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Optical Immunosensor Based on Nanostructured ZnO Thin Films for Agricultural Purposes

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***Abstract* — Optical, structural and adsorptional properties of ZnO nanostructured thin films have been studied for the development of highly sensitive optical immunosensor. Photoluminescent properties of ZnO thin films, deposited by atomic layer deposition (ALD) on the silicon substrates were applied for determination of Grapevine virus A-type proteins (GVA-antigens). The immobilization of anti-GVA antibodies on the surface of ZnO films resulted in the increase of photoluminescence intensity of NBE peak of ZnO and appearance of new photoluminescence band in the region of 400-550 nm. The GVA-antigen detection was performed by the evaluation of changes and behavior of a photoluminescence band around 425 nm appeared after immobilization of GVA antibodies on the surface of ZnO thin films. The sensitivity of the obtained label-free biosensor towards GVA-antigens was in the range from 1 pg/ml to 10 ng/ml. The possibility to detect GVA-antigens without additional labels (e.g enzymes or fluorescent dyes) has been demonstrated. Some aspects of the mechanism of interaction between ZnO and TiO_2 nanostructures and proteins are discussed.**

Keywords — ZnO; photoluminescence; biosensor; immunosensor; protein based immune complexes.

I. INTRODUCTION

Immunosensors are the class of biosensors based on the reaction between antibody and antigen by formation of an immune complex. The interaction between antigen-antibody couple is mostly highly specific and selective one [1,2]. Immunosensors are bio-analytical devices dedicated for selective determination of biological targets, which are mainly based proteins [3,4]. The main challenges in the development of immunosesnors are: (i) selection of the most selective biomolecules, which are able to form complex with analyte; (ii) proper immobilization of biological materials, which are providing selective recognition of analyte [5,6] and (iii) the application of the most efficient analytical transduction system [1]. Different biomolecules can be applied for the formation of biological recognition layer dependently to what kind of material belongs the analyte. If the analyte is antibody then antigens, which can be recognized by these antibodies, are the most suitable for the formation of biological recognition layer [7-10]. If the analyte is protein against which the selective-antibodies are available, then these antibodies are very well suitable for the formation of biological recognition layer [3,11,12].

Among the most efficient immobilization methods the application of conducting polymers for the entrapment of proteins, which exhibit biological recognition properties seems very promising [5-8]. However such immobilization method has significant drawbacks related to random orientation of immobilized biomolecules and coverage of some binding-able sites within polymeric matrix [5,6]. Therefore, the self-assembled-monolayers based technologies are applied as alternative methods for the immobilization of proteins on the surface of signal transducer [3,13], however this method also mostly provides only random orientation of immobilized molecules due to number of sites ($-NH_2$ and/or -COOH) suitable for the covalent immobilization of proteins. Very good immobilized orientation can be achieved by pre-modification of surface with protein G which is followed by antibody immobilization [14], or immobilization of separated fragments of antibodies [3,4,9,15-19]. Calculation and evaluation of thermodynamic parameters allows to predict sensitivity and reusability of newly designed immunosensors based on immobilized antibodies [20]. In addition to the selection of proper biomolecules and most suitable immobilization method, it is very important to apply the sensitive signal transduction methods. In the development of

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immunosensors many different types of signal transducers can be applied, e.g. for non-transparent samples electrochemical [5,21] methods or resonant oscillators such as quartz (QCM) [11] or capacitive micromechanical transducers (cMUTs) [22,23] transducers are very well suitable. Atomic force microscopy based transducers also seems very promising in the determination of immune-complexes [24]. However mentioned methods mostly suffers from relatively low sensitivity, therefore, optical methods are applied due their better sensitivity. Optical methods of detection based on light absorbance, reflectance or other optical signal offers fast and accurate detection of the target analyte. The main advantage of the optical transduction is a contactless detection of biomolecular interaction, i.e. without contamination and/or significant damage of bio-samples. Among optical methods such techniques as ellipsometry [9,14,15,25], or surface plasmon resonance [26-28] should be mentioned, because both here mentioned techniques are well suitable for label free determination of formation of immune-complex. However both these techniques require relatively sophisticated equipment therefore are not very easy in practical application and are relatively expensive. Therefore, other techniques, which are much cheaper and more sensitive are required for this purpose. Here photoluminescence based transducers seems very suitable [1]. In order to design a label-free photoluminescence (PL) based sensor the photoluminescent substrate suitable for the immobilization of biological recognition part is required. Such PL-able substrates can be based on metal oxides [29]. Among such oxides Zinc oxide (ZnO) and Titanium dioxide (TiO_2) based PL-substrates seems the most promising. Nanostructured Zinc oxide (ZnO) layers are known as materials with unique combination of physico-chemical (i.e. optical, electrical, adsorptional etc.) properties [30] suitable for a wide range of various biosensors [31-33]. The biocompatibility, high chemical stability and strong adsorption ability of the given metal oxide along with the abundance in nature make ZnO very suitable for the immobilization of biomaterials, which are applied as biological recognition parts in biosensors [34]. Being wide band gap semiconductor (Eg ~ 3.37 eV) with good optical properties, ZnO nanostructures are broadly applied in PL-based optical biosensors [30]. Optical properties of ZnO in particular, an intense photoluminescence at room temperature, allow the use of this material as transducer in optical immunosensors [35]. Out of many optical approaches, the PL-based methods are among the most simple and sensitive ones [1].

In this research an influence of GVA immune complex (Grapevine virus A-type proteins) on the PL spectra of nanostructured ZnO films has been analysed and applied for the development of PL-based immunosensors.

II. ZnO-based immunosensor for the determination of GVA antigens

ZnO thin films of 110 nm thicknesses were formed by atomic layer deposition (ALD). The SEM images show a conformal coating of the Silicon substrates by ZnO films with a rough surface of the samples. GIXRD analysis indicated a hexagonal wurtzite structure of ZnO. The detailed characterization of structural and surface properties is described by authors in [31]. The principle of the PL-based biosensor action is based on the changes of the PL signal before and after immobilization of the biosensitive layer on the surface and after the interaction of immobilized bio-recognition elements with the analyte (Fig. 1). As one can see, the PL-based biosensors allow the detection of biological interaction without any additional labels such as fluorescent dye or quantum dots, what makes the procedure much easier. Photoluminescence spectra of the ZnO films were characterized by NBE peak at 378 nm and weak DLE emission in the visible range (Fig. 2, line *a*).

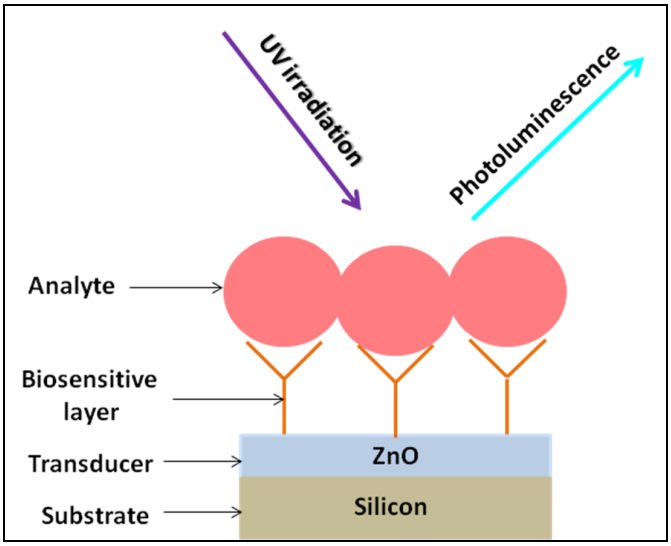

Fig. 1. The scheme of optical (PL-based) immunosensor.

A. Functionalization of ZnO films

The GVA proteins used in the experiment were provided by National Scientific Centre "Institute of Viticulture and Wine Making named after V. Ye. Tairov" (Odesa, Ukraine). The anti-GVA antibodies were dissolved in the PBS, pH=7.4, and this solution was equally distributed on the surface of the as grown ZnO film modified substrates with the size of 5×5 mm and incubated for 1 hour in humid environment at room temperature. After this, the samples were washed with PBS and then dried in air for 1 hour at room temperature. The same protocol was further used for GVA antigens specimen dilutions. The biosensitive layer was formed by the immobilization of anti-GVA antibodies on the surface of ZnO which resulted in the increasing of NBE peak intensity and appearance of a new PL band in the range from 400 to 550 nm (Fig. 2, line *b*). No NBE peak shift was observed. The optimal concentration of anti-GVA antibodies immobilized in the biosensitive layer was determined experimentally to be at 1/200 dilution of initial anti-GVA containing solution.

B. Immunosenor performance

The sensitivity of ZnO based immunosensor towards target analyte GVA-antigen marked as "Ag+" was tested using the concentrations of GVA-antigen in the range from 1 pg/ml to 1 µg/ml. The interaction between $ZnO_{110\ nm}$/anti-GVA and GVA-antigen resulted in the decrease of the NBE peak

intensity for ZnO_{110nm}/anti-GVA/Ag+-based structures (Fig. 3). However, the initial variation of the NBE peak intensity for all ZnO_{110nm} samples disabled the application of NBE intensity changes as a sensor signal. For this reason, the spectra were normalized. The response of the biosensor was based on the change in the intensity of the PL band at 425 nm corresponding to the protein related luminescence, caused by immobilized anti-GVA. The response of the as formed immunosensor was observed at the antigen concentrations from 1 pg/ml to 10 ng/ml, where the intensity of protein related PL line decreased with the increase of the antigen concentration. The further increase of the antigen concentrations led to the increasing PL intensity and full saturation of the PL signal at the 1 µg/ml.

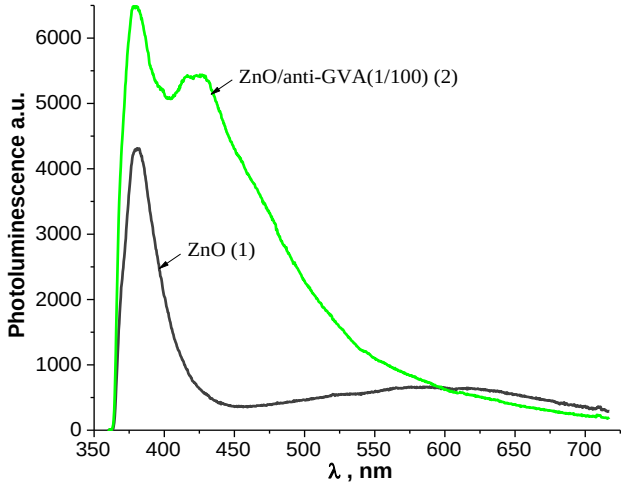

Fig. 2. PL spectra of ZnO 110nm and ZnO 110nm functionalized with anti-GVA using 1/100 diluted initial anti-GVA solution.

The biosensor response (S) was calculated using the equation:

$$S = (I_{Ab} - I_{Ab\text{-}Ag})/I_{Ab} \quad (1)$$

where: I_{Ab} is a PL intensity at 425 nm of ZnO_{110nm} with immobilized anti-GVA; I_{Ab-Ag} is the PL intensity at 425 nm of ZnO_{110nm} with immobilized anti-GVA and GVA-antigen.

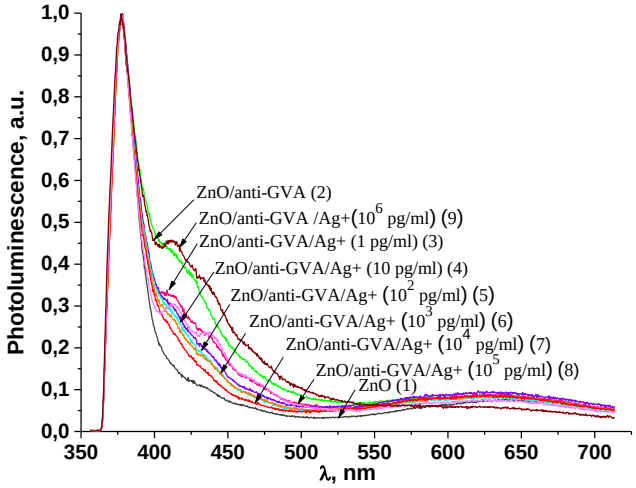

Fig. 3. PL spectra of ZnO100nm/anti-GVA/Ag+ imunosensor after the incubation in different GVA-antigen concentrations containing samples (Ag-positive or Ag+).

The sensitivity of the obtained biosensor to the GVA antigen was in the range from 1 pg/ml to 10 ng/ml. The selectivity of the immunosensor was checked by the incubation of ZnO/anti-GVA-based structure with control-specimen "Ag-", isolated from the non-infected grapevine plants and did not contain any GVA proteins. Such incubation resulted in the decrease of NBE peak intensity and PL emission in the region of 400-500 nm. However, unlike the case of GVA Ag+ specimen, PL lines, related to the GVA emission, have overlapped with each other and intersected at wavelength around 425 nm. Therefore, no specific biosensor response was observed in the absence of GVA antigens.

C. Analysis of the results

In the case of direct adsorption of the biosensitive layer (anti-GVA antibodies) the binding between ZnO and the biomolecules occurs due to Van der Waals forces, electrostatic interaction and hydrogen bonds [3]. The increase of the PL intensity of the NBE peak after the formation of the biosensitive layer could result from the charge transfer between anti-GVA molecules and the conductance band of ZnO [3,7,9].

Quenching of the main ZnO peak and the decrease of the PL intensity related to anti-GVA antibodies (proteins) after their interaction with target-analyte (GVA antigen), may be induced by several reasons [3,36,37]:

1) The surface reaction with a quencher may introduce non-radiative surface defects;

2) Charge transfer from a radiative material to a quencher [36];

3) Collisional PL quenching mechanism is responsible for the recognition of the analyte [37].

The appearance of the protein related luminescence band in the range of 400–500 nm can be caused by radiative transitions from defect states in the forbidden gap to valance band of ZnO. From the other side, the increase of PL peak at 425 nm observed at high concentrations of anti-GVA antibodies is in the same region as the blue emission peak of Zinc Sulfide (ZnS) [38,39]. This fact indicates that during the adsorption process the disulfide bonds formed between the chains of anti-GVA, at least partly dissociate and forms strong complexes (-O-Zn-S-anti-GVA) with Zn atoms from the ZnO structure [31].

III. Conclusions

The results presented in the given report indicate that photoluminescent properties of nanostructured ZnO films can be applied for determination of the presence and concentration of bio-analytes, such as GVA-antigens. Considering the physical and chemical properties of ZnO (i.e. high chemical stability, good biocompatibility, etc.) it has great prospects to be used as transducer material in optical biosensors, since its

photoluminescence is easily excited and registered as a biosensor signal, and the interaction with proteins changes the optical properties of ZnO.

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