Journal of Nanoscience and Nanoengineering
Vol. 2, No. 1, 2016, pp. 15-19
http://www.aiscience.org/journal/jnn

On the New Phenomenon of Fluorescence Self-Quenching in Organic Monolayers at the Air-Liquid Interface and on Solid Supports

George R. Ivanov*

Laboratory of Nanoscience and Nanotechnology, Department of Physics, University of Architecture, Civil Engineering and Geodesy and Advanced Technologies Ltd., Sofia, Bulgaria

Abstract

The novel phenomenon of fluorescence self-quenching was earlier discovered by us. It was observed in monolayers from fluorescently head labeled phospholipids, mainly Nitrobenzoxadiazole Dipalmitoyl Phosphatidyl Ethanolamine (DP-NBD-PE), at the air-liquid interface. Here we report that the morphology and film structure is preserved during Langmuir-Blodgett (LB) film transfer on the solid support as observed with fluorescence microscopy. The self-quenching phenomenon in the solid phase is interpreted as radiationless energy transfer when NBD chromophores are below the critical distance R_c of 0,94 nm in the solid phase. Possible applications for high sensitivity and selectivity biosensors are discussed.

Keywords

Fluorescence Self-Quenching, Langmuir Films, Langmuir-Blodgett Films, Phospholipids, Biosensors, Fluorescence Microscopy

Received: September 17, 2015 / Accepted: February 11, 2016 / Published online: March 1, 2016

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 or sensitivity is required. In this paper 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 from sensor application point of view - 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. Here we focus only on one of them, on understanding of the fluorescence self-quenching phenomenon.

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. 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. [1]. 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 polarity 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

* Corresponding author
E-mail address: george@at-equipment.com

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° [2] and therefore the real environment of the chromophores may be different for absorption and emission. Overview of the spectral characteristics of NBD was made [3]. The fact that NBD labeled phospholipids can serve as a flexible matrix for incorporation of selectively reacting with analyte in a solution biomolecules and the high sensitivity of the chromophore to the dielectric constant of its surrounding drew our attention to this molecule.

2. Materials and Methods

DP-NBD-PE (Sigma) was supplied in chloroform solution. The claimed purity was above 98% and it was used without further purification. MilliQ filtered ultra-pure water was used. Fluorescence microscopy was performed on air-water interface using epifluorescence, mercury lamp illumination and high sensitivity camera or on LB film monolayers deposited either on microscope slides or quartz glass substrates. For better cleaning a mid sized trough machined from bulk Teflon from Advanced Technologies Ltd. (Sofia, Bulgaria) was used. The accuracy of the area measurement was above 0.001 nm^2/molecule and the precision of surface pressure measurement was above 0.1 mN/m with 10 times higher resolution. This molecule shows good quality LB film deposition at different surface pressures. Multiple LB film layers also deposit well unlike the corresponding phospholipids. Slow compression and dipping speeds were used in order to avoid kinetic effects.

3. Results and Discussion

On Fig. 1 is shown the isotherm of a Langmuir film from DP-NBD-PE at 20°C. The isotherm is characterized with an almost horizontal region starting at 7 mN/m where the main phase transition from liquid-expanded to liquid-condensed phase occurs. In this region there is a phase coexistence between the two phases. Along with these measurements the monolayer was studied with fluorescence microscopy both at the air-water interface and on deposited LB films. The fluorescence microscopy results for 20°C at the air-water interface are published and discussed in detail elsewhere [4]. This was the first time that fluorescence self-quenching in organic monolayers at the air-water interface (Langmuir films) was described. Fluorescence microscopy for Langmuir films measured at 5°C was also published [5]. Here on Fig. 2 for the first time we publish fluorescence microscopy data for LB film monolayers on solid support.

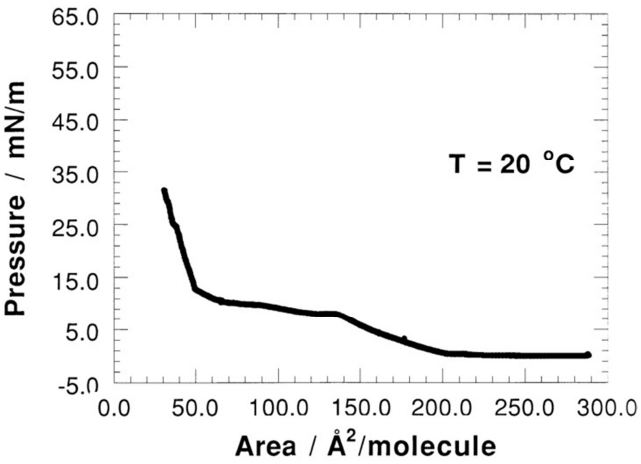

Fig. 1. An isotherm at 20°C for a Langmuir film from DP-NBD-PE. A phase transition from liquid-expanded to liquid-condensed film is seen at 7 mN/m. Another phase transition from liquid-condensed to solid phase is seen at 25 mN/m.

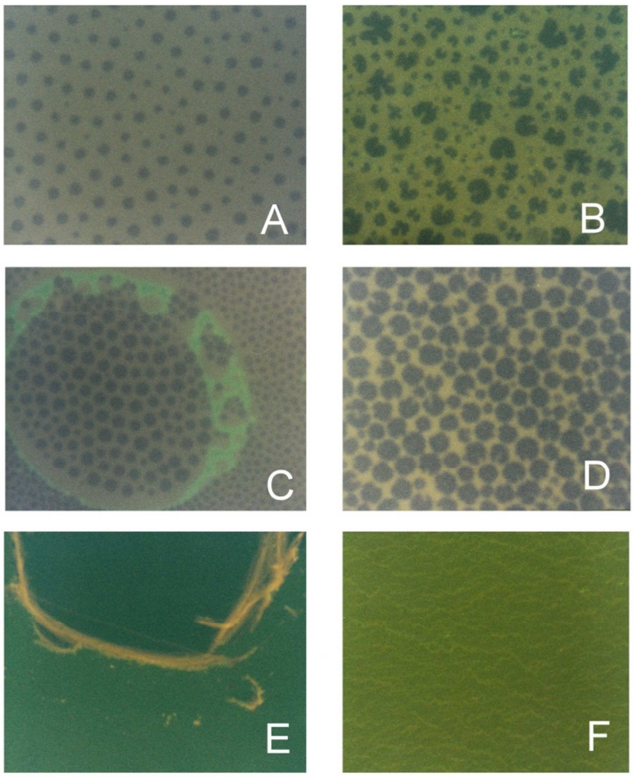

Fig. 2. Fluorescence microscopy data of an LB film monolayer from DP-NBD-PE. Picture width is 0.9 mm. From A to D deposition was carried at room temperature and increasing surface pressures from 8 to 22 mN/m. Picture E shows a collapse in the film deposited at 26.9 mN/m and 7°C. Picture F shows the same film as in E but measured 30 hours later. One can see that the collapse has developed and spread in the entire film.

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 [5] 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. Comparison of the results from Fig. 2, other unpublished data and fluorescence microscopy observations of Langmuir films [4, 5] shows complete preservation of the domain shape and size during LB film transfer. One can expect that features are preserved also at sub micrometer level and features observed with Atomic Force Microscopy on LB film monolayers are also present in Langmuir films and not due to film transfer.

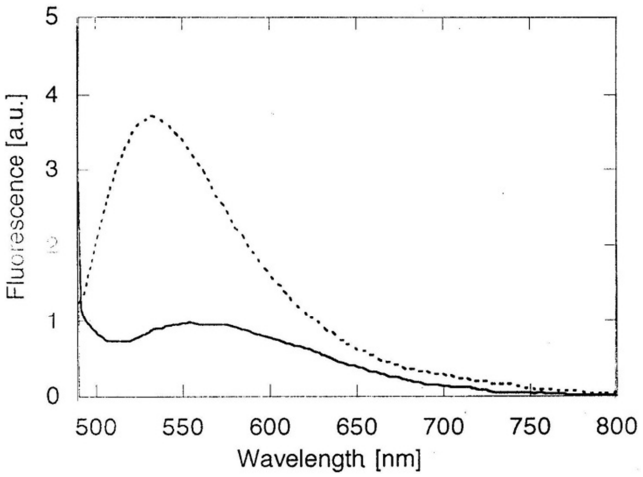

Fig. 3. Fluorescence spectrum of DP-NBD-PE in chlorophorm solution (dashed line) and deposited as LB film monolayer in the solid phase. Both the red shift of the spectrum and high fluorescence self-quenching can be seen in the LB film.

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 effect is also presented in the fluorescence spectra for DP-NBD-PE molecules shown on Fig. 3. For an LB monolayer deposited in the solid phase at high surface pressures the fluorescence is almost completely self-quenched. This new phenomenon can be used 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 detection apart from the influence on the fluorescence peak maximum, intensity and lifetime from the polarity change of the surrounding medium. The possibility for practical applications of this new phenomenon requires its better understanding.

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 and for understanding of fluorescence self-quenching. The presence of large paramagnetic metal ions can be monitored by the fluorescence quenching of the NBD chromophore. Morris et al. [6] 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 [7]. A method was proposed [8] for measuring the position of NBD chromophores in the biomembrane by quenching its fluorescence by spin-labeled at 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 DP-NBD-PE – 1,42 nm. In the case of tail NBD labeled lipids 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 [9]. Detailed analysis of the fluorescence lifetime characteristics of NBD-aminohexane acid (NBD-NH(CH_2)5$C0_2$H) at low concentrations in solvents of different polarity and donor hydrogen connection strengths was conducted [10]. This substance has aminoalkane side chain similar to the chains in which NBD chromophores is connected 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. The so-called Fluorescence Lifetime Imaging Microscopy (flimscopy) was developed which initially used 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, and 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 simulates the biological membrane, and provides improved stability against denaturation of the protein, produces 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 [11] and urea by ureaze [12]. 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 neighbouring heads. Structural changes in the membrane can lead to an analytical signal in the form of change of fluorescence intensity due to fluorescence self-quenching 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 [13 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 [14, 15]. 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 self-quenching 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 [16] 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 [17]. More recently NBD labeled molecules were used as sensors for phosphatidylserine containing membranes [18], cysteine/homocysteine [19] and for selective chemical sensing of hazardous compounds and drugs of abuse [20].

4. Conclusions

We were the first to start investigating systematically films at air-water interface and on solid support prepared by the LB method composed from only fluorescently NBD-labeled phospholipids. Previous research has shown that this is the most promising fluorophore label for sensor applications. Here we have shown that domain shape and structure on the water is preserved during film transfer on solid supports. 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. Here we focus on understanding on one of these phenomena - fluorescence self-quenching in Langmuir films. Self-quenching not only drastically decreases fluorescence intensity but also leads to a decrease in fluorescence lifetimes by an easily measurable change of over 100%. Thus we have 2 independent channels to discriminate the effect in a sensor application. The self-quencheing was understood in terms of molecular conformational change when molecule go from liquid to solid phase which leads to more dense molecular packing in the solid phase and radiationless energy transfer between the closely spaced molecular heads below the critical NBD chromophore distances R_c of 0,94 nm. So any change in the molecular environment, e.g. a change in an embedded enzyme, which leads to this conformational change can be easily measured.

Acknowledgments

This work is supported by a contract with the Centre for Research and Design of the University of Architecture, Civil Engineering and Geodesy in Sofia, Bulgaria number BN-176/15.

References

[1] Monti, J. A., S. T. Christian, and WA. Shaw, (1978). J. Lipid Research, Vol. 19 p. 222

[2] Thompson, N. X., H. M. McConnell and T. P. Burghardt, (1984). Biophys. J., Vol. 46, p. 739

[3] Suzuki, H. and H. Hiratsuka, (1988). In: Nonlinear Optical Properties of Organic Materials, SPIE Vol. 971, p. 97

[4] Ivanov, G. R. (1992). First observation of fluorescence self-quenching in Langmuir films. Chem. Phys. Lett., Vol. 193, p. 323

[5] Ivanov, G. R., G. Georgiev and Z. Lalchev - Book chapter "Fluorescently Labelled Phospholipids - New Class of Materials for Chemical Sensors for Environmental Monitoring", in "Relevant Perspectives in Global Environmental Change", Julius Ibukun Agboola (Ed.), (2011) ISBN: 978-953-307-709-3, InTech, Rijeka

[6] Morris, S. J., D. Bradley, and R. Blumenthal. (1985). Biochim. Biophis. Acta, Vol. 818, p. 365

[7] Rajarathnam, K., J. Hochman, M. Schindler, and S. Ferguson-Miller. (1989). Biochemistry, Vol. 28, p. 3168

[8] Chattopadhyay, A. and E. London. (1987). Biochemistry, Vol. 26, p. 39

[9] Arvinte, T., A. Cudd, and K. Hildenbrand. (1986). *Biochim. Biophys. Acta*, Vol. 860, p. 215

[10] Lin, S. and W.S. Struve. (1991). Photochem. Photobiol., Vol. 54, p. 361

[11] Brennan, J. D. and UJ. Krull. (1992). *Chemtech,* Vol. 22, p. 227

[12] Brennan, J. D., R. S. Brown, A. Delia Manna, K. M. R. Kallury, P. A. Piunno, and U. J. Krull. (1993*). Sens. Act. B,* Vol. 11, p. 109

[13] Brennan, J.D., K.K. Flora, G.N. Bendiak, G.A. Baker, M.A. Kane, S. Pandey and F.V. Bright. (2000). Phys. Chem. B, Vol. 104, p. 10100

[14] Brown, R. S., J. D. Brennan, and U. J. Krull. (1994). J. Chem. Phys., Vol. 100, p. 6019

[15] Shrive, J. D. A., J. A. Brennan, R. S. Brown, and U. J. Krull. (1995). Appl. Spectroscopy, Vol. 49, p. 304

[16] Nikolelis, D. P, J. D. Brennan, R. S. Brown, and U. J. Krull. (1992). Anal. Chim. Acta., Vol. 257, p. 49

[17] Brennan, J. D., K. M. R. Kallury, and U. J. Krull. (1994). *Thin Solid Films*, Vol. 244, p. 898

[18] Lakshmi C., R. G. Hanshaw, B. D. Smith. (2004) Tetrahedron 60 (49) pp 11307-11315

[19] Chen Y-H, J-C. Tsai, T-H. Cheng, S-S. Yuan, Y-M. Wang. (2014), Biosensors and Bioelectronics, 56, pp 117-123

[20] Sharma P. S., F. D'Souza and W. Kutner. (2012), Trends in Analytical Chemistry 34, pp 59-77