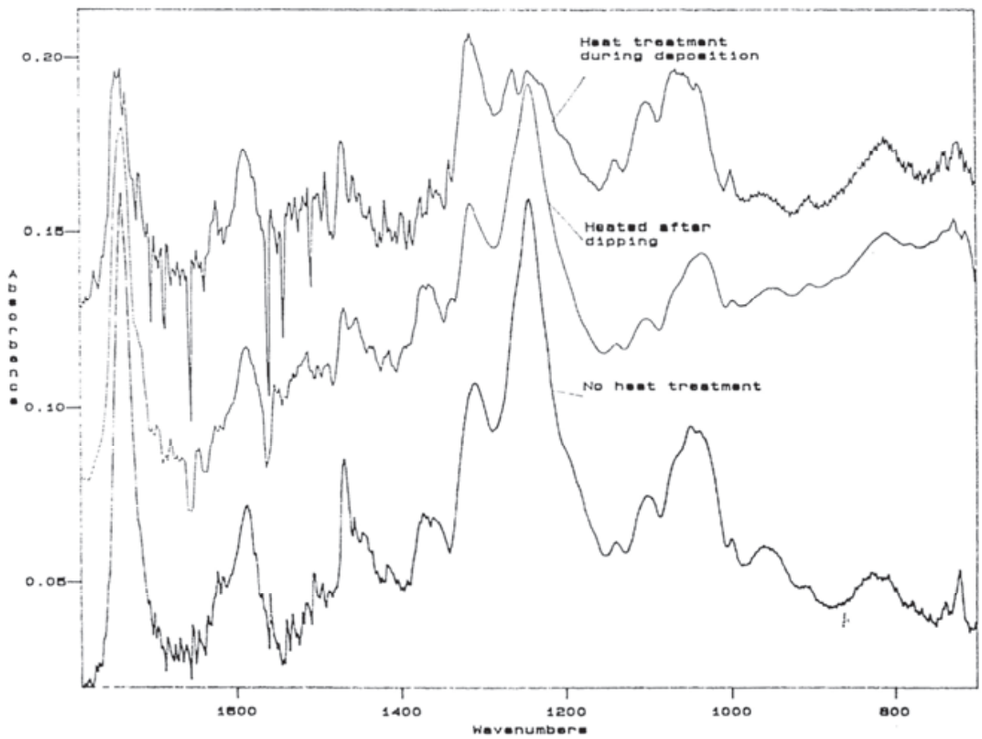

Fig. 6. The influence of heat treatment on multilayer LB films from DP-NBD-PE on the infrared spectra.

film obtained by high speed deposition and heat treatment of each bilayer during the deposition. The most important change is the significant broadening of the absorption lines at 1244 cm^{-1} (which is a mixture of lines Y_w CH_2 and Y_w P=0) and especially at 1738 cm^{-1} (which corresponds to the Ys C=0) when heat treatment is performed. Particularly noticeable is this broadening when each bilayer is heated. The broadening of these lines indicates greater spread in the orientation of the corresponding parts of the DP-NBD-PE molecule. This is an expected result when heat treatment is performed.

Additional information about the molecular arrangement and orientation of the chromophore head may be obtained from polarized absorption spectroscopy in the visible region. Simple NBD-derivatives have three main lines of absorption in the visible and near UV region - at around 420 nm, at 306-360 nm, and 225 nm (Lancet and Pecht, 1977). The first line corresponds to the line of 460 nm for NBD-labeled lipids and is due to intramolecular charge transfer (Paprica et al., 1993), which is accompanied by a large (~ 4 Debye) change in dipole moment (Mukherjee et al., 1994). The line absorption at 306-360 nm (for NBD-lipids ~ 335 nm) corresponds to a transition $\pi^* \leftarrow \pi$. Absorption spectra of NBD labeled lipids are shown in Fig. 7. The chloroform solution of a tail NBD labeled dipalmitoyl phosphatidylcholine has a maximum absorption at 475 nm. DP-NBD-PE in chloroform solution has a maximum at 457 nm. When deposited as LB film it has absorption maximum at 460 nm and a pronounced shoulder at 492 nm. This shoulder is probably due to J-aggregation of molecules in which the optical dipoles are arranged like a brick wall (Czikklely et al., 1970 a, b). J-aggregates are characterized by red shift of the absorption spectrum by about 30 nm and therefore this is the most likely interpretation. The presence of a large percentage of J-aggregates in the condensed phase in which deposition was carried out, may explain the fluorescence quenching in the solid phase.

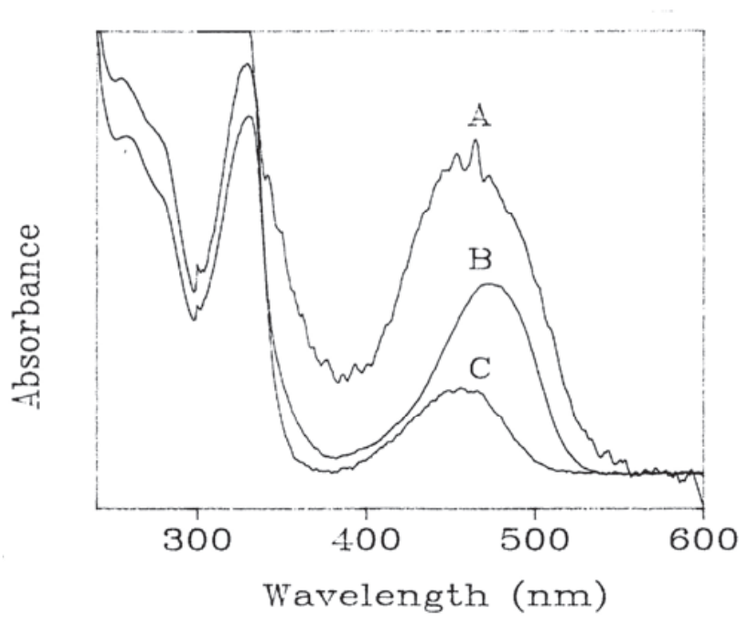

Fig. 7. UV-VIS absorption spectra of: A – LB film from DP-NBD-PE deposited at 35 mN/m and NaCl in water; B – chlorophorm solution of dipalmytoyl phosphatidyl choline labeled in the tail with NBD; C – chlorophorm solution of DP-NBD-PE.

On Fig. 8 and Fig. 9 are shown the polarization absorption spectra of two samples of LB films from DP-NBD-PE with identical thickness. Both normal incidence of the beam and incidence at 45° angle is used (index 45) to the substrate. The beam polarization is either parallel to the direction of withdrawal of the substrate (index p) or perpendicular to it (index s). Results for the area integral under the curves are summarized in Table. 2.

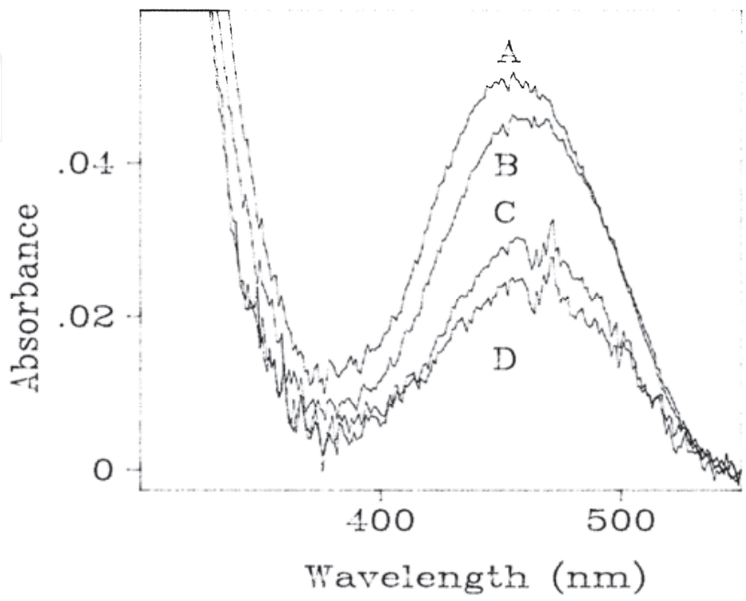

Fig. 8. Polarization spectroscopy of sample G5 - 11 LB layer structure of DP-NBD-PE deposited at Π=47 mN/m and fast withdrawal. A - p45; B - s; C - p; D - s45.

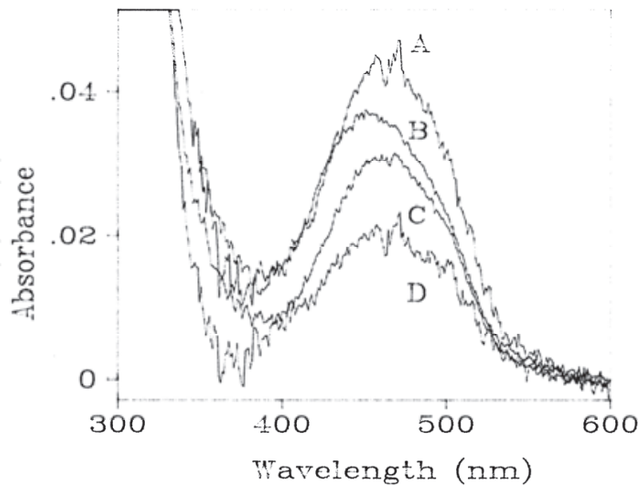

Fig. 9. Polarization spectroscopy of sample G9 - 11 LB layer structure of DP-NBD-PE deposited at Π = 35 mN/m with heat treatment and the presence of NaCl in the water. A - s45; B - p45; C - s; D - p.

LB film	p	s	p45	s45
G5	10,38	11,39	4,06	2,17
G9	1,93	2,27	2,55	3,35

Table 2. Integral areas of the 460 nm line of the absorption spectra at different polarization of the incident light for 2 different 11 layer LB films from DP-NBD-PE. G5 and G9 are the same films from Table 1. G9 was heat treated during the deposition.

From the polarization data it is seen that in both cases the optical dipoles of the molecules orient themselves with a slight advantage in the direction perpendicular to the direction of withdrawal (**s** component is larger than **p** component). From the data in table. 2 order parameter for the molecules $\langle \cos^2 \theta \rangle$ can be calculated. The value for G5 film is 0.48. If the arrangement is perfect the value should be 1. We can see the weak ordering of molecules or more precisely of their chromophore heads.
Fluorescence spectrum of LB multilayer structure of DP-NBD-PE deposited on a glass substrate in the solid phase was compared with the spectrum in chloroform solution in Fig. 10. Excitation was at the maximum absorption at 465 nm. About five-fold decrease in the fluorescence in the solid phase can be seen. When deposition is carried out in the liquid phase below surface pressure of 8 mN/m fluorescence is similar to that in a solution. In solid phase deposition the fluorescence quenching is almost complete. This is not due to reduced absorption, as absorption is increased by 10% in the solid phase deposited film. Interestingly, the addition of cobalt ions in the water subphase leads to almost complete recovery of fluorescence to its level in the solution (line not shown). This can be used in chemical sensors for heavy metal detection.

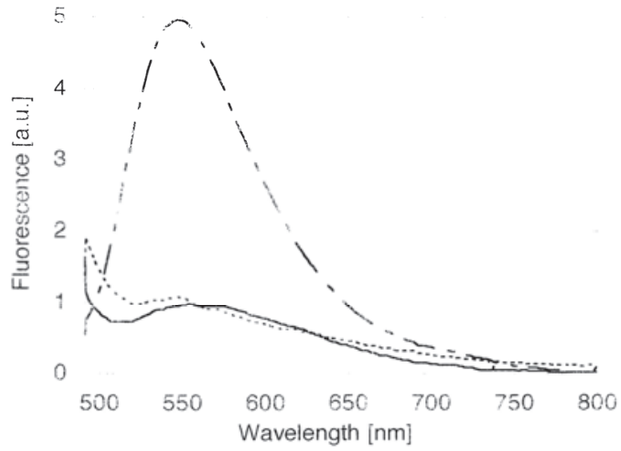

Fig. 10. Fluorescence spectroscopy of DP-NBD-PE of an LB film deposited in a solid phase (lower 2 curves) and in a solution. The spectra of an LB film deposited in a liquid phase is similar to the spectrum in solution.

An important question in view of sensor applications are the mechanisms of fluorescence selfquenching in NBD-labeled lipids. There is a similar study for octadecyl-rhodamine molecule (MacDonald, 1990), commonly used in Resonance Energy Transfer (RET) studies with NBD-molecules. The most obvious possibility for a mechanism to quench the fluorescence is the collision between an excited molecule and a quencher molecule. For this

process is important the local concentration of fluorescently labeled molecules in the liquid phase. Calculations show that for collisions to occur with molecules with diffusion rate of 10 $\mu m^2/s$ and fluorescence lifetime of 4 ns then the distance between them must be less than 0.3 nm. And such small distance is impossible for molecules with 2 tails in a liquid phase, even assuming an increase in local concentration. In monolayers that undergo phase transition liquid - solid state diffusion rate decreases more than three orders of magnitude: from 50 $\mu m^2/s$ to 0,03 $\mu m^2/s$ (Peters and Beck, 1983). Fluorescence lifetime of dilauroil and dimiristoil NBD-PE in liposomes of egg lecithin is 6-8 ns (Arvinte et al., 1986). Thus, in the liquid phase diffusion during the excited state of the DP-NBD-PE is about 3 nm and in solid phase it is only about 0,02 nm. From the isotherm of DP-NBD-PE (Fig. 2) it can be seen that at room temperature the average area per molecule in the solid phase is 0,45 nm, and in liquid phase is 1,4 nm. Upon an assumption of cylindrical adjacent tightly packed molecules the distances between the centers of molecules in liquid phase is 1,33 nm and 0,78 nm in the solid phase. Obviously the collisional quenching mechanism of fluorescence is not applicable in solid phase. However, it is not clear why there was no significant fluorescence quenching in the liquid phase. The answer probably lies in the observation of Lin and Struve (1991) for extremely fast fluorescence lifetime of the NBD chromophore in water - 0,933 ns. Indeed, at the air-water interface the NBD-group in DP-NBD-PE molecule is positioned entirely in the water as shown by molecular conformational modeling (see Fig. 14). Another possible explanation is the dependence of the lifetime on the concentration of DP-NBD-PE (Brown et al., 1994). At all concentrations the lifetime is a two exponent function and the average ranged from 8,66 ns at 0,1% concentration; 5,39 ns at 10%; 1,32 ns at 40%; 0,97 ns at 50%. At 100% concentration (we work with films composed only from this molecule) the lifetime will probably be even smaller. The above mentioned distances between molecules in the liquid phase are several times larger than the distance which an excited molecule can diffuse for these small lifetimes. This explains why there is no fluorescence quenching in the liquid phase.

Therefore, as a possible mechanism for fluorescence quenching remains energy transfer. For octadecyl-rhodamine molecule the distance at which energy transfer to monomer or dimer of the same molecule is 50% (Förster radius) is 5,5-5,8 nm for a transfer to monomer and 2,7 nm for a transfer to dimer. This calculation is done using a formula that takes into account the spectral overlap of the excitation and emission spectra for a given molecule. For NBD-molecules the Förster radius R_0 is 2,55 ± 0,15 nm (Brown et at., 1994) . Research that shows anomalous long distance energy transfer (Draxler et al., 1989; Fromhertz and Reinbold, 1988) should also be taken into account. In LB films, they observed 20% efficiency of energy transfer over distances of 150 nm. Depending on mutual orientation of molecules, this distance may decrease to 30 nm. If the molecules, among which energy transfer takes place are different, then the lifetime of the donor molecule should increase with increasing its concentration. But in the case of octadecyl-rhodamine it decreases. Therefore, the basic mechanism of fluorescence quenching in octadecyl-rhodamine is emissionless energy transfer to the dimers of the same molecules. This lipid associated fluorescence label forms pre-bonded dimers. However this mechanism does not explain why fluorescence quenching in DP-NBD-PE molecules occurs only in the solid phase.

As a most probable reason for the fluorescence quenching for DP-NBD-PE a model was developed that takes into account the likelihood of static quenching by forming emissionless traps consisting of pairs of statistical DP-NBD-PE molecules that are at critical distance R_c for trap formation (Brown et al., 1994; Shrive et al., 1995). Calculations give a value for R_c of

0,94 nm for this molecule. This value is greater than the intermolecular distances in solid phase layers of DP-NBD-PE and less than the distances in the liquid phase at room temperature. The addition of cobalt ions leads to increased intermolecular distances of up to 0,93 nm, which may explain the recovery of fluorescence intensity of the solid phase in presence of this ion which is known as good fluorescence quencher. Thus, this model explains fluorescence quenching in the solid phase, the lack of quenching in the liquid phase and the recovery of fluorescence in the solid phase with the addition of cobalt ions in the water. Given the anomalous long-distance energy transfer, observed in LB films (Draxler et al., 1989; Fromhertz and Reinbold, 1988) it is clear that critical is the formation of traps, which is precisely what we observed.

In order to have a better understanding of the morphology of the LB films with submicrometer resolution we performed Atomic Force Microscopy (AFM) measurements. Fig. 11 shows AFM images with cross-sections along selected lines. On Fig. 11 A deposition was carried out at 7 mN/m, slightly above the transition from liquid to solid phase. The liquid – solid phase coexistence is clearly seen. Cross section through one of the solid domains reveals cylindrical structure with a height of 5,8 nm and a width of several tens of nanometers. Taking into account the height of the monolayer (3,1 nm), these structures can be interpreted as a bilayer in the solid phase. These structures are observed systematically in all our experiments. They occur above the main liquid-solid phase transition. Deposition in the liquid phase (Fig. 12) showed that there are no structures outside of the normal silicon wafer bumps of less than 0,5 nm in the monolayer.

Cylindrical structures with bilayer height are observed also when the depositions are carried under the equilibrium spreading pressure of 19,6 mN/m (see Fig. 4). If the deposition at higher pressures is carried out these cylinders grow in height initially up to 13 nm for deposition at 33 mN/m (Fig. 11 B) and grow up to 35 nm, and in some cases to a hundred nanometers at 43 mN/m (Fig. 11 C). However, if the monolayer is allowed to relax under normal laboratory conditions for some time (50 days in this case), those cylinders again become with bilayer height (Fig. 11 D).

These three-dimensional cylinders can not be obtained due to the deposition process and/or interactions with the substrate because their height depends on the deposition pressure. Layering in the vertical direction of the DP-NBD-PE molecule can be observed in these high structures, suggesting that the cylinders are made of DP-NBD-PE molecules rather than impurities. The question remains why it is energetically more favorable for part of the molecules to accumulate on one another and not to attach to the adjacent solid phase. Possible answer is that this can be due to kinetic effects if we are compressing the layer or depositing at higher speeds. Against this explanation is the lack of such structures in the liquid phase deposition under the same conditions (Fig. 12). Furthermore, our previous data from fluorescent microscopy at the air-water interface does not show the presence of kinetic effects at these speeds. Against this explanation is the fact that high structures relax to bilayer cylinders with time (Fig. 11 D). Data from Stark spectroscopy measurements show that DP-NBD-PE molecules tend to form centrosymmetrical non-polar structures. Thus for the second layer in the bilayer structures the molecules most probably flip over and we have a tail – tail contact. This is the first time that such 3D structures are observed when deposition is carried below the ESP. These structures are stable over time at least for several months. Their presence is very important for sensor applications because they ensure simultaneously high contact area and low film thickness. Thus high sensitivity at fast

reaction times can be achieved (no slow diffusion needed). These self assembled 3D structures are part of our efforts in the hybrid assembly approach which combines the self-assembly technology with high performance robotic tools such as precise manipulators with submicron resolution and mechatronic handling (Kostadinov, 2010; Dantchev and Kostadinov, 2006).

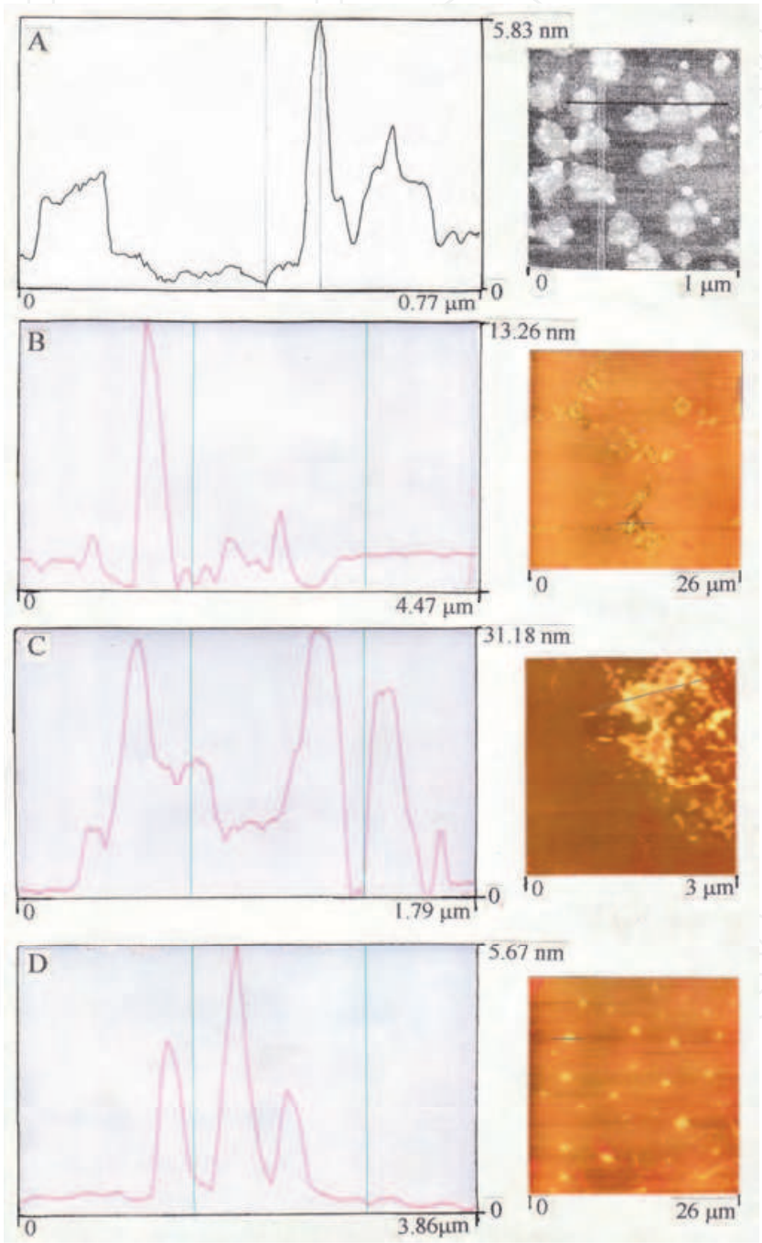

Fig. 11. AFM pictures and crosssections of LB monolayers from DP-NBD-PE deposited from pure water at 20^0 C and surface pressures of: (A) 7 mN/m; (B) 33 mN/m; (C) 43 mN/m; (D) 33 mN/m 50 days after the deposition.

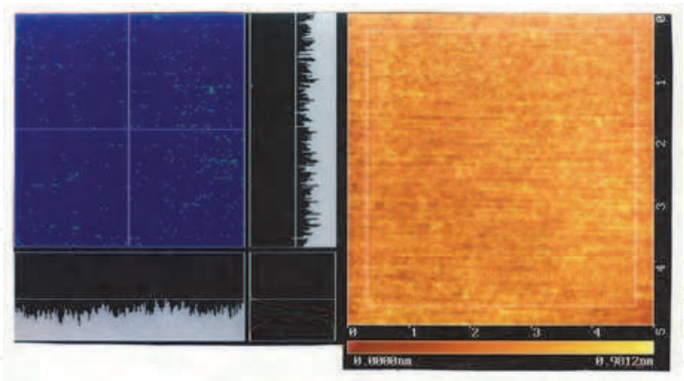

Fig. 12. AFM pictures and crosssection (left at a level of 1,04 nm) of LB monolayers from DP-NBD-PE deposited from pure water at 20^0 C and surface pressures of 3,7 mN/m. Scan size is 5 μm.

6. Molecular modelling of the DP-NBD-PE molecule at simulated air-water interface

In order to get a better understanding of the 3 newly discovered phenomena in LB films from DP-NBD-PE we have performed molecular conformational analysis at simulated air-water interface. The two most probable conformations from this analysis are shown on Fig. 14 and their characteristics are summarized in Table. 3.

Conformation	Height [nm]	phi - pho distance Δ [nm]	Area per molecule – single molecule [nm^2]	Area per molecule – in a monolayer [nm^2]
A – liquid phase	2,31	0,938	1,18	0,81
B – solid phase	3,25	1,396	-	0,69

Table 3. Characteristics of the two most probable conformations for the DP-NBD-PE molecule at the air-water interface. The conformation in the solid phase is obtained only when interactions with surrounding molecules are taken into account.

The solid phase conformation A data correspond very well to the experimental data from the previous paragraphs. The average area per molecule is 0.66 nm^2 if the isotherm from Fig. 2 is extrapolated to zero surface pressure, which almost coincides with the measured value of 0,69 nm^2. The height of the molecule measured with different methods (including small angle X-ray diffraction) is 3,1 nm, which taking into account for some interdigitation of tails in the LB multilayer structure, matches the obtained here value of 3,25 nm. Also the predictions from the measurements of molecular orientation from polarized FTIR data are fulfilled. Particularly impressive is that the benzene ring is indeed perpendicular to the substrate.

The liquid phase conformation in Fig. 13 A corresponds to the liquid film of DP-NBD-PE provided that the tails are even flatter and not in *all-trans* conformation. Indeed, the area per molecule in the liquid phase at zero pressure is about 2 nm, which is more than the 0,81 nm predicted by our model. Also AFM measurements showed a difference in height between the liquid and solid phase of about 1,6 nm. If the height of the molecule in the solid phase of 3,1 nm then for the height of the molecule in liquid phase remains 1,5 nm, which is less than

the 2,31 nm in our model. So the assumption of greater tilting of the tails, leading to a lower height of the molecule and larger area is fully justified. Scanning surface potential microscopy measurements show that there is a big difference in the surface potential of the monolayer in liquid and in solid phase, most likely due to the different orientation of the strong dipole in the NBD-group. Indeed, the orientation of this group for the conformations on Fig. 14 show an angle of almost 90 ° between them, which may explain these results. In the liquid phase conformation A just over half of the chromophores are above the air-water interface, while the NBD group in the solid phase conformation B is deeply immersed in the water. The difference in dielectric constants of the environment in both cases leads to different fluorescence quenching by the water and may explain some phenomena observed by the fluorescence microscopy.

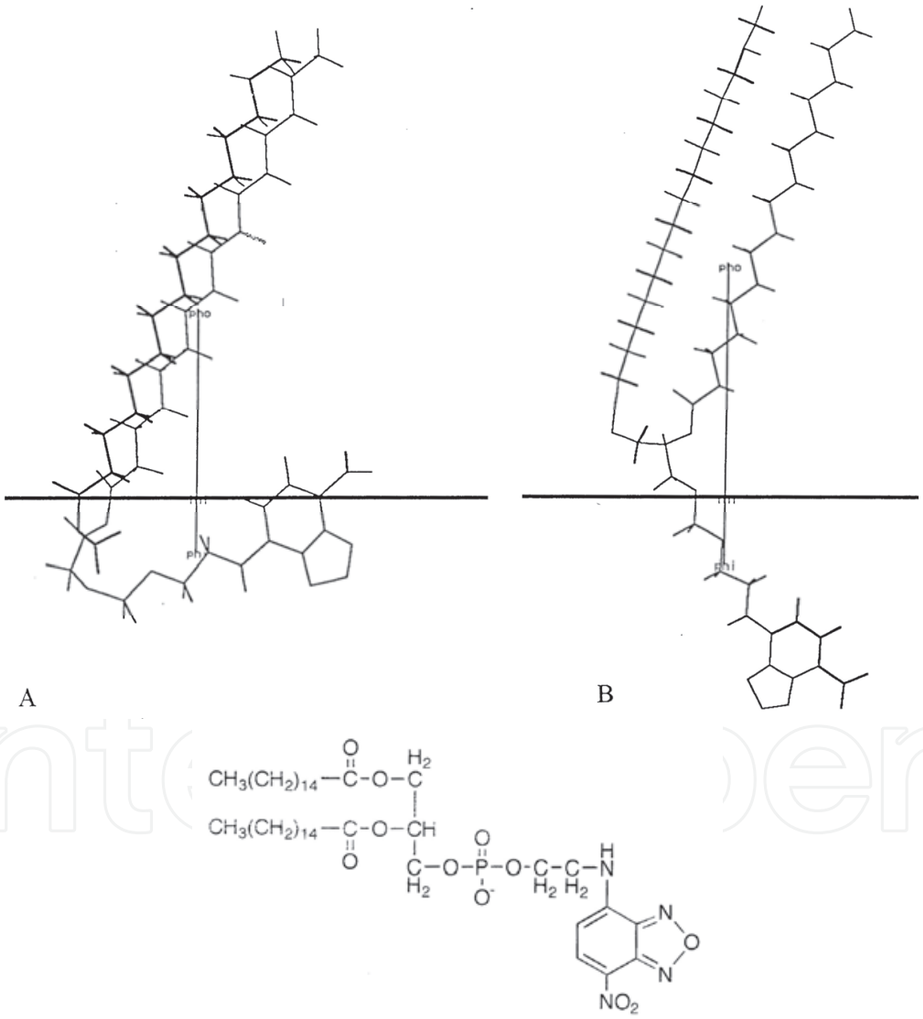

Fig. 13. The most probable conformations of DP-NBD-PE obtained from molecular conformational analysis. (A) The conformation in the liquid phase; (B) the conformation in the solid phase. Also shown are the hydrophilic (phi) and hydrophobic (pho) centers. The line that connects them is the phi-pho distance Δ in Table 3. The chemical formula of the compound is shown below.

The hydrophilic-hydrophobic balance Φ for DP-NBD-PE was calculated to be 0,55. The distance Δ between the hydrophilic and hydrophobic center in the liquid phase conformation was 0,938 nm, while in the solid phase conformation it is 1,396 nm. According to the classification of Brasseur, 1990 (vol. 1, p. 210) both conformations fall in the zone with $\Phi > 0,2$ and $\Delta > 0,43$, which is characteristic of molecules that can self assemble in organized structures. Another prediction of Brasseur is that due to the large difference in Δ of the two conformations at the molecular level they can not mix and have to form two phases. Exactly this is what we observe in our experiments and there is coexistence between liquid and solid phase and well seen phase separated domains.

7. Conclusions

We were the first to start investigating systematically films at air-water interface and on solid support from fluorescently NBD-labeled phospholipids. Previous research has shown that this is the most promising fluorophore label for sensor applications. In our investigations we use the most advanced method for preparing supramolecular architectures from organic molecules – the method of Langmuir-Blodgett film deposition and research. This is a true nanotechnology process.

Over the years we have discovered 3 new phenomena in these molecules which make them a promising candidate for chemical and biochemical sensor applications when fast response times, high sensitivity and selectivity are required. We were the first to observe fluorescence self quenching in insoluble monolayers at the air-water interface. Self quenching not only drastically decreases fluorescence intensity but also leads to a decrease in fluorescence kinetics times by an easily measurable change of over 30 %. Thus we have 2 independent channels to discriminate the effect in a sensor application. This phenomenon was understood in terms of molecular conformational change which leads to more dense molecular packing in the solid phase and radiationless energy transfer between the closely spaced molecular heads. So any change in the molecular environment which leads to this conformational change can be easily detected.

The second new phenomenon describes the influence of heavy metals on fluorescent intensity in this type of molecules. Usually large paramagnetic metal atoms are strong fluorescence quenchers. But when they are dissolved in the water subphase during the deposition process the opposite effect was observed – the fluorescence intensity was increased. This was explained by the fact that these large atoms effectively increase intermolecular distances in the head of the molecules where they attach and thus decrease the fluorescence self quenching described above. This effect can be used for heavy metal detection.

The third new phenomenon describes the possibility to deposit monolayers at some special conditions in which there is not only coexistence of solid and liquid phase but higher, bilayer or tens of nanometer high cylinders are deposited. This structure was very stable at least within several months period. It allows a much greater contact surface between the fluorescence molecules and the substances to be detected. Thus, high sensitivity sensors can be obtained without increasing their thickness. When the thickness is small so are the diffusion lengths which limit the sensor reaction time. Thus, very fast sensors with high sensitivity can be obtained. The possibility to mix selectively reacting proteins in this flexible phospholipid matrix can provide an unmatched selectivity. These properties are very important for environmental monitoring.

8. Acknowledgments

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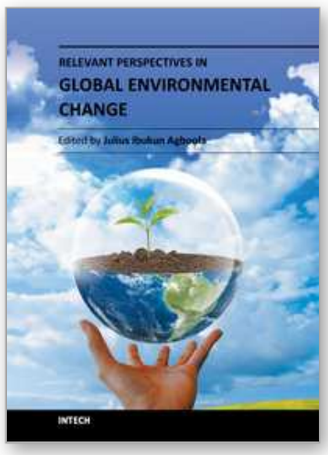

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Over the years, environmental change has sharpened significant dynamic evolution and knowledge in organizational structures of organisms, from cellular/molecular to macro-organism level including our society. Changes in social and ecological systems due to environmental change will hopefully result in a shift towards sustainability, with legislative and government entities responding to diverse policy and management issues concerning the building, management and restoration of social-ecological systems on a regional and global scale. Solutions are particularly needed at the regional level, where physical features of the landscape, biological systems and human institutions interact. The purpose of this book is to disseminate both theoretical and applied studies on interactions between human and natural systems from multidisciplinary research perspectives on global environmental change. It combines interdisciplinary approaches, long-term research and a practical solution to the increasing intensity of problems related to environmental change, and is intended for a broad target audience ranging from students to specialists.

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