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# Towards Application of $Al_2O_3$/ZnO Nanolaminates in Immunosensors: Total Internal Reflection Spectroscopic Ellipsometry based Evaluation of BSA Immobilization

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**Abstract:** In this research, $Al_2O_3$/ZnO nanolaminates were evaluated for possible application in the design of optical immunosensors. Total internal reflection ellipsometry (TIRE) was utilized to study the optical response during the formation of bovine serum albumin (BSA) based monolayer on the surface of $Al_2O_3$/ZnO nanolaminates, which were pre-modified with a N-(3-aminopropyl) triethoxysilane (APTES) layer. The influence of the thicknesses of $Al_2O_3$ and ZnO layers on the performance of $Al_2O_3$/ZnO nanolaminates based structure has been assessed. The research has shown the noticeable contribution of multiple reflections from the interfaces between $Al_2O_3$ and ZnO for the enhancement of the optical response in total internal reflection configuration. $Al_2O_3$/ZnO nanolaminates of 200 nm total thickness based on four 50 nm thick alternating $Al_2O_3$ and ZnO layers have shown better sensitivity than the nanolaminate based on two 100 nm oxide-layers. Real-time monitoring of the ellipsometric parameters $\Psi(\lambda)$ and $\Delta(\lambda)$ has shown that the BSA was successfully covalently attached to the nanolaminate/APTES surface. The surface concentration of immobilized BSA was evaluated from the real-time data of the ellipsometric parameters $\Psi(\lambda)$ and $\Delta(\lambda)$. Expected advantages and disadvantages of $Al_2O_3$/ZnO nanolaminates during expected application in optical immunosensors are discussed.

**Keywords:** Total Internal Reflection Ellipsometry, Spectroscopic Ellipsometry; Thin films; Total internal reflection; Nanostructures; Immunosensor.

## 1. Introduction

Optical biosensors are now widely used in various applications [1–3]. One of possible optical configuration, which is used in the design of biosensors (immunosensors), which are dedicated for the determination of antigen and antibody specific interaction, is the exploitation of total internal reflection (TIR) phenomenon [4]. This makes possible the monitoring of biomolecule immobilization processes on surfaces and their specific interaction with other biomolecules in opaque media [5]. The same TIR is also used in surface plasmon resonance (SPR) based biosensors [6] and in some infrared irradiation based techniques [7]. Ellipsometry also can be used in such TIR

mode [8]. The combination of ellipsometry and the SPR phenomenon (also called Total Internal Reflection Ellipsometry (TIRE)) uses a prism, which enables to induce total internal reflection. TIRE provides better sensitivity to surface changes, when compared with traditional ellipsometry configurations and SPR even in intensity interrogation modes [9]. The exploitation of the SPR phenomenon demands a semitransparent metal film mostly based on gold, silver or copper. The application of a glass prism as an optical element to obtain internal reflection provides the possibility to study surface modification or antigen-antibody interaction without the light beam passing through the liquid medium where these processes occur. Total internal reflection configurations also have been used for photonic crystal applications for optical biosensing [10,11]. However, only several publications have been dedicated to study dielectric thin films in internal reflection mode [12]. An enhancement of the ellipsometric optical response due to multiple reflection inside the additional dielectric tantalum oxide surface layer with a high refractive index in internal reflection configurations has been reported [13–15].

Due to recent technological achievements, there is significant interest in the application of zinc oxide (ZnO) based thin films and nanolaminates as materials for the design of optical biosensors [16–18]. ZnO-based thin films and nanolaminates can be deposited using various methods [17,18]. They are bio-compatible, non-toxic and chemically stable [19]. The high isoelectric point of ZnO can also be used for the immobilization of proteins with lower isoelectric points through the formation of electrostatic interactions [20]. Additionally, ZnO-based nanomaterials demonstrate controllable wettability, which allows the reduction of the volume of the sample, a quick response, increased sensitivity and signal-to-noise ratio of the developed biosensors [21]. Moreover, ZnO is compatible with complementary metal oxide semiconductor technology and therefore is suitable for the production of integrated circuits for devices with integrated small biosensors [16]. Thus, ZnO-based multi-layered structures (nanolaminates) have been selected as substrates for optical biosensors operating in total internal reflection ellipsometry mode.

The aim of this study has been: (i) to evaluate TIRE-based sensitivity of several different $Al_2O_3$/ZnO nanolaminates and single $Al_2O_3$ or ZnO layers; (ii) to determinate advantages and limitations of nanolaminates in the applicability in the design of optical biosensors. Ellipsometric parameters $\Psi$ and $\Delta$ in total internal reflection configuration have been measured in order to demonstrate properties of such layered structures and possible applicability in optical biosensors design.

## 2. Material and methods

### *2.1 Materials*

N-(3-aminopropyl)triethoxysilane (APTES, 99%) and 1-ethyl-3-(3-diaminopropyl)carbodiimide) hydrochloride (EDC) were obtained from Sigma-Aldrich (Germany). N-hydroxysuccinimide (NHS) was gotten from Merck (Germany). Bovine serum albumin (BSA, fraction V) was obtained from Carl Roth GmbH&Co (Karlsruhe, Germany). Phosphate buffered saline (PBS) tablets (0.14 M NaCl, 0.0027 M KCl, 0.01 M phosphate buffer pH 7.4) were purchased from AB Medicago (Sweden). All chemicals were of analytical grade or better. All aqueous solutions were prepared in UHQ water (conductivity less than 1 μS/cm), purified by DEMIWA rosa 5 (WATEK, Czech Republic).

Two types of samples were investigated, which were deposited on 1 mm thick glass plates: (i) $Al_2O_3$/ZnO nanolaminate having total thickness of 200 nm consisting of four alternating 50 nm $Al_2O_3$ and ZnO layers, (ii) $Al_2O_3$/ZnO nanolaminates having total thickness of 200 nm consisting of two 50 nm $Al_2O_3$ and ZnO layers. The $Al_2O_3$/ZnO nanolaminates on the glass substrates were formed using the atomic layer deposition (ALD) method. The procedure was performed in a home-

made ALD reactor at a fixed temperature of 100°C. All deposition conditions were described in our previous publications [22–24].

### *2.2 Functionalization of the $Al_2O_3$/ZnO nanolaminates' surface by silanization*

The top layer of both here evaluated nanolaminates was based on ZnO, which was modified by (3-Aminopropyl)trimethoxysilane. The salinization of the nanolaminates with APTES from vapor phase was performed according to the procedure described previously [25]. To minimize the influence of humidity, the silanization was performed in a glovebox in an inert atmosphere. A few drops of APTES were deposited in a small vial, which was then placed next to the nanolaminate located in a glass Petri dish. The Petri dish was then covered and isolated from contact with air with heat resistant insulation tape. The covered Petri dish was then placed in an oven and kept overnight at 90°C. Afterwards, the modified nanolaminate was washed with toluene, ethanol and distilled water and then dried in an oven at 110°C. In this way, the surfaces of the $Al_2O_3$/ZnO nanolaminates were functionalized with amino groups, which are necessary for covalent immobilization of BSA. The water contact angle on the modified and unmodified nanolaminates was measured with a KSV Instruments CAM200 optical goniometer. A drop of distilled water was placed on the surface with a micro syringe, captured with a camera for 10 times and then images were fitted. This measurement was repeated for 3 times on the same surface.

### *2.3 Immobilization of BSA on silanized surface of $Al_2O_3$/ZnO nanolaminates and on $Al_2O_3$ and ZnO layered structures*

In order to immobilize covalently BSA on the salanized surfaces of the $Al_2O_3$/ZnO nanolaminates, the carboxyl groups of the BSA were activated using a mixture of EDC and NHS aqueous solutions by incubation for 15 min. BSA solution of 0.1 mg/ml concentration was prepared in PBS, and the final concentrations of EDC and NHS in the BSA solution was 400 mM and 100 mM, respectively. The activated carboxyl groups of BSA were exposed to the amino groups on the pre-modified $Al_2O_3$/ZnO nanolaminate surfaces. BSA immobilization kinetics were registered using TIRE. The influence of the non-activated BSA adsorption on the registered signal was also evaluated on silanized and not-silanized $Al_2O_3$/ZnO nanolaminates.

### *2.4 TIRE measurements*

The ellipsometric experiments were conducted using a rotating compensator based ellipsometer J. A. Woollam M2000X (Lincoln, USA). The TIRE experiments were carried out at a 70° angle of incidence with the illumination being at wavelengths in the 210 nm – 1000 nm spectral range. This was conducted using a BK7 glass 70° angle prism connected *via* a refractive index matching fluid with the glass plate coated with the nanolaminates (Fig. 1). In the TIRE experiment, a liquid handling system with a custom-built Teflon chamber was used in which the nanolaminate surfaces were placed. The connecting valve was then opened, allowing the buffer solution to be injected into the chamber. This chamber was filled with PBS, pH 7.4, which was needed for the interaction of the proteins with the modified surface. The BSA solution (0.1 mg/ml) in PBS, pH 7.4, was then injected into the Teflon chamber in order to form a thin protein layer.

The spectra of the ellipsometric parameters $\Psi(\lambda)$ and $\Delta(\lambda)$ were registered in their dynamic acquisition mode at rate of one spectra per second. After the initial ~15 minutes of baseline measurements, the solution of BSA with the activated carboxyl groups was introduced into the Teflon chamber containing silanized $Al_2O_3$/ZnO nanolaminates, which contained amino groups. Ellipsometric parameters $\Psi(\lambda)$ and $\Delta(\lambda)$ in TIRE configurations have different sensitivities [26]. The ellipsometric parameter $\Delta(\lambda)$ was used for the real time signal registration because of its higher

sensitivity towards surface changes during BSA immobilization. Simulations were conducted using Complete EASE software from J. A. Woollam Company. The ellipsometric measurements of the created optical models were conducted taking into account the methodology for the evaluation of absorbing films presented in other researches [27].

## 3. Results and discussion

### *3.1 Simulations*

In order to apply efficiently $Al_2O_3$/ZnO nanolaminates in optical immunosensor design, the performance of differently nanostructured laminates should be evaluated. Therefore, in this research, the simulation of the ellipsometric parameters has been conducted to determine the influence of several parameters such as thicknesses and number of layers on the sensitivity of optical system. Foremost simulations were done in an external reflection (reflection ellipsometry) setup. Conventional reflection ellipsometry simulations showed that the optical response –of the ellipsometric parameters increased for the nanolaminate consisting of 50 nm thick layers of $Al_2O_3$ and ZnO, while for thicker and thinner layers of $Al_2O_3$ and ZnO in nanolaminates, these optical parameters decreased. Thus, for further studies, nanolaminates with $Al_2O_3$ and ZnO layers of 50 nm thickness were chosen in order to improve the sensitivity of the system. However, as it is mentioned in introduction, the TIRE configuration is more appropriate for bio-sensing applications due to the possibility to conduct optical measurements in liquid and/or in an opaque medium without light passing through analyte containing solution [5]. In TIRE configuration, a better signal-to-noise ratio is achieved and the light scattering effect is minimized. Thus in the next part of investigations, the four types of $Al_2O_3$/ZnO nanolaminates formed on the glass substrates were examined in the TIRE configuration: (i) four 50 nm alternating $Al_2O_3$ and ZnO layers based nanolaminate of total 200 nm thickness, (ii) two 100 nm $Al_2O_3$ and ZnO layers based nanolaminate of total 200 nm thickness, (iii) a single 200 nm ZnO film and (iv) a single 200 nm $Al_2O_3$ film. As it is mentioned above, all metal oxide based samples were of the same total thicknesses (200 nm) and ellipsometric parameters were compared for all four samples in order to find out the differences in the sensitivity (Fig.2 a,b,c,d). The analysis of the maximal shift of ellipsometric parameter $\Delta$ of the conducted simulations showed that the multilayered structures of the $Al_2O_3$/ZnO with 50 nm layers demonstrated twice better sensitivity during the simulated 10 nm protein layer adsorption in comparison to the sensitivity of single ZnO film of the same total thickness (200 nm). As can be seen from Figures 2a, 2b, 2c and 2d, the changes in the ellipsometric parameter $\Delta$ for numerically simulated deposition of 10 nm layer of protein formed on nanolaminate consisting of four alternating 50 nm layers of $Al_2O_3$ and ZnO and on single 200 nm ZnO layer were $\Delta$=11.8° and $\Delta$=5.44°, respectively. For the single 200 nm ZnO and the single 200 nm $Al_2O_3$ layers, these values were $\Delta$=6.02° and $\Delta$=1.4°, respectively. The protein refractive index dispersions were modelled with the Cauchy function and following values for this function were chosen: A=1.45, B=0.01 and C=0. The contribution of a few nanometers APTES layer has been also taken into account. In order to determine the contribution of the layered structure, two samples with different numbers and thicknesses of $Al_2O_3$ and ZnO layers, but with the same total amount of aluminum and zinc oxide were analyzed in more detail. From the optical response of these two samples, it can be clearly seen (Fig. 2a, 2b) that a structure based on four 50 nm $Al_2O_3$ and ZnO layers showed better sensitivity towards simulated deposition of 10 nm protein layer ($\Delta$=11.8°) in comparison to that simulated for structure based on two 100 nm $Al_2O_3$ and ZnO layers ($\Delta$=5.44°). Because the amounts of both materials were the same in these simulated samples, it is reasonable to assume that enhancement of the optical response is induced by the higher number of layers, which are forming nanolaminates. Such effect is observed due to the multiple reflection of light from the interfaces between the $Al_2O_3$ and ZnO layers. Therefore, for the

TIRE-based analysis of protein layer formation, the structure of the $Al_2O_3$/ZnO nanolaminates based on four 50 nm $Al_2O_3$ and ZnO layers was chosen.

### *3.2 Optical properties of $Al_2O_3$/ZnO nanolaminates*

In this part of investigations, the ellipsometric spectra of the $Al_2O_3$/ZnO nanolaminates were recorded in a total internal reflection configuration. The spectra of the ellipsometric parameters $\Psi(\lambda)$ and $\Delta(\lambda)$ for the nanolaminates/APTES are shown in Figure 3. From this Figure, it is seen that optical losses due to ZnO absorption manifested themselves as peaks in the $\Psi(\lambda)$ and $\Delta(\lambda)$. The peak for $\Psi(\lambda)$ has been observed at the $\lambda \approx 400$ nm when an external angle of the light incidence to the prism was equal to 70 degrees. The optical dispersions of the refractive indexes of $Al_2O_3$ (Fig. 4a) and ZnO (Fig. 4a) were evaluated by regression analysis from the multilayer model and were then used as fixed parameters in the evaluation signals registered during BSA covalent immobilization.

In this study, the multi-layer model represents the five-phase structures of the nanolaminates consisting of four 50 nm alternating layers of $Al_2O_3$ and ZnO deposited on glass substrates. In the regression analysis, the starting values of the optical dispersion were taken from the Complete EASE database and the thicknesses of the layers, which were treated as free fitting values, initially were applied as 50 nm. The optical constants of the $Al_2O_3$ layers in the nanolaminates were determined using the Cauchy dispersion function. The regression results showed that the thicknesses of the $Al_2O_3$ layers in the nanolaminates were 49.72±0.56 nm and 50.20±0.43 nm for first and second bilayer, respectively. Meanwhile, the optical constants for the ZnO layers were determined by using PSemi-M0 and two Gaussian peaks functions. The obtained dispersions of the ZnO and $Al_2O_3$ optical constants are presented in Figure 4a. The determined thicknesses of the ZnO layers were 50.19±0.47 nm and 49.77±0.61 nm for the first and second ZnO layer, respectively. The refractive index and extinction coefficient of the APTES layer was also determined by the Cauchy function. The obtained optical constant dependences of the wavelength are presented in Figure 4b. The evaluated thickness of the (3-Aminopropyl)trimethoxysilane layer using these optical constants was 6.99±0.79 nm. For the six phases structure of the nanolaminates with the APTES layer formed on the top of the ZnO surface, the modelled data were fitted to the experimental ellipsometric results with a mean square error (MSE) value of MSE=12.72.

### *3.3 The evaluation of $Al_2O_3$/ZnO nanolaminates as possible substrates for optical immunosensors*

As it has been noted above, for the immobilization of BSA, the nanolaminate (ZnO) was modified by APTES. Using this procedure, the surface was functionalized by amino groups, which are required for covalent immobilization of BSA onto the ZnO surface. In this experiment, BSA was used as a model protein to imitate the formation of protein-based layer on the ZnO surface. To estimate the influence of not specific adsorption of BSA, silanized surface of nanolaminates was treated by not activated BSA.

The refractive index of covalently immobilized BSA was determined by Cauchy dispersion function in the same way as it was used for the evaluation of (3-Aminopropyl)trimethoxysilane layer (Fig. 4b). The lowest point of $\Psi(\lambda)$ spectrum was slightly red-shifted, producing an additional decrease of amplitude of the lowest point, because the ZnO surface was modified by additional BSA layer. A similar tendency was observed for the $\Delta(\lambda)$ parameter in the vicinity of the absorption band of the ZnO (Fig. 3). Due to the formation of the BSA layer on the top of the ZnO surface, the ellipsometric parameter $\Delta(\lambda)$ changed up to 12 degrees at $\lambda$=390 nm, while the maximum shift for $\Psi(\lambda)$ was 1.7 degrees at $\lambda$=370 nm. The regression analysis showed that such changes in ellipsometric parameters correspond to the 7.3±0.67 nm thickness of the BSA layer. A detailed

understanding of the protein immobilization process on the solid-liquid interfaces can be gained by analyzing the evolution of the ellipsometric parameter $\Delta$(t) over time (Fig. 5a). The TIRE method in its dynamic data acquisition mode is suitable for the evaluation of changes of both refractive index and thickness over time. It should be noted, that in some particular cases, obtained physical quantities can be related to particular information about immobilized biomolecules [7]. One of the most common approaches, which are applied for analytical signal acquisition in various types of immunosensors, is the evaluation of surface concentration changes. For this purpose, the dynamic TIRE data of the refractive index and thickness, which were registered during the formation of the BSA layer, were recalculated into changes of the surface concentration using de Feijter's formula [28] (Fig. 5b). While both parameters – film thickness and refractive index – were determined from regression analysis. Surface concentration (ng/cm$^2$) has been calculated by formula:

$$\Gamma = \frac{d(n-n_{buffer})}{\Delta n/\Delta C} \times 100 \qquad (1)$$

where $\Delta n/\Delta C$=0.18 $\frac{cm^3}{g}$ [29] is the refractive index increment for the layer material, depending on the protein concentration in the buffered solution, $d$ is the thickness (nm) of the protein layer obtained from regression analysis, $n$ is the refractive index of the protein layer obtained from regression analysis and $n_{\text{buffer}}$ is the refractive index of the buffered solution. The evolution of the surface concentration was calculated at intervals of about 4 minutes and the fast change of the surface concentration of immobilized BSA proteins was registered up to 27 minutes when analytical signal becomes steady.

## 4. Conclusion

The spectroscopic dynamic TIRE method was utilized for the characterization of the real time formation of the BSA layer on the surface of the functionalized $Al_2O_3$/ZnO nanolaminates. The conducted simulations of the optical response of structures based on $Al_2O_3$/ZnO layered nanolaminates showed a twice higher variation of the ellipsometric parameter $\Delta$ during the simulation of the formation of additional protein layers on the surface of $Al_2O_3$/ZnO/$Al_2O_3$/ZnO nanolaminate, which was based on four 50 nm oxide-layers, in comparison to the variations observed on the surface of $Al_2O_3$/ZnO nanolaminate based on two 100 nm oxide-layers. The increased sensitivity of the ellipsometric parameters can be explained by the multiple reflections of the light waves in a condition of total internal reflection, especially for ellipsometric parameter $\Delta$. These studies showed that the number of bilayers and the thicknesses of the films have a noticeable influence on the sensitivity of these kinds of optical systems. Therefore, the refractive index dispersion of the layers needs to be optimized by taking into account the materials from which they are made. Such multiple total internal reflections are very commonly used in ATR-FTIR spectroscopy [7]. However, to the best of our knowledge, the exploitation of multiple TIR effect with spectroscopic ellipsometry in the UV-VIS spectral range for the optical immunosensor design is being used for the first time. It should be noted, that the TIRE method with dielectric multilayer structures is less sensitive to surface changes than the TIRE with thin metal films, which utilizes the SPR effect. This observation can be explained by the different localization of the electric field on the sensing surfaces. However, the biocompatibility, the photoluminescence features of ZnO [30] and the optical properties of ZnO/Au nanostructures [31], such as the simultaneous real time monitoring of SPR[3] or localized SPR [32] effects in VIS and the photoluminescence peak of ZnO in the UV range, offers possibilities of constructing advanced optical immunosensors.

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**Captions**

**Fig. 1.** Principal experiment scheme.

**Fig. 2.** Simulated TIRE spectra (solid curves) of (a) nanolaminate based on four 50nm thick $Al_2O_3$ and ZnO layers, (b) nanolaminate based on two 100nm thick $Al_2O_3$ and ZnO layers, (c) single 200 nm ZnO layer and (d) single 200 nm $Al_2O_3$ layer. The dashed curves represent the changes of ellipsometric parameters $\Psi(\lambda)$ and $\Delta(\lambda)$ after the formation of 10 nm thick layer of the proteins on the surface of above mentioned oxide-based layers modelled by Cauchy function.

**Fig. 3.** The TIRE data of the ellipsometric parameters' $\Psi(\lambda)$ and $\Delta(\lambda)$ (points) and the fitted curves (solid lines) of the six phases structure of the nanolaminate based on four 50 nm thick $Al_2O_3$ and ZnO layers with the APTES layer on the ZnO surface ($\Delta$ – ○; $\Psi$ – □) and with the BSA layer immobilized on the APTES surface ($\Delta$ – ●; $\Psi$ – ■).

**Fig. 4.** a) ZnO and $Al_2O_3$ optical dispersion of the complex refractive index in $Al_2O_3$/ZnO based nanolaminates; b) refractive index (solid curve) of bovine serum albumin covalently immobilized on a ZnO surface modified with APTES. Refractive index dispersion of APTES film (dashed curve) formed on a ZnO surface.

**Fig. 5.** a) The kinetics covalently BSA immobilization on the surface of APTES-modified $Al_2O_3$/ZnO nanolaminate, parameter $\Delta$ at 390 nm wavelength; b) the surface concentration of BSA calculated from TIRE data using equation *1*.

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