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Biosensor based on Langmuir-Blodgett film with alcohol oxidase enzyme

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Abstract: **The work examined the sensor properties of a biofilm based on phospholipid molecules 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine with immobilized molecules of the enzyme alcohol oxidase to vapors of ethyl and isopropyl alcohols. The immobilization of alcohol oxidase enzyme molecules was carried out using a Langmuir monolayer process of phospholipid molecules. The sensor coating was obtained using the Langmuir-Schaeffer technology, in which the substrate is oriented parallel to the monolayer. The analysis of microimages of the film surface obtained by atomic force microscopy, allows to present of enzyme molecules in it was established. The study of the sensory properties of the formed coatings was carried out using acoustoelectronic technology. The presence of the enzyme in the sensor coating led to an increase in the amplitude and phase responses of the acoustic delay line when interacting with vapors of the detected substance. The maximum amplitude and phase responses were recorded when the film interacted with ethanol vapor and were 1.5 dB and 19°, respectively. The work showed that the formed sensor coating has selective sensitivity to ethanol vapor. This allows us to conclude that it is possible to use this sensor coating to create an acoustoelectronic ethanol biosensor. Increasing the sensitivity of such biosensors can be achieved by varying technological parameters such as the number of layers in the film, as well as the amount of immobilized enzyme in each layer.**

Keywords: **Langmuir-Blodgett films, monolayers with immobilized enzyme, acoustoelectronic sensor, biosensor, ethanol sensor**

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Contents

1. INTRODUCTION

The active development of the medical and chemical industry requires the creation of new effective systems of technological parameters process flow monitoring. The slightest change in the concentration of precursors and chemical reagents can take a significant influence on the result of the technological process. In addition, the slightest leakage of certain chemicals can have toxicological effects on plant personnel and the environment. Such substances include saturated monohydric alcohols, which have a wide variability of effects on the human body [1,2]. In this regard, the task of determining the concentration of alcohol vapors in the environment becomes particularly relevant.

There are two types of most commonly used sensors for determining alcohol vapors in the gas phase. The first is an optical type of sensor, based on changes in the absorption and refractive coefficients of the sample being studied. The second type of sensors is based on changes in the electrical conductivity of the system. The main disadvantage of both types of sensors is their low selectivity and difficulty in determining the elemental composition of a mixture of different alcohols [3,4]. Carrying out enzymatic reactions on a touch coating is one of the ways to solve this problem [5].

There are a number of techniques that allow the immobilization of enzyme molecules in the sensor layer [6-8]. One of the effective approaches is the Langmuir-Blodgett method, which allows the formation of a highly ordered film of surfactant molecules with the simultaneous immobilization of enzyme molecules in it [9,10]. Such films can be used as a sensitive touch coating in the manufacture of various types of sensors [11]. A promising direction in sensors is the use of acoustoelectronic technologies, which are based on changes in the characteristics of an acoustic wave when interacting with the external environment.

In an acoustoelectronic device, an acoustic wave propagates in a sound pipe, the surface of which is in contact with the environment. With a certain change in the gas composition in the environment, the characteristics (amplitude, phase, speed) of the acoustic wave in the sound pipeline change, which are recorded by the measuring device [12]. A fairly common acoustoelectronic device is an acoustic delay line (ALD), in which a gas-sensitive sensor coating is located on the surface of a piezoelectric sound pipe between an electrode system of interdigital transducers (IDTs) [13,14]. The parameters of the acoustic wave in the delay line (amplitude, frequency, phase, speed) depend quite strongly on the properties of the sensor coating. Almost all acoustoelectronic sensors are based on this principle. Thus, changes in the properties of the sensor coating when interacting with the external environment, for example, such as density, elasticity, viscosity, conductivity, are the basis for the development of acoustoelectronic sensors for various purposes.

The use of this technology using LB films with immobilized proteins as a sensor coating, which biochemically selectively interact with various organic and inorganic substances, makes it possible to create a whole family of various new generation enzymatic biosensors [15-18].

The purpose of this work was to study the sensory properties of a film of phospholipid molecules (1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine – DPPE) with the immobilized enzyme alcohol oxidase (AO) to vapors of ethyl and isopropyl alcohol.

2. EXPERIMENTAL PART

2.1. Creation of sensor cover based on the Langmuir-Blodgett films with immobilized enzyme alcoholoxidaze

To form Langmuir-Blodgett films and immobilize the enzyme in them, a solution of 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE, 99%, Sigma Aldrich) in chloroform (99%, Sigma Aldrich) with a concentration of 10^{-3} was used M/l. The formation of a multilayer DPPE film with immobilized AO enzyme was performed on an LB Trough Nima KSV KN2001 installation (Nima KSV, Finland) at an aqueous subphase temperature of 22°C. An aqueous solution of AO molecules with an enzyme concentration of 0.015 mg/ml was used as a subphase.

The formation of a sensor coating based on a Langmuir-Blodgett multilayered films of DPPE phospholipid molecules with an immobilized enzyme occurred according to the following procedure. A 60 μL aliquot of a DPPE solution in chloroform was dropped onto the surface of the aqueous subphase. After 120 minutes allocated for the adsorption of the enzyme on the surface of the aqueous subphase [19], the DPPE monolayer was compressed by movable barriers with a constant rate of area decreasing equal to 1.5 cm^2/min. During the compression of the monolayer by movable barriers, the dependence of the surface pressure on the area occupied by one molecule in the monolayer (compression isotherm) was automatically recorded. Compression isotherms of DPPE monolayers formed on the aqueous subphase in the presence and absence of dissolved AO molecules are shown in **Fig. 1**. To assess the effect of AO enzyme adsorption on the surface properties of monolayers, parameters such as specific area per molecule in the liquid-condensed phase were used monolayer (A_0) and monolayer compression modulus (k) [20]. The quantity A_0 is numerically equal to the

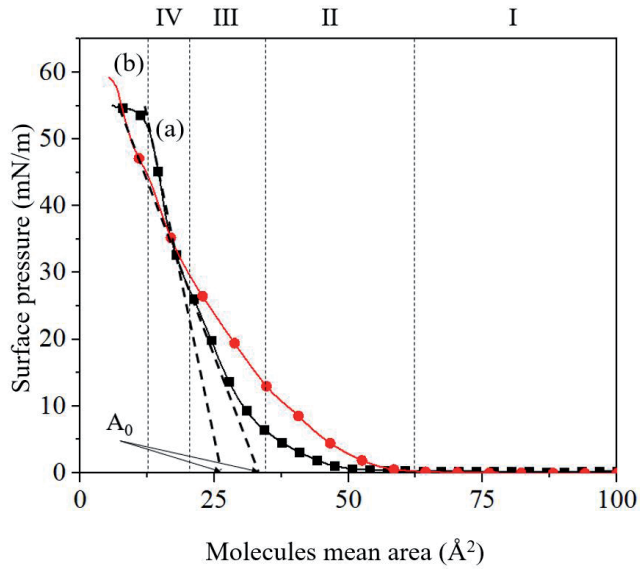

Fig. 1. *Compression isotherms of a DPPE monolayer on a subphase in the absence (a) and at the presence of dissolved AO enzyme molecules (b), where I, II, III and IV are the gas, liquid-expanded, liquid-condensed and condensed phases of the monolayer, respectively.*

coordinate of the intersection point of the straight line drawn through the condensed phase of the monolayer and the abscissa axis. The monolayer compression modulus (k) is determined from the following relation:

$$k = -A_0 \frac{d\pi}{dA}, \qquad (1)$$

where A_0, A, π are the specific area per molecule in the non-tilted condensed phase of the monolayer, the specific area per molecule and surface pressure, respectively.

The transfer of the formed monolayers to solid substrates was carried out using the Langmuir-Schaeffer method (horizontal lift) at the surface pressure value of 40 mN/m. The substrate oriented parallel to the monolayer was take into a contact with the water surface, and the monolayer was adsorbed on the surface of the substrate. The process of monolayer transfer was repeated after the drying of the formed films during the 10 minutes. So a six-layer film with immobilized enzyme molecules was formed. The plates of lithium niobate plates and mica

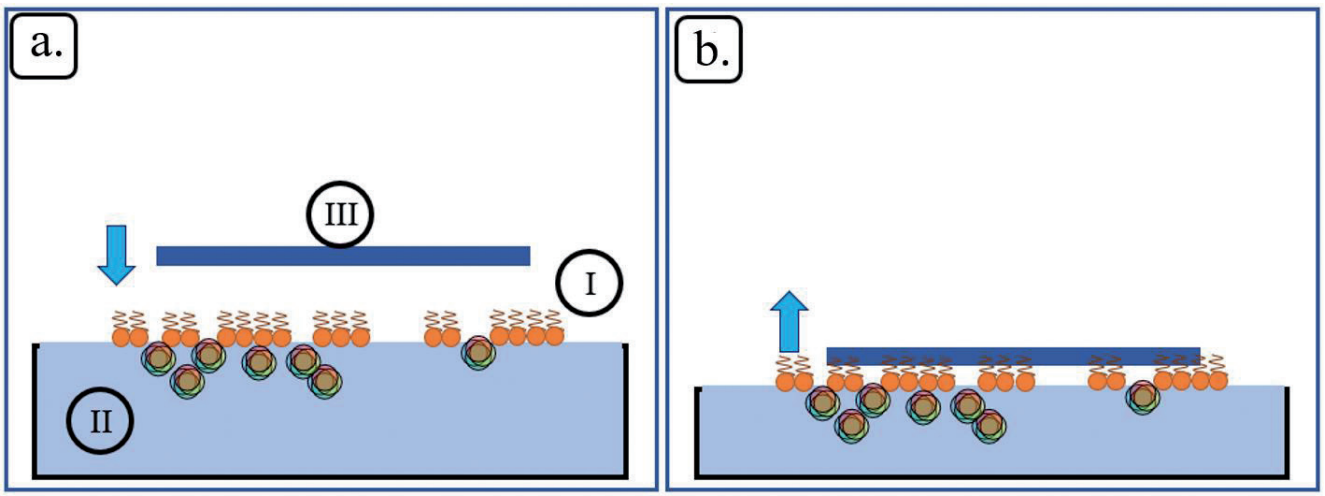

Fig. 2. *Schematic representation of the process of forming a sensor film using the Langmuir-Schaeffer technology (a. – immersion of the substrate, b – separation of the substrate from the surface of water with an adsorbed monolayer), where I is a monolayer with immobilized AO enzyme molecules, II is an aqueous subphase with a dissolved enzyme AO, III–substrate.*

plates were used as substrates. The process of transferring a Langmuir monolayer onto a solid substrate is schematically shown in **Fig. 2**.

Drying of the sensor coatings was carried out for 2 hours in a desiccator. Since the sensitive film was formed on the entire surface of the plate, after the LB film had dried, the electrode structures of the acoustic delay line were mechanically cleaned using chloroform (99% Sigma Aldrich) and ethyl alcohol (95% Sigma Aldrich). The formed coating was localized in the space between the transducers and did not affect the process of excitation and recording of the acoustic wave.

2.2. Studying by atomic force microscopy of the formed sensor covering morphology

The morphology of DPPE films with immobilized AO enzyme transferred onto mica substrates was studied by atomic force microscopy using an NT-MDT Solver setup (Russia, Zelenograd, NT-MDT). Scanning was carried out in intermittent contact mode with a frequency of 1 Hz. NSG10 probes (NT-MDT, Russia) with a radius of curvature of the probe tip of 10 nm were used for scanning. Surface images obtained from studying the films are shown in **Fig. 3**.

To quantify the properties of the film surface, the average film roughness (R_a) was

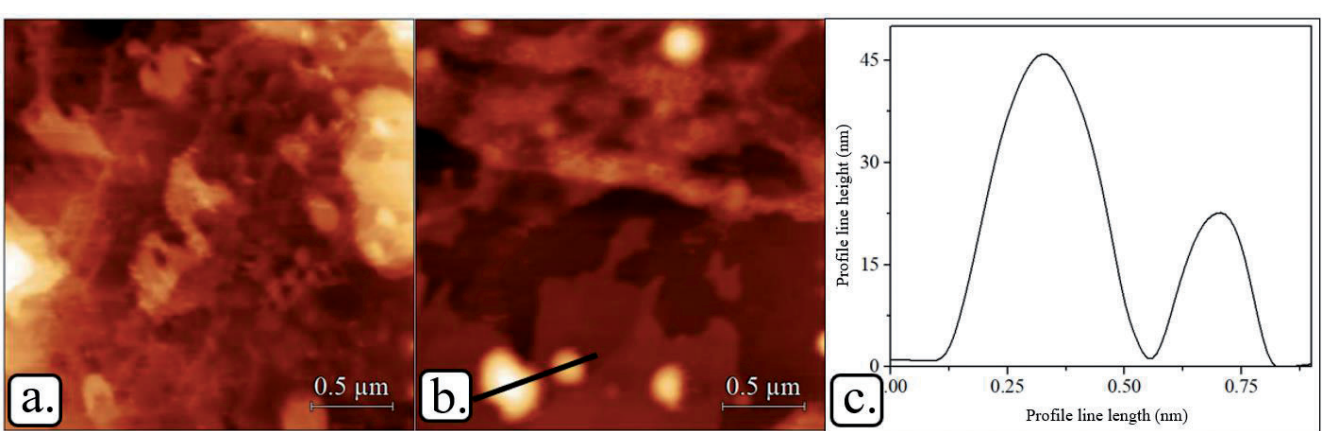

Fig. 3. *Images of the surface of the DPPE film formed in the absence (a) and in the presence (b) of AO enzyme molecules dissolved in the subphase, and the profile line of the film surface with included aggregates of enzyme molecules (c).*

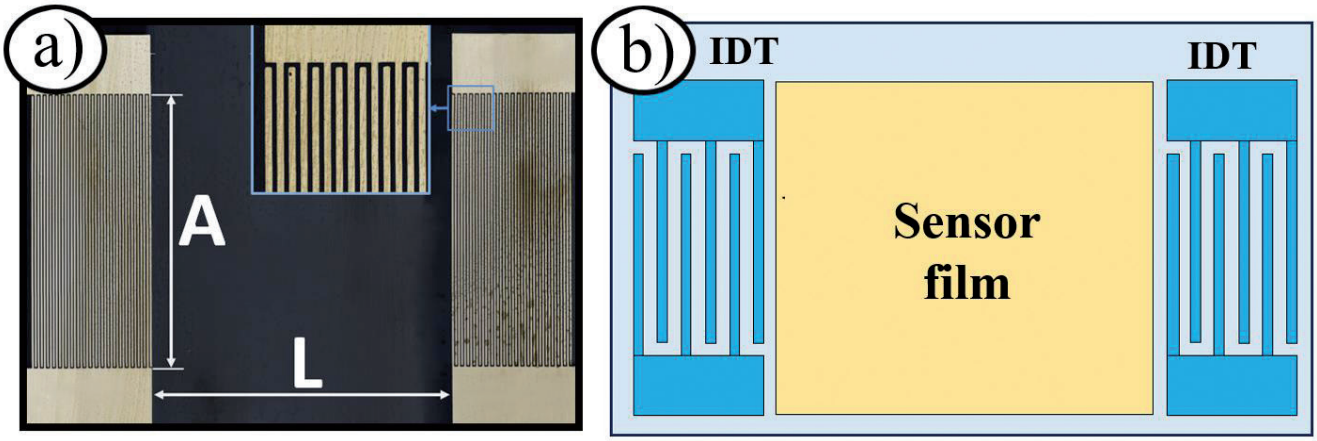

Fig. 4. *Optical image of the electrode structures of the created delay line (a), schematic image of the location of the sensitive sensor coating on the surface of the lens (b).*

calculated. The calculation was performed automatically using software Gwyddion 2.62 [21,22].

2.3 Studying of the sencoric properties of the created films by acoustic electronic method

2.3.1. Creating an Acoustic Delay Line

The acoustic delay line was formed on a piezoelectric substrate made of lithium niobate 128YX, dimensions 25×15 mm and thickness 2 mm. Electrode structures in the form of interdigitated converters (IDTs) were manufactured using maskless photolithography and magnetron sputtering of aluminum. **Fig. 4***a* shows an optical image of the formed electrode structures of the acoustoelectronic delay line obtained using an OlympusLext laser confocal scanning microscope. Transducer aperture A = 9 mm, distance between transducers L = 10 mm. The operating wavelength is determined by the distance between adjacent IDT electrodes and was 200 μm. Each interdigitated transducer contained 20 pairs of pin electrodes. Fig. 4*b* shows a schematic representation of the area where the sensitive coating is located on the surface of the delay line.

Fig. 5 shows the amplitude-frequency dependences of the S_{12} parameters of the acoustic delay line in the absence of a coating, with a DPPE coating formed, and with a DPPE+enzyme coating.

It is clear from the graphs that the coating does not have a significant effect on the type and level of the acoustic signal.

2.3.2. Measuring stand for biosensor research

The study of the gas-sensitive properties of the formed coatings was carried out using an automatic measuring complex. It schematic

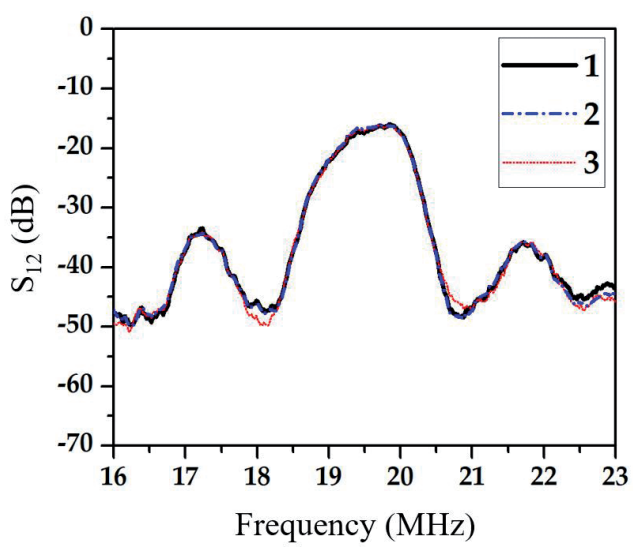

Fig. 5. *Frequency dependences of the S_{12} delay line parameters in the absence of a coating (1), with a formed DPPE coating (2) and a DPPE+enzyme coating (3).*

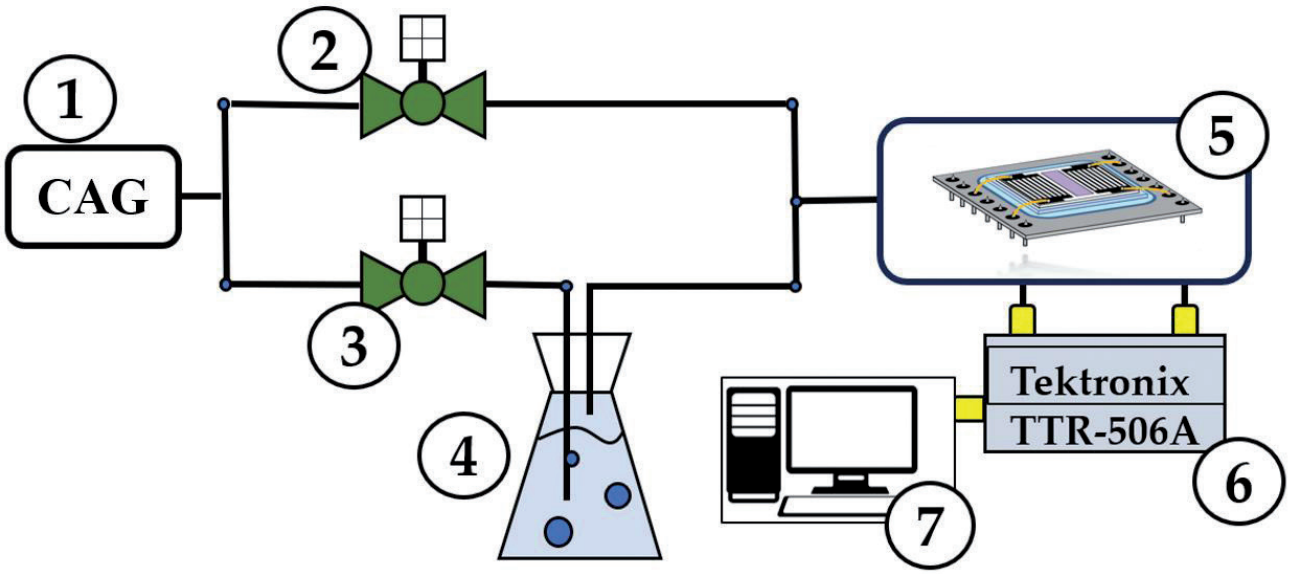

Fig. 6. *Schematic representation of the measuring stand (1) clean air generator (2) dry air flow meter (3) dry air flow meter for supply to the bubbler (4), measuring chamber (5), vector network analyzer (6) and personal computer (7).*

representation is provided in **Fig. 6.** The flow of clean, dried air from the clean air generator (1) is supplied through stainless steel lines to flow meters (2) and (3), the flow meter (2) regulates the flow of dry air used to purge the measuring chamber and diluting the test gas mixture flow. The flow meter (3) regulates the flow of dry air supplied to the aerator (pore size 20 microns) of the bubbler (4). When bubbling, dry air is saturated with vapors of the test liquid (propanol and ethanol) and at the outlet of the bubbler a mixture of dry air and vapors of the test substance is obtained at a concentration close to saturated. Adjusting the ratio of gas flows passing through flow meters (2) and (3) made it possible to supply a gas mixture with the desired concentration of the test substance into the measuring chamber (5). Using a Tektronix TTR-506A vector network analyzer and a personal computer, changes in the acoustic signal were recorded when exposed to different concentrations of the tested substances.

Detailed information on the measuring stand is presented in the works: [15,23].

3. RESULTS AND DISCUSSION

3.1. Effect of adsorption of the enzyme alcohol oxidase on the surface properties of DPPE monolayers

Fig. 1 shows compression isotherms of DPPE monolayers formed on the subphase in the absence and presence of dissolved AO enzyme. In the absence of AO enzyme molecules dissolved in the subphase, phospholipid DPPE molecules form a stable monolayer. On the compression isotherm, gaseous, liquid-expanded (II), liquid-condensed (III) and condensed phases (IV) can be distinguished. In the condensed phase, the values of A_0 and k take values of 25.2 Å^2 and 106.68 mN/m, respectively. The addition of dissolved AO enzyme molecules to the subphase affects the surface properties of the DPPE monolayer, so A_0 increases to 35.7 Å^2, and k decreases to 68.5 mN/m. There is a shift in the compression isotherm towards larger areas occupied by one molecule. This effect is explained by the incorporation of AO enzyme molecules into the Langmuir monolayer of DPPE. In the presence of AO molecules dissolved in

the subphase, the gas phase-liquid-expanded phase phase transition occurs at large areas per molecule. This effect is associated with the adsorption of AO molecules on the surface of water, which leads to an increase in the total number of molecules located on it [19]. A decrease in k and an increase in A_0 shows that the adsorption of AO molecules has an expanding effect on the structure of the DPPE monolayer.

3.2. Morphology of Langmuir-Blodgett DPPE films with immobilized alcohol oxidase (AO) enzyme

Fig. 3 shows images of the surface of a DPPE film formed in the absence and presence of AO enzyme molecules dissolved in the subphase, obtained by studying it using atomic force microscopy. Multilayer Langmuir-Blodgett films have a developed morphology. This is due to the formation of pores in the film during the evaporation of water captured during the transfer of monolayers [15] on a solid substrate. In Fig. 3*a* it is seen the 6-layer DPPE film, formed on the subphase in the absence of dissolved AO molecules, has a developed surface with an average roughness of about 3 nm. The surface roughness of a six-layer DPPE film with immobilized AO enzyme molecules is on the order of 5 nm. On the surface of the film, individual aggregates of AO enzyme molecules are visible with heights from 15 to 40 nm and occupied areas of the order of 0.2 μm^2. The minimum heights of the observed aggregates are values comparable to the dimensions of the AO molecule, which are 10 nm×10 nm×10.5 nm [24]. The presence of adsorbed aggregates of AO enzyme molecules leads to structural changes in the film, which leads to an increase in the roughness of the film surface.

3.3. Sensor properties of Langmuir-Blodgett DPPE films with immobilized enzyme alcohol oxides (AO)

The alcohol oxidase enzyme catalyzes the oxidation of short-chain aliphatic alcohols to their corresponding aldehydes according to the following scheme [25]:

$$RCH_2OH + O_2 \xrightarrow{\text{alcohol oxidase}} RCHO + H_2O_2. \quad (2)$$

During reaction (2), a change in conductivity occurs in the region where the AO enzyme is localized. In the samples under study, the en enzyme is localized in the LB layer up to 15 nm thick, and a change in its conductivity will affect the electrical properties of the acoustic wave probing it. **Fig. 7** shows the concentration dependences of the change in the amplitude of the acoustic wave (ΔS_{12}) in the acoustic delay line at a frequency of 19.7 MHz during the interaction of an air mixture containing

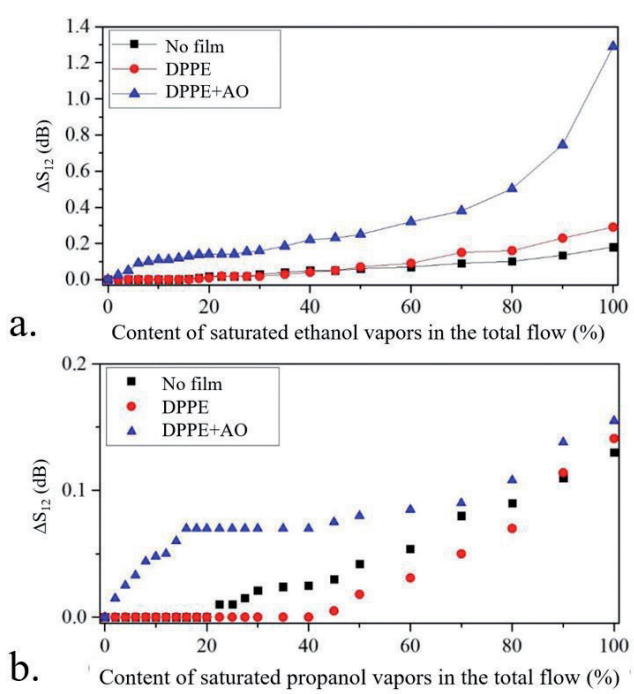

Fig. 7. *Concentration dependences of the change in the amplitude of the acoustic wave in the acoustic delay line at a frequency of 19.7 MHz for ethanol (a) and propanol (b) vapors for pure LZ, LZ with a deposited DPPE film and LZ with a deposited DPPE film with immobilized AO enzyme.*

various concentrations of ethanol or propanol vapor with the formed sensor coatings.

The attenuation of an acoustic wave in an acoustic laser without and with an applied coating that does not contain immobilized AO enzyme molecules when interacting with ethanol and propanol vapors is 0.15 dB. The presence of immobilized AO enzyme molecules in the film coating leads to an increase in the attenuation of the acoustic wave from 0.2 dB to 1.3 dB at 100% ethanol vapor content in the sample flow. The attenuation of an acoustic wave in an acoustic laser coated with a film coating with an immobilized AO enzyme upon interaction with propanol vapor is also 0.15 dB.

Fig. 8 shows the concentration dependences of the change in the phase of the acoustic wave in the acoustic delay line at a frequency of 19.7 MHz for ethanol and propanol vapors for pure LZ, LZ with a deposited DPPE film, and LZ with a deposited DPPE film with immobilized AO enzyme. The impact of propanol vapor on the LZ with an applied sensor coating without an enzyme and with an enzyme leads to a phase shift of the acoustic wave by 2 and 3, respectively. Thus, the formed sensor coating has virtually no phase sensitivity to propanol vapor.

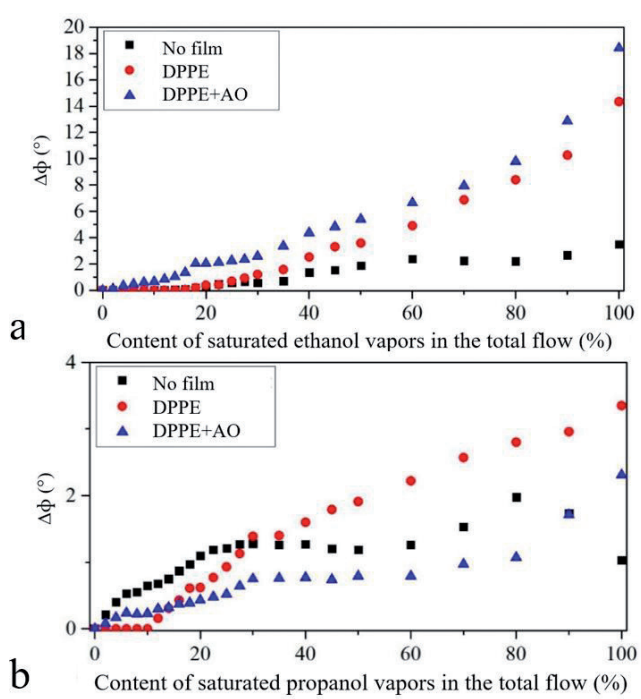

Fig. 8. *Concentration dependences of the change in the phase of the acoustic wave in the acoustic delay line at a frequency of 19.7 MHz for ethanol (a) and propanol (b) vapors for pure LZ, LZ with an applied DPPE film and LZ with a deposited DPPE film with immobilized AO enzyme.*

The effect of ethanol vapor on a laser without a film coating leads to a change in the phase of the acoustic wave by 3°. The presence of a film coating on the surface of the LZ without and with an immobilized enzyme leads to an increase in the maximum phase shift to 14° and 19°, respectively. In this case, in the range of ethanol vapor concentrations in the total sample flow from 0 to 20%, the presence of a DPPE film with an immobilized enzyme leads to an increase in the phase response to 2. Thus, it was shown that the formed sensor coating has selective sensitivity to ethanol vapor. The selectivity of the formed sensor coating is based on the differences in the catalytic activity of the AO enzyme used in relation to the alcohols used [26].

CONCLUSION

The work studied the morphology and sensory properties of Langmuir-Blodgett films of phospholipid DPPE molecules with immobilized AO enzyme molecules to vapors of ethyl and isopropyl alcohols. The presence of the AO enzyme in the formed film coatings was demonstrated using atomic force microscopy. In the course of studying the gas sensitivity of the formed sensor coatings, it was shown that LB films have the greatest sensory response to ethyl alcohol vapor. Thus, the maximum change in the amplitude and phase of the acoustic wave in the acoustic delay line in the presence of ethanol vapor was 1.5 dB and 19°, respectively. In this case, in the range

of ethanol concentrations in the sample flow from 10 to 70%, the amplitude response has a character close to linear.

This allows us to conclude that it is possible to use this sensor coating to create an acoustoelectronic ethanol biosensor. Increasing the sensitivity of such biosensors can be achieved by varying technological parameters such as the number of layers in the film, as well as the amount of immobilized enzyme in each layer.

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