



## Photoluminescence immunosensor based on bovine leukemia virus proteins immobilized on the ZnO nanorods

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### ARTICLE INFO

#### Keywords:

Optical immunosensor  
Bovine leukemia virus (BLV)  
ZnO nanorods  
Photoluminescence  
Antigen-antibody complex

### ABSTRACT

Bovine leukaemia virus (BLV) proteins gp51, which are serving as antigens for specific antibodies against BLV proteins (anti-gp51), were applied as biological recognition part in the design of immunosensor devoted for the determination of anti-gp51. The efficiency of the immobilization of BLV proteins gp51 on ZnO nanorod (ZnO-NR) modified glass (ZnO-NR/glass) surface was evaluated. The formation of antigen-antibody complex on the ZnO/glass modified by the BLV proteins gp51 (gp51/ZnO-NR/glass) was investigated by the determination of changes in ZnO photoluminescence. The applicability of gp51/ZnO-NR/glass in the design of photoluminescence based immunosensor was evaluated. Bovine serum albumin (BSA) was applied for the modification of sensing gp51 layer in order to form gp51&BSA layer with advanced selectivity. Polyallylamine hydrochloride (PAH) was applied in order to improve the immobilization of gp51 and BSA based sensing layer (gp51&BSA) on the surface of ZnO-NR/glass. PAH was applied during the formation of gp51&BSA/PAH/ZnO-NR/glass structure. Some aspects of the mechanism of interaction between biomolecules (gp51, BSA and anti-gp51) and ZnO-NR during the preparation and action of gp51&BSA/ZnO-NR/glass- and gp51&BSA/PAH/ZnO-NR/glass-based immunosensors have been discussed.

### 1. Introduction

Express determination of retroviral infections among humans and animals is an important global problem. Bovine leukemia virus (BLV) induces one of several recently known retroviral infections, which affects cattle all over the world and is endemic in many dairy herds [1]. Cattle of all ages may be infected by BLV but the most of them might be infected soon after the birth by ingestion of milk from an infected cow. Therefore the main way to prevent the spread of diseases caused by retroviral infection is the timely diagnosis [2]. Recently used BLV detection methods are time- and labor-consuming and therefore they do not meet recent requirements. In order to improve diagnosis a number of different BLV infection detection methods were developed, including polymerase chain reaction [3] and different types of the immunological

methods such as enzyme-linked immunosorbent assay, agar gel immunodiffusion, radioimmune assay, immunoblot test [4,5], etc. The traditional immunological methods have high specificity and sensitivity, but the most of them are time consuming and/or require some additional expensive materials, such as the labeled antibodies [6]. Because BLV is one of the most common infectious of cattle and is endemic in many dairy herds, thus control and eradication programs based on fast, simple, and sensitive BLV detection method are highly desirable. These requirements can be successfully solved using immunosensors [7–10].

Practical application of electrochemical immunosensors, even if they demonstrate high sensitivity to analyte and are less sensitive to the non-specific adsorption of interfering materials, still have several drawbacks: (i) sometimes additional redox mediators are required in

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<https://doi.org/10.1016/j.snb.2019.01.054>

Received 7 August 2018; Received in revised form 21 December 2018; Accepted 12 January 2019

Available online 14 January 2019

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order to generate amperometric response, (ii) the signal of such devices depends on characteristics of aliquot (e.g. ionic strength, pH, etc), (iii) the regeneration of surface in order to use it for multiple analyte detection is not always successful [6,7,11,12]. Therefore, in some cases label-free immunosensors are more preferable. Several surface plasmon resonance (SPR) [7], optical ellipsometry [8], acoustic [9] and photoluminescence (PL) [13] based BLV sensors were developed. Most of above-mentioned sensors are optical, where metal and metal oxide based structures are applied. Some metal oxide based nanorods can provide a possibility to detect analytical signal of immunosensor directly (without the usage of additional reagents) and rapidly (within 3–5 minutes), inexpensively (cost of single analysis is less as a one US dollar) [14]. Among many metal oxides zinc oxide (ZnO) also has attracted interest of biosensorics related researches due to suitable semiconducting properties [15], which are based on wide and direct optical band gap (3.3 eV), high exciton binding energy (~60 meV) and possibility to tune a band gap in the range from 2.1 to 3.5 eV. Because of here mentioned unique properties the ZnO is regarded as one of the most prospective materials for photonics, optoelectronics and biosensorics [15–17]. The wurtzite-based ZnO nanorods (NRs) showed high photoluminescence in UV range (370–390 nm), which is related to excitonic emission band [18] and such NRs have been used for PL based detection of *Salmonella* [18]. Additionally, ZnO nanorods with the layer of the immobilized antibodies were successfully used for interdigitated capacitive immunosensors [19], for aptamers [20] and lipid films-based electrochemical sensors [21], and some other analytical systems.

Recent developments in optoelectronics offer LEDs with output power of 0.4–1 mW, which are in the interest of biosensorics as low power intensity light source suitable for excitation of photoluminescence. However traditional detection part of the based biosensor consist of monochromator and photomultiplier, which are required in order to enhance the low intensity signal, what makes the cost of the detection system quite high. This drawback can be solved by the application of optical fiber based technique [22]. Recently, we have developed an fiber optic-based system based on photoluminescence measurements suitable for biomedical applications [22]. It is low cost, portable, computer driven technique. Despite of this, the optical fiber system has lower spectral resolution comparing to the traditional methods, what is significant drawback during intensity measurements [22].

In this paper we have evaluated the application of ZnO nanorods (ZnO-NRs) in the design of optical fiber based photoluminescence immunosensor, which is dedicated for the determination of specific antibodies produced in cattle as humoral immune response against BLV antigens that mainly consist of protein gp51.

## 2. Experimental

### 2.1. Materials

BLV proteins gp51, and two cattle serums (i) 'BLV-positive', which has specific antibodies against BLV proteins, and (ii) 'BLV-negative', which is free from specific antibodies against BLV proteins gp51, all were purchased from "Leykonad", Poltava, Ukraine. Glass slides, polyallylamine hydrochloride (PAH), butanol and bovine serum albumin (BSA) were obtained from Sigma-Aldrich (St. Louis, USA). All solutions were prepared using distilled and deionized water (Milli Q-plus-Millipore system).

### 2.2. Preparation and characterization of ZnO-NRs sensitive layer

ZnO nanorod powder was synthesized as reported previously [22]. Colloidal suspension of ZnO-NRs in butanol (concentration 1 mg/ml) was prepared. The solution of ZnO-NRs (20  $\mu$ L) was deposited on the cleaned glass substrate in order to form ZnO-NRs layer on the glass surface. The samples were dried at room temperature and annealed in

muffle furnace at 450 °C in air for 3 h. Scanning electron microscopy (SEM) imaging of the prepared samples was performed by Hitachi S4800 SEM (Mannheim Office) (Japan).

Optical properties of the ZnO-based nanorods were studied by PL spectroscopy. Nitrogen laser of  $\lambda = 337$  nm LGI-21 from Polyaron (Moscow, Russia) with 0.4 mW output power was used as an excitation source. The PL spectra were studied by optical fiber spectrometer, HR2000+ from Ocean Optics (Dunedin, USA) in the wavelength range of 360–420 nm.

Raman spectrometer ALFA300R from Witek (Ulm, Germany) with 532 nm excitation laser source was used for transmittance Raman measurements. The Raman spectra of the samples were recorded in the range of 50–2000  $\text{cm}^{-1}$ .

### 2.3. Functionalization of the ZnO-NRs/glass by proteins and the evaluation of immunosensor response

BLV proteins gp51, which served as an antigen (Ag) for anti-gp51 antibodies, were used in the development of biological recognition part of immunosensor. BLV proteins were immobilized on ZnO-nanorod (ZnO-NR) modified glass (ZnO-NR/glass) surface. The functionalization procedures, which were applied for modification of PL-based transducer surface are shown in Fig. 1.

The ZnO-NR/glass slides were washed with ethanol, dried and then rinsed with physiological (0.85%) solution of NaCl. Initial BLV protein gp51, which was acting as antigen (Ag) in this bioanalytical system, containing sample was diluted to the concentration of 0.5 mg/L with the solution of NaCl and such diluted solutions were incubated on the surface of ZnO-NR/glass substrates for 20 min in order to get layered gp51/ZnO-NR/glass structure. Then gp51/ZnO-NR/glass surface was rinsed with the physiological solution of NaCl and dried in air at room temperature. In order to prevent non-specific adsorption of proteins, the gp51/ZnO-NR/glass slides were incubated in 0.5 mg/mL solution of BSA for 20 min (Fig. 1A). The PL-spectra were registered before and after each incubation step.

*The deposition of polyallylamine hydrochloride (PAH) and investigation of modified surfaces.* In order to get PAH-modified ZnO-NR/glass (PAH/ZnO/glass) aqueous solution of 20  $\mu$ g/mL of PAH was incubated for 20 min on the surface of ZnO-NR/glass substrate and then it was washed with distilled water, and dried in air at room temperature. The immobilization of gp51 and BSA on the PAH/ZnO-NR/glass was performed similarly to the procedure, which is described for ZnO-NR/glass substrate (Fig. 1B) and in such way gp51&BSA/PAH/ZnO-NR/glass-based immunosensing structure was formed. PL-spectra were recorded before and after each modification step.

During the determination of antibodies against BLV proteins gp51 (gp51), the gp51/ZnO-NR/glass and gp51/PAH/ZnO-NR/glass samples were incubated for 20 min in 'BLV-positive' cattle serum containing anti-gp51 and in 'BLV-negative' cattle serum aliquot not-containing anti-gp51. Before the incubation both 'BLV-positive' and 'BLV-negative'

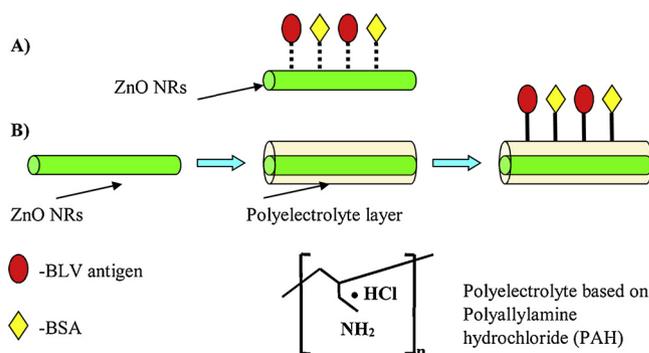
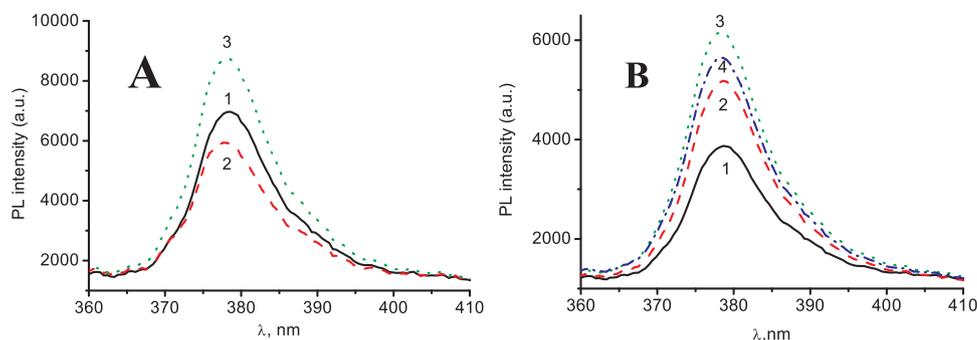


Fig. 1. Scheme of gp51 protein and BSA immobilisation on the surface of ZnO-NR/glass (A) and on - PAH/ZnO-NR/glass (B).





**Fig. 4.** PL-spectra of bare ZnO-NR/glass substrate (A): 1 – ZnO-NRs/glass, 2 – gp51/ZnO-NRs/glass, 3 – gp51&BSA/ZnO-NRs/glass; and PAH-modified ZnO nanorods (B): 1 – ZnO-NRs/glass, 2 – PAH/ZnO-NRs/glass, 3 – gp51/PAH/ZnO-NRs/glass, 4 – gp51&BSA/PAH/ZnO-NRs/glass after the immobilization of BLV-antigen (gp51) and the blockage of remaining free binding sites with BSA. The curves are average of 3 measurements at each stage of modification.

charged state, whereas ZnO-NRs surface after the adsorption of gp51 proteins is charged positively. Therefore, due to electrostatic interaction the adsorption of gp51 proteins on bare ZnO-NR/glass substrate exhibited opposite effect to the intensity of PL-signal of ZnO-NRs in comparison to that induced by BSA proteins.

Polyelectrolyte – PAH – is also involved into variation of ZnO surface charge. PAH is supposed to provide better electrostatic attraction of gp51 to the ZnO-NRs surface via the polycationic layer of PAH [31–34] and the formation of polyelectrolyte layer on the ZnO surface resulted the increase of ZnO photoluminescence due to the passivation of centers, corresponding to non-radiative recombination or/and the decrease of the depletion layer in surface region of ZnO-NRs.

The surface of PAH layer has been charged positively [30]. Therefore, BLV-antigens have been bound to PAH via negatively charged groups such as  $-\text{COO}^-$ ,  $-\text{COH}$ ,  $-\text{OH}$ , etc. The increase of PL the most probably is related to electrostatic interactions between ZnO-NRs, PAH and BLV-antigens, because gp51 are not able to transfer charge to the ZnO-NRs.

Before the investigation of bovine serum samples the response of immunosensor towards ‘BLV-negative’ and ‘BLV-positive’ (anti-gp51 antibodies containing) serum probes were tested (Fig. 5A,B). From the data obtained for ZnO-NR/glass samples, the non-specific adsorption of materials, which are present in the ‘BLV-positive’ probe, results in the decrease of PL-signal, whereas the non-specific adsorption of materials, which are present in the ‘BLV-negative’ probe, results in to the increase of the PL-signal (Fig. 5A). Non-specific interaction of ‘BLV-positive’ and ‘BLV-negative’ probes with PAH/ZnO-NR/glass surface resulted in the increase of the PL-signal intensity (Fig. 5B). Comparing the changes of ZnO-NR/glass- and PAH/ZnO-NR/glass-based immunosensor signals during the functionalization by proteins we can assume that the formation of PAH/ZnO-NR/glass structure, resulted in the change of interaction between the PL-centers of ZnO-NRs and proteins (gp51 and BSA), which are forming bioselective layer (Figs. 4 and 5) [18,24].

The interaction of ‘BLV-negative’ probe with the surface of bare ZnO-NR/glass structure resulted in the increase of the PL-signal. The interaction of ‘BLV-negative’ probe with PAH/ZnO-NR/glass structure resulted in the increase of the PL signal.

The responses of biofunctionalized gp51&BSA/ZnO-NR/glass and gp51&BSA/PAH/ZnO-NR/glass structures towards ‘BLV-positive’ and

‘BLV-negative’ aliquots are shown in Figs. 6 and 7, respectively. The decrease of the PL-signal was observed when both, above mentioned, gp51-modified structures were incubated in ‘BLV-positive’ aliquots. It is worth to mention that after specific interaction observed signal, which is based on PL quenching, is more significant than the signal, which is based on non-specific interaction of ‘BLV-positive’ aliquot with ZnO-NR/glass and PAH/ZnO-NR/glass structures, which were not modified by gp51 that are specifically binding anti-gp51 antibodies present in ‘BLV-positive’ aliquot. Both gp51&BSA/ZnO-NR/glass and gp51&BSA/PAH-ZnO-NR/glass structures after the incubation in ‘BLV-negative’ aliquots showed the increase of the PL-signal, while after the incubation in ‘BLV-positive’ aliquots the signals of both (gp51&BSA/ZnO-NR/glass and gp51&BSA/PAH-ZnO-NR/glass) structures has decreased.

The response of developed immunosensors was calculated according to the following equation, which was reported earlier [18,35]:

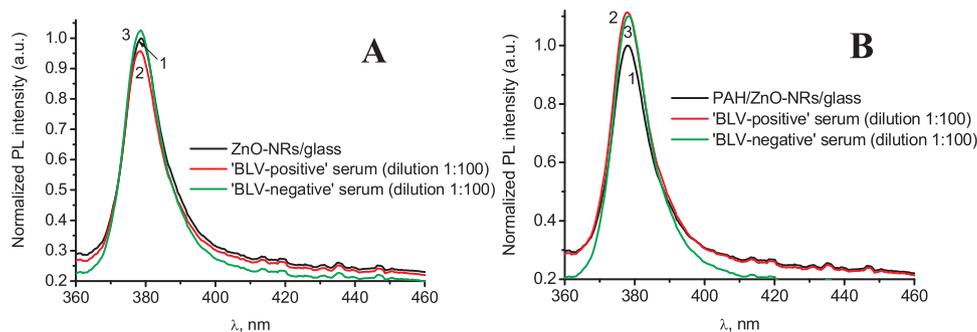
$$S = 1 - I_{\text{norm}}(C), \quad (1)$$

where  $I_{\text{norm}}(C)$  is a normalized PL intensity after the treatment of gp51&BSA/ZnO-NR/glass structure with ‘BLV-positive’ or ‘BLV-negative’ probe at corresponding probe concentration  $C$ .

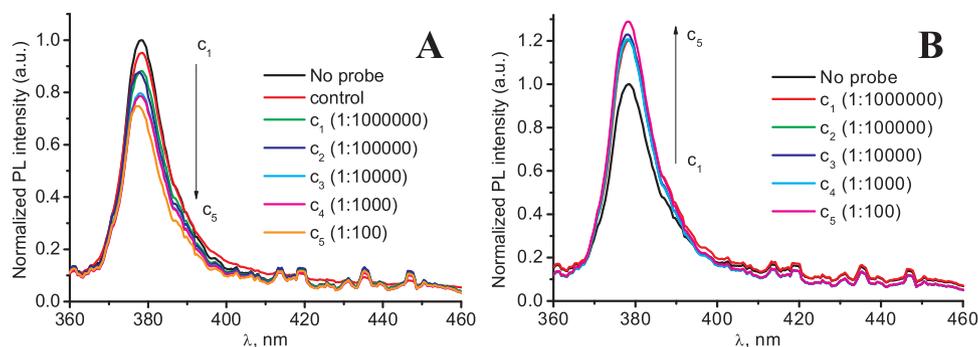
The calibration curves of ZnO-NR/glass and PAH/ZnO-NR/glass based structures towards ‘BLV-positive’ and ‘BLV-negative’ BLV probes are shown in Fig. 8A and B, respectively. In presented results some differences of PL-signal of these two ZnO-NR/glass and PAH/ZnO-NR/glass based structures after interaction with ‘BLV-positive’ and ‘BLV-negative’ probes are observed.

The incubation of gp51&BSA/ZnO-NR/glass and gp51&BSA/PAH/ZnO-NR/glass based structures in ‘BLV-positive’ aliquots resulted in the decrease of PL due to interaction of immobilized gp51 proteins with anti-BLV antibodies. Form shifts of PL-spectra maximums we can conclude that the dominating effect on photoluminescent centres of ZnO-NRs based on electrostatic interaction between ZnO-NRs and immobilized gp51 in similar way as it was determined in the case of interaction between  $\text{TiO}_2$  and gp51 [36].

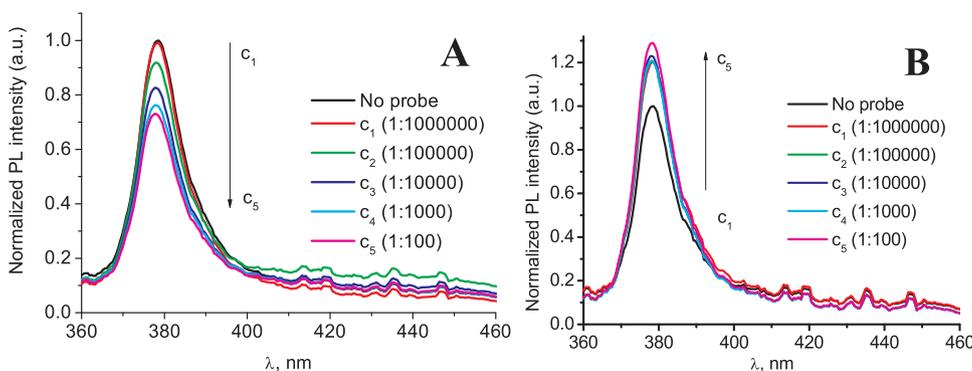
We can conclude that no significant changes between 1:1,000,000 and 1:100,000 dilution of probes were observed for gp51&BSA/ZnO-NR/glass based immunosensor (Fig. 8A), whereas for immunosensor, which was based on gp51&BSA/PAH ZnO-NR/glass, the PL-signal at the



**Fig. 5.** Control measurements of ‘BLV-positive’ and ‘BLV-negative’ serum probes on ZnO-NR/glass (A): 1 – ZnO-NRs/glass, 2 – BLV-positive’ probe/ZnO-NRs/glass, 3 – BLV-negative’ probe/ZnO-NRs/glass and PAN/ZnO-NR/glass (B): 1 – PAH/ZnO-NRs/glass, 2 – BLV-positive’ probe/PAH/ZnO-NRs/glass, 3 – BLV-negative’ probe/PAH/ZnO-NRs/glass. The curves are average of 3 measurements at each stage of modification.



**Fig. 6.** Response of immunosensor based on gp51/ZnO-NR/glass structure towards ‘BLV-positive’ probe (A), which contains specific anti-gp51 antibodies, and towards ‘BLV-negative’ probe which does not contain any specific anti-gp51 antibodies (B). The curves are average of 3 measurements at each stage of modification.



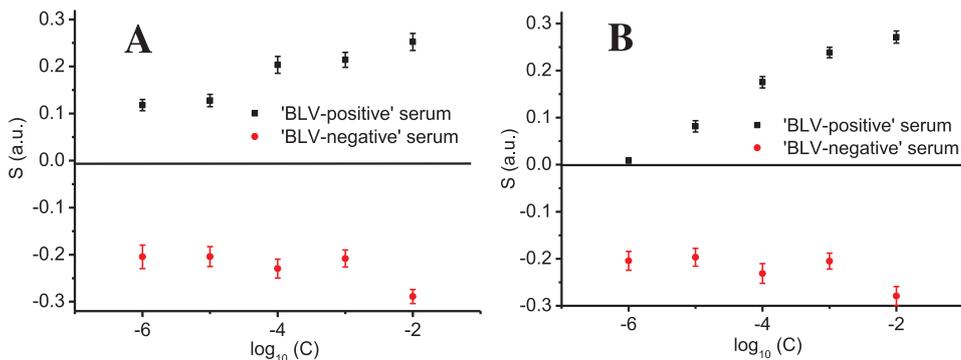
**Fig. 7.** Response of immunosensor based on gp51&BSA/PAH/ZnO-NR/glass structure towards ‘BLV-positive’ probe (A), which contains specific anti-gp51 antibodies, and towards ‘BLV-negative’ probe which does not contain any specific anti-gp51 antibodies (B). The curves are average of 3 measurements at each stage of modification.

same concentration level has increased up to 10%.

It is worth to mention, that the direct and polyelectrolyte – PAH – assisted immobilization of BLV-antigens on the ZnO-NR/glass surface provided to gp51&BSA/ZnO-NR/glass-based immunosensor advanced sensitivity against BLV antibodies [18]. However, random orientation of BLV-antigens significantly reduced the sensitivity of gp51&BSA/ZnO-NR/glass-based immunosensor towards anti-gp51 antibodies and analytical signal saturation was observed even at the dilution of ‘BLV-positive’ serum at 1:10,000. Contrary to this, gp51&BSA/PAH/ZnO-NR/glass-based immunosensor showed good linearity of signal in the serum dilution range of 1:1,000,000 – 1:1000 of ‘BLV-positive’. The developed gp51&BSA/PAH/ZnO-NR/glass-based immunosensor can well differentiate ‘BLV-positive’, and ‘BLV-negative’ serums in relatively high dilution range from 1:1000 to 1:100,000. Selectivity tests of gp51&BSA/ZnO-NR/glass- and gp51&BSA/PAH/ZnO-NR/glass-based sensors are represented supplementary material figure S2.

**4. Conclusion**

Novel photoluminescence system based on gp51&BSA/ZnO-NR/



**Fig. 8.** Calibration plots of immunosensor signal *S* based on gp51&BSA/ZnO-NR/glass (A) and gp51&BSA/PAH/ZnO-NR/glass (B) modified surfaces towards differently diluted ‘BLV-negative’ and ‘BLV-positive’ serums with dilution *C* (The plots are shown in semi logarithmic scale). Here  $S = 1 - I_{norm}(C)$  (according to Eq. (1)), where  $I_{norm}(C)$  is a normalized PL intensity after the treatment of gp51&BSA/ZnO-NR/glass structure with ‘BLV-positive’ or ‘BLV-negative’ probe at corresponding probe concentration *C*;  $\log_{10}(C)$  – is a logarithm of concentration, taking into account that the concentration of initial (undiluted sample) is equal to 1.

glass- and gp51&BSA/PAH/ZnO-NR/glass-based immunosensor for the express detection of specific antibodies against BLV protein gp51 has been reported. The system is able to discriminate between ‘BLV-positive’ and ‘BLV-negative’ blood serum probes in the dilution range from 1:1000 to 1:100,000. The proposed mechanism of PL-signal generation is mostly based on electrostatic interaction between immobilized/interacting biomolecules and ZnO-NRs.

**Acknowledgements**

The conducted research has been partially supported by project H2020-MSCA-RISE-2017, ‘Novel 1D photonic metal oxide nanostructures for early stage cancer detection’ (Project number: 778157); Ukraine-Latvia bilateral project ‘Algoritma optimizēšana audzēju progresēšanas un ārstēšanas efektivitātes bioķīmisko raksturlielumu noteikšanai ar biosensoriem’ (LV-UA/2016/6, contract nr. ZD2016/20322). I.I. acknowledges the financial support from the National Science Centre of Poland by the SONATA 11 project UMO-2016/21/D/ST3/00962. A.R. and A.R. are grateful to Ukrainian-Lithuanian bilateral research project ‘Application of hybrid nanostructures which are based

on TiO<sub>2</sub> or ZnO and modified by biomolecules, in optoelectronic sensors” Lithuanian Research Council project No P-LU-18-53.

## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:10.1016/j.snb.2019.01.054.

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