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To cite this article: George R Ivanov and Ivan Avramov 2019 J. Phys.: Conf. Ser. 1186 012007

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Langmuir-Blodgett Films from Fluorescently Labelled Phospholipids Deposited on Surface Acoustic Wave Devices

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Abstract. Over the years several new effects in fluorescently labelled Langmuir monolayers or Langmuir-Blodgett (LB) films, deposited on solid support plates, have been reported. Mostly layers from Dipalmytoyl Phosphatidyl Ethanolamine head labelled with Nitrobenzoxadiazole (DPPE-NBD) have been studied. This molecule behaves exactly as the DPPE molecule which is part of biological membranes. So it is expected that DPPE-NBD molecules can serve as an appropriate matrix for implementation of selectively reacting proteins or enzymes while preserving their function. This combination can act as an active layer in biosensor applications for operation in either gas or liquid environments and this molecule could further increase the system sensitivity in such applications. Addition of the chromophore NBD head group also stabilizes the film and allows for multilayer deposition which is not possible for phospholipids.

In this study, LB monolayers were deposited on glass substrates at different surface pressures and with or without Cadmium ions in the water subphase. They were studied using fluorescence spectroscopy and Atomic Force Microscopy (AFM). In view of possible biosensor applications, LB layer by layer deposition was performed on Rayleigh Surface Acoustic Wave (SAW) resonant devices working at 440 MHz. These can effectively be used to transduce the biosensor signal from the active layer by providing a mass proportional frequency output. Layers from DPPE-NBD that might also be suitable for possible SAW based biosensor applications were deposited at a surface pressure of 10 mN/m. AFM spectroscopy reveals the phase coexistence of liquid and solid phases as well as higher bilayer nanosized cylinders. Fluorescence intensity was strongly self-quenched at this pressure and fluorescence lifetime spectroscopy shows a complex behaviour with a 3-rd order exponential decay. A frequency downshift of 10 KHz per monolayer was measured with the coated SAW device which results in a deposited mass of about 1 ng/monolayer.

1. Introduction

One of the best methods for the creation of supramolecular architectures in a well-controlled manner is the method of Langmuir and Blodgett [1]. The authors of this method received the Nobel Prize in the thirties of the last century but the interest in it has been growing significantly starting from the eighties. This can be seen from the exponential growth of publications and from international conferences named after the method. This interest can be attributed in part to the fact that optoelectronics and molecular electronics have become frontier areas of material science. In both areas there are limitations to
inorganic materials and therefore, ordered organic materials become increasingly important to a variety of practical applications. Another major area where LB films have promising applications is the area of chemical and biological sensing and this is our focus based on 3 new effects studied in LB monolayers of fluorescently labelled phospholipids [2, 3, 4].

Some of the most precise chemical sensors nowadays utilize bulk acoustic wave (BAW) resonators and surface acoustic wave (SAW) devices as highly sensitive microbalances measuring extremely small quantities of chemical analytes attached to their active surface area. If that area is coated by a bioactive layer that can trap certain biological substances, a selective biosensor is obtained. In the case of a BAW resonator as a gravimetric sensor device the relationship between its frequency shift and the attached mass is given by the Sauerbrey equation. The sensor’s frequency shift is proportional to the square of the frequency but this equation assumes that the attached mass is rigid and is able to follow the substrate deformations during oscillation. However, when measuring big organic molecules in liquids this is not the case, moreover, the selected resonant frequency of the BAW resonators rarely exceeds 5-10 MHz for optimum operation in liquids. These factors limit the usability of BAW sensors for biosensing applications. SAW devices typically operate in the 30 MHz to 1 GHz range and provide much higher sensitivity compared to their BAW counterparts but, with exception of the leaky SAW mode, their operation is more appropriate for gas phase environments.

In this study we tested Rayleigh surface acoustic wave (RSAW) two-port resonators (TPR) for their ability to tolerate LB films as possible selective biosensing layers. There are several reasons why we decided to use these devices for such applications:

1. Compared to leaky wave delay lines that are widely used as biosensors, RSAW TPR have much lower insertion loss and higher Q factor resulting in substantially lower noise and detection limits in the sensor system;
2. They occupy much less substrate area and cost less accordingly;
3. They are fabricated on thermally compensated quartz cuts and feature superior thermal stability – an issue of great practical importance in real-life sensor systems.

2. Materials and Methods
DipalmitoylPhosphatidylEthanolamine head labelled with NitroBenzoxaDiazole (DPPE-NBD) was purchased from Avanti Polar Lipids (USA) in chloroform solution and was with 99.5 % claimed purity. A 5th generation of a Langmuir-Blodgett system designed by Advanced Technologies Ltd. (Bulgaria) [5] was used for this experiment. The system has several world innovations but what is important for the experiment described here is that the noise of the surface pressure sensor is less than 0.03 mN/m and constant surface pressure control accuracy is in the ± 0.1 mN/m range. Thus a precise measure on the surface area change during the LB deposition is possible and hence an estimate of the deposited mass of the substance. LB film monolayers were deposited initially on microscope cover glass slides which were tested to be smooth enough for Atomic Force Microscopy (AFM) studies. Optical spectroscopy measurements were performed in the Institute of Electronics – BAS on Horiba Scientific Fluorolog-3 spectrophotometer. AFM measurements were performed in the Faculty and Chemistry and Pharmacy in Sofia University on a Bruker Nanoscope V instrument. The test RSAW device used here, operate at 440 MHz and use a gold electrode structure to avoid possible corrosion when coming in contact with various liquids. Design details and uncoated performance are described in [6] in detail.

3. Results and Discussion
The isotherm of the DPPE-NBD molecule just prior to deposition of the first layer on the SAW device is shown on figure 1. The liquid-expanded and liquid-condensed phase coexistence region starts at around 12 mN/m at a temperature of 25° C. The surface pressure of this region strongly depends on temperature. The Langmuir film isotherm from DPPE-NBD was studied in detail using additional surface potential measurements and Brewster angle microscopy [7]. In the phase coexistence region
circular domains of the solid phase coexist with the liquid phase. With increasing surface pressure, the solid phase domains grow in diameter and eventually make a contact with each other. At this point the surface pressure starts to increase sharply.

LB monolayers were deposited on an up stroke on glass slides at a slow withdrawal speed of 0.02 mm/s and measured by fluorescence spectroscopy (figure 2) and AFM (figure 3). The deposition was carried out at the following conditions: sample 1 (NBD_PE_1) from pure water subphase and deposition surface pressure of 5 mN/m - in the liquid phase; sample 2 (NBD_PE_2) – pure water and pressure of 11.5 mN/m; sample 3 (NBD_PE_3) – pure water and pressure of 35 mN/m; sample 4 (NBD_PE_4) with a presence of CdCl in the water subphase with a concentration of 1 µg/l (5 times below the allowable concentration in the drinking water) and pressure of 8 mN/m; sample 5 (NBD_PE_5) – same subphase as the previous sample and pressure of 35 mN/m. The strong self-quenching of the fluorescence can be seen on figure 2 when the molecules come in close proximity to one another in the solid phase (sample 2). The fluorescence is restored at higher pressures because the monolayer contains very high 30 to 100 nm in height cylinders [4]. So large part of the molecules escaped in the vertical dimension and they seem to be in the liquid phase with strong fluorescence. It can be seen in the case of sample 4 that Cadmium ions dequench the self-quenched fluorescence which is another unexpected new phenomena for this molecules.

![Figure 1. Surface pressure isotherm of a Langmuir film from DPPE-NBD at 25°C prior to the deposition on the SAW resonator. The chloroform was allowed to evaporate for 25 minutes before the compression was started. In order to avoid kinetic effects the compression velocity was very low and equal to 0.04 mm/s.](image1)

![Figure 2. Fluorescence spectroscopy of LB monolayers from DPPE-NBD deposited at different surface pressures and samples 4 and 5 with Cd^{2+} ions in the water subphase.](image2)

The phase coexistence region of the solid and the liquid phase can be clearly seen on the AFM picture and cross-section of sample 2. Also, bilayer height cylinders can be clearly seen. This is another one of
the new effects for this molecule that we observed. It should be noted that the Equilibrium Spreading Pressure for this molecule is at 19.6 mN/m [4] so this monolayer deposited at 11.5 mN/m should be in thermodynamic equilibrium. From the point of view of sensor applications this bilayer cylinders with diameters in the 50 to 200 nm range lead to a much better developed surface which can significantly increase the sensitivity of a future sensor. Further, our previous AFM experiments show that the bilayer cylinders remain stable for at least 50 days [4].

As a next step, we made subsequent depositions of LB layers on the surface of the RSAW resonant device. Surface pressure in the middle of the “horizontal” plateau of the liquid – solid phase coexistence region was used. The same monolayer substance was used for all subsequent depositions. The first monolayer was deposited on the upstroke. It was found to have strong adhesion to the device surface and could not be removed later on from the device even by using the glue technique. Subsequent layers were removed from the device with this technique. A very slow deposition speed of 0.02 mm/s was used with a 1 minute relaxation time before reversing the direction of substrate movement.

Results for the surface pressure as a function of time diagram and mean area per molecule as a function of time diagram are shown on figure 4 for the deposition of layer 2 and 3. Initially the Langmuir film monolayer is compressed to 1 mN/m to compensate for the monolayer relaxation during the measuring process of the 1st LB monolayer. Then, the constant surface pressure maintenance software is switched on and it can be seen that above 1000 seconds, the surface pressure is very stable. Also, the monolayer is very stable and there is almost no change in the area-per-molecule before the deposition starts around 1200 seconds. Then, one can see the change in area per molecule when deposition of the both – the 2nd and 3rd LB monolayers was performed. From here, one can calculate the amount of substance deposited on the entire substrate. The immersed in the water subphase plate on which the resonator was attached had a surface area of around 145 mm². Assuming an active resonator area of 0.1 mm² it can be calculated that around 1 ng of DPPE-NBD was deposited with these 2 layers. A more precise evaluation on the active resonator surface is needed in order to compare results from this calculation with results from the resonant frequency change.

Figure 3. AFM of an LB monolayer from DPPE-NBD – sample 2 described in the text.
Figure 4. The deposition of layers 2 and 3 from DPPE-NBD on the RSAW resonator: a) surface pressure as a function of time; b) mean are per molecule as a function of time.

Figure 5. Frequency (upper curves) and group delay (lower curves) responses of the two-port RSAW resonator with gold electrode structure after a): deposition of a single LB monolayer and b) deposition of two more monolayers on top of the first one.

Table 1. Electrical characteristics of the RSAW devices after the LB layer depositions.

| Depositions          | Resonant frequency $f_o$ (MHz) | Insertion loss $IL$ (dB) | LM suppression (dB) | Group delay $\tau_g$ (ns) | Loaded $Q_L$ |
|----------------------|--------------------------------|--------------------------|---------------------|--------------------------|--------------|
| First LB layer       | 439.68                          | 17.54                    | 2.2                 | 2084                     | 2880         |
| Two more LB monolayers | 439.66                          | 16.83                    | 6.3                 | 2032                     | 2807         |

In figure 5 we compare the electrical characteristics of one RSAW TPR after the first monolayer deposition (figure 5 a)) and then after two more layer depositions on top of the first one (figure 5 b)). The frequency and group delay data were measured with a vector network analyzer and the characteristics indicative of the resonant behavior after coating are summarized in table 1. The frequency downshift between the two depositions is 20 kHz which, according to the mass sensitivity of 20 kHz/ng of these devices, derived in [8], results in about 2 ng deposited mass. Keeping in mind that two LB layers were deposited in the case of figure 2 b), we can conclude that the absolute weight of the phospholipids monolayer used is about 1 ng. We also observe an improvement of the longitudinal mode (LM) suppression in the acoustic devices between figure 5 a) and b) meaning that the LB film is a highly nonlinear elastic material affecting the wave propagation properties. A slight improvement in insertion...
loss and a negligible degradation in loaded Q ($Q_L = \pi f_0 \tau_g$) indicate that monolayers of phospholipid LB films are very well tolerated by RSAW TPR. This makes them suitable for biosensing applications.

4. Conclusion
LB films of DPPE-NBD were deposited on glass slides and studied with fluorescence spectroscopy and AFM. The present study confirms previously observed new effects of fluorescence self quenching in the solid phase and dequenching if there are Cadmium ions in the water. Also, bilayer height cylinders were observed. Successful deposition of LB layers on RSAW device was performed. For possible biosensor applications only one LB monolayer is needed. Present experiment shows that it is firmly attached to the surface of the SAW device, thus robust and stable over time sensors are to be expected. The change of frequency can easily be measured by connecting the SAW device to an oscillator loop and measuring its frequency shift with a frequency counter. In this study, we measured a 10 kHz per monolayer frequency down shift which is an excellent sensitivity for possible biosensor applications keeping in mind that such sensor oscillators provide a short time stability of a few parts in $10^{-10}$/s. Also, an unexpected improvement in insertion loss was observed which means that the thin LB nanofilm techniques is very well tolerated by SAW resonant devices.

Acknowledgments
This work was in part supported from contract BN-204/17 with Centre for Research and Design from the University of Architecture, Civil Engineering and Geodesy.

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