Enzyme Biosensing Based on Zinc Oxide Nanostructures as Active Surface

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Abstract. Ag/ZnO mesostructures deposited onto substrates different were analysed in order to use ZnO as bioactive surface. This paper presents the results obtained at the eNDE of strips gratings deposited on different substrates used as bioactive surface using the EM sensor with MM lens in order to improve the emphasizing of the evanescent waves appeared when the slits of MSG are filled with immobilized enzymes.

1. Introduction
Nanomaterials prepared from metals oxides, shaped into nanoparticles have been widely used for their ability as electrode biosensing materials to enhance the efficiencies of electrochemical biosensor [1]. Metal oxide nanoparticles can increase efficiency of photochemical reactions and greatly improve the catalytic activity of enzymes to generate novel photo-electrochemical systems [2]. Some oxides nanoparticles (ex: ZnO, TiO2, SnO2, Fe2O3, MnO2, etc.) can easily act as enhancing agents for effective acceleration of electron transfer between electrode and detection molecules, so leading to more rapid current response for target molecules (Figure 1) [3-5]. Nanostructures of ZnO (and other nanomaterials) including metal oxide semiconductor-metal hybrids and inorganic-organic hybrids have also used as matrices for enzymes immobilization [6-9].

Nanoparticles and nanostructured thin films have been studied in terms of their gas sensing abilities and biosensing element [10-13] where it was shown that a rough surface with a larger surface area had a positive influence on the sensor response [14, 15]. ZnO nanostructures formed an attractive matrix for GOx/ChOx immobilization, which exhibits a high affinity, high sensitivity, and fast response for glucose/cholesterol detection. The content of the paper is oriented toward development biosensing of glucose and cholesterol based on ZnO material by immobilizing of glucose oxidase and cholesterol oxidase on the surface of zinc oxide nanostructures. ZnO nanostructures as enzyme matrices show significant potential for use in biosensors. Relatively recent researches revealed that metallic structures with sub-wavelength features in the EM field at RF and microwave frequency range have potential for producing unusual EM response [16, 17], creation of high impedance surface [18], have an effective plasmonic behavior [19], high index of refraction and negative refractive index [20, 21]. The diffraction limits using a sensor with MM decrease until 300 nM instead of 100 μm in the case of conventional sensors and biosensors [12, 22, 23].
2. Materials and methods

A wide range of techniques to deposit thin films can be used [24-29] but in this paper the vacuum evaporation technique is used to deposit zinc oxide nanostructured thin films in order to obtain for investigate as biosensing layer in MSG structures. A zinc metallic film was deposited on ITO/glass and SiO$_2$/Si at room temperature. The conditions for Zn metallic film and Ag strips deposition was presented in [30], where the pressure in the deposition chamber was maintained at 10$^{-5}$ Torr and the substrate temperature was kept at 300 K. The Ag/ZnO/different substrates structures are shown in Figure 2. The XRD patterns were made on a diffractometer using CuKα radiation ($\lambda = 1.5406 \text{ Å}$) in order to identify the phase composition of the samples (Figure 3). The samples were analysed in the range of $2\theta = 5^0 \sim 80^0$ with a scanning angle rate of 0.02 and a 2 s/step count time.

Figure 2. MSG structure.

Figure 3. XRD ZnO thin film.

The XRD results demonstrate that there are two peaks in the pattern at 34.4$^0$ and 36.2$^0$, which corresponds with the (002) and (101) planes of ZnO, respectively. All of the indexed peaks in the obtained pattern are well-matched with that of bulk ZnO which confirms that the synthesized products are many crystalline and possesses a wurtzite hexagonal structure.

No other peak related to impurities was detected in the pattern within the detection limit of the X-ray diffraction further confirms that the obtained products are pure ZnO.

The AFM images (Figures 4a and b) emphasize the polycrystalline granular aspect and of ZnO layer with bound enzyme show many globular structures, confirming immobilization of enzymes. The analyses find that the surfaces are without inclusions and defects can be used as bioactive surface.
Figure 4. The AFM images of: (a) ZnO/ITO/glass with immobilized enzyme glucose; (b) ZnO/SiO$_2$/Si with immobilized enzyme cholesterol.

3. Experimental set-up

3.1. Biosensing mechanism

It was studied the influence of nanoparticle size on the performance of prepared biosensors and nanoparticles with smaller size were found to be more suitable for enzyme immobilization.

There are three modes of the enzymes oxidation referred to as the first, the second and the third generation glucose/cholesterol biosensors depending on the electron transfer mechanisms [31]. The reduction rate of the oxygen is proportional to the glucose/cholesterol concentration that is quantified by either measuring the augmentation of hydrogen peroxide or decrement of the oxygen concentration.

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\begin{align*}
\text{Glucose} + O_2 & \xrightarrow{GO} \text{Gluconic acid} + H_2O_2 \\
\text{Cholesterol} + O_2 & \xrightarrow{CHO} \text{Cholest-4-en-3-one} + H_2O_2 \\
H_2O_2 & \rightarrow O_2 + 2H^+ + 2e^- 
\end{align*}
\]  

(1)

Since, the kinetics of the enzyme reaction is monitored by the rate of formation of product or the disappearance of a reactant. If either the product or reactant is electro-active, then the progress of the reaction can be monitored using amperometry [32]. Due to the presence of water (H-OH) it is a high probability that cholesterol oxidase/glucose oxidase will accept a proton from (H-OH) converting it to ion which in turn will interact with a ZnO nanostructures and produce a potential change at the electrode. The sensing mechanism for our glucose/cholesterol biosensors consist by the voltage applied across the two electrodes which causes a current to flow via electron tunnelling through the potential barrier between the nanoparticles. The current for the mixture decreases proportionally with the number of glucose/cholesterol molecules.

3.2. Electromagnetic sensing. Evanescent waves and FDTD simulation

The sensor based on MM lens described in [33-36] improving the spatial resolution to obtain the electromagnetic images using Fourier optics [37] and experimental set-up presented in [12, 39] were used at working frequency of 474 MHz. The simulation of system functioning was carried on XFDTD 6.3 software produced by REMCOM [40].

In Figure 5a is presented the result of simulation with XFDTD where the $E_y$ component is displayed. In [30] it has been presented the behaviour of the field with air in the slits, it can be shown that for uricase between the strips, the amplitude of the electric field has the same behaviour as in [21]
but the amplitude decreasing due to electrical permittivity high of the uricase. 
Thus, the symmetrical maxima appear in the middle of the slits, decreasing to the minimum value on the strips edges. Inside the strips, other pair of maxima appears, followed by the decreasing to middle of the strip. The Figure 5b shows the dependency of e.m.f. amplitude induced in the reception coil of sensor allows the correct emphasizing of extremely thick conductive strips and eventual interruptions.

Figure 5. Numerical results for electric-field amplitude distribution near the strips; the field values are normalized to the amplitude of the incident field.

The realized samples presented in §2 having Ag 1.2 mm width strips and 0.8 mm width slits, h = 14 μm deposited on different substrates (ITO/glass and SiO2/Si), without and with enzymes were fixed on a support on a system that assures XY displacement – Newmark USA, controlled by PC through codes written in Matlab 2014b. The distance between screen aperture and the surface to be examined has been maintained at 20 μm ± 1 μm. The sensor with MM lens made by two conical Swiss rolls [35, 41] is coupled to Network/Spectrum/Impedance Analyzer Agilent 4395A and is maintained fixed during the measurements. The acquisition through IEEE 488 interface and storage of data are made by the same PC. Considering the middle of the slits as reference point, symmetrically to left and right, 1mm distance have been scanned, along the length of strip 1mm distance have been scanned, thus a surface of 1x2 mm² is scanned, both on the sample without enzyme as well as with enzymes. The spatial resolution of the system was verified according to [34]. For MSG with features compatible with the value of incident field, TEz polarized wave acts, at normal incidence for MSG. The results obtained at the scanning of 1x2 mm² of MSG structures with 10 μm steps in both directions at frequency of 474 MHz were employed.

4. Results and discussions
Much recent attention has been focused on amperometric measurements because they can yield a linear relationship between sensor output and analyte concentration. Figure 6 shows the relation between the current and enzyme concentration for both architectures considered as biosensing materials. It can be observed that the current increases with the concentration and saturation appears at higher concentrations of enzymes. The inset is calibration curve at $\lambda=0.6$ m.

Thus, the Ag/ZnO/ITO/glass architecture exhibits good performance as biosensing material for glucose (Figure 6a) as well as Ag/ZnO/SiO2/Si architecture exhibits for cholesterol (Figure 6b). The response time is fast, which exhibits a high electron communication feature of the used ZnO thin films (Figure 6c). The calibration curves were obtained in the range of 50-1350 mg/dl (1.3-34.9 mM) of enzymes (Figure 6b) and the current response showed a linear dynamic range of 100-1050 mg/dl (2.6-27.1 mM). The tested sensor configuration showed large dynamic ranges with an output response that was linear versus $[1/$concentration$]$ of the enzymes activity with sensitivity at lower response current as shown in inset in Figures 6a,b. Adding 0.05 mM of ascorbic acid and uric acid to 0.5 mM glucose
and cholesterol only generated some noise shown in Figure 6c. The morphology of the nanostructure significantly affects biosensor electrochemical properties where ZnO nanostructures have been investigated for application in enzymes biorecognition materials.

Figure 6. Experimental results in test solutions (a) ZnO/ITO/glass and ZnO/SiO₂/Si; (b) Variation in the current with enzymes concentration; (c) Calibration curve showing the study of interferences with time trace line of output response change with time for both architecture biosensing.

5. Conclusions
In conclusion, we have demonstrated a simple fabrication procedure for a highly sensitive electrochemical glucose and cholesterol biosensing based on ZnO nanostructures as MSG structures. The paper presents an extension of the performances of the EM sensor with MM lens for experimental emphasizing of evanescent modes appeared in mesostructures in subwavelength regime, excited with TEz polarized EM waves in RF range. The study focuses on the apparition of evanescent modes for the case when the space between strips of MSG structures are filled with enzymes, confirming the results according to which the amplitude of the signal in the reception coils is increased when an enzymes (glucose and cholesterol) is present in slits. The redox current improves for the bioelectrodes based on ZnO film grown on different substrates. The good performance in terms of improved sensitivity, stability, selectivity, reproducibility, negligible interference and rapid response by our proposed biosensing materials also makes it suitable for externally integrating/interfacing a biosensing element to commercial devices, adding to the advantages of simplicity and low cost for the enzymatic detection of biochemically important substances.

6. References
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