

Antibody Immobilization in Zinc Oxide Thin Films as an Easy-Handle Strategy for *Escherichia coli* Detection

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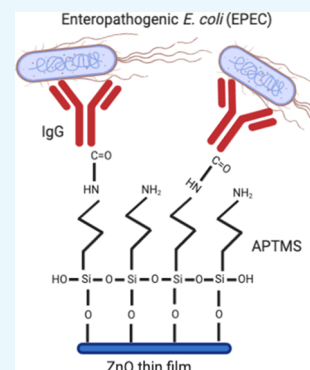


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ABSTRACT: The antibody immobilization compatible with low-cost materials and label-free strategies is a challenge for biosensor device fabrication. In this study, ZnO thin film deposition was carried out on corning glass substrates by ultrasonic spray pyrolysis at 200 °C. The thin films were analyzed as platforms for enteropathogenic *Escherichia coli* (*E. coli* EPEC) antibody immobilization. The modification of thin films from the functionalization and antibody immobilization steps was visualized using Fourier transform infrared spectroscopy (FTIR) spectroscopy, and surface changes were observed by atomic force microscopy. The obtained FTIR spectra after functionalization showed a contribution of the amino group (NH₂) derived from silane (3-aminopropyltrimethoxysilane). The antibody immobilization showed an amide I conserved signal corresponding to the C=O stretching vibrations and the amide II signal related to the N–H scissor vibration mode. In this way, the signals observed are correlated with the presence of antibody immobilized on the film. The ZnO film morphology changes after every stage of the process and allows observing the antibody distribution on the immobilized surface. In order to validate the antibody recognition capability as well as the *E. coli* EPEC detection *in situ*, polymerase chain reaction was used.



1. INTRODUCTION

The study of antibody immobilization on semiconductors has a growing interest for several applications, mainly in the field of biomedicine, such as immunoassays and biosensors.¹ Immunosensor devices for application in early detection of diseases, toxins, or analytes of biological interest is a technology with promising development in the future.^{2,3} On the other hand, enteropathogenic *Escherichia coli* (EPEC) is the chief cause of watery and potentially fatal juvenile diarrhea in the developing world, being considered a pathogenic microorganism of clinical concern.⁴ Most of the research about immunosensors is focused on antigen–antibody immobilization on sensor surfaces.^{5–7} The antibody–antigen interaction has been basically used to determine analyte concentrations and for bacterial identification as well.⁸ This has been possible because of the high sensitivity, affinity, and specific recognition that can be achieved. Commonly, the immobilization strategies are mainly focused on expensive materials such as nanoparticles, gold electrodes, platinum, and graphite as well as the use of highly crystalline materials.⁹ To overcome these limitations, the use of zinc oxide (ZnO) for antibody immobilization in biosensors has been studied.^{10–12} Some authors study biosensors for the enhancement of biomolecule detection in ZnO thin-film transistors.¹³ Other groups have reported ZnO nanosurfaces for C-reactive protein detection,¹⁴ cysteine-functionalized ZnO nanoparticles, using potato extract as a nontoxic and economical reducing agent,¹⁵ and electrochemical biosensors for detection of cardiac biomarkers, using the stoichiometric surface compositions of nanotextured

ZnO thin films.¹⁶ However, the conventional techniques often used for ZnO thin-film deposition (sputtering, plasma-enhanced chemical vapor deposition, ablation laser, among others) have some disadvantages if low-cost platforms are required, such as expensive reagents, the need of high vacuum, and low compatibility with large-area substrates. To solve these issues, it is necessary to find out easier and low-cost alternatives for thin film deposition. In order to employ solution-based low-cost technologies for low-cost electronic devices, the deposition temperature and post-thermal treatments are required to be equal to or lower than 200–300 °C.¹⁷ For instance, spray pyrolysis is an inexpensive and quite simple technique that can be operated at atmospheric pressure without a vacuum system. Moreover, deposition can be developed on large surfaces.¹⁸ As far as the authors know, an antibody immobilization method that uses ZnO thin films at low temperatures has not been reported. Therefore, the present work focuses on the use of ZnO thin films deposited at 200 °C as a low-cost platform for antibody immobilization as well as an easy-handle *E. coli* EPEC detection. Thin-film deposition, functionalization, and antibody immobilization

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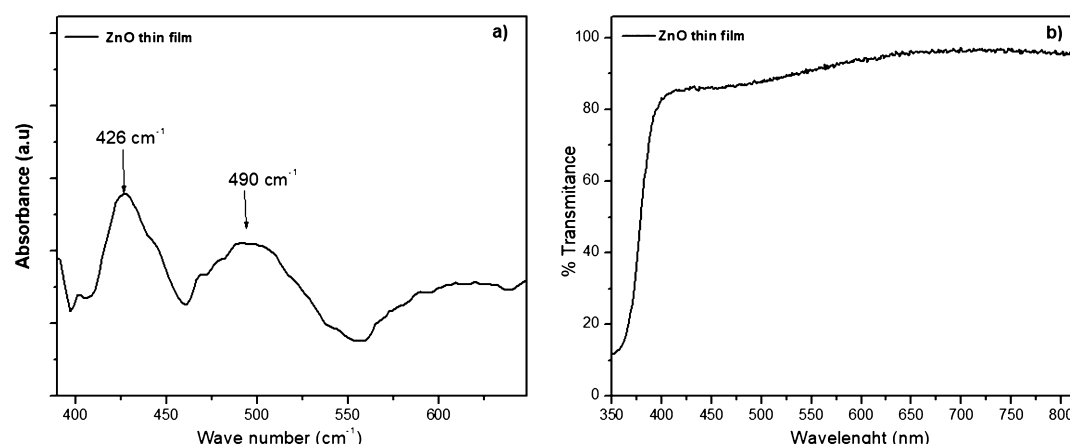


Figure 1. Optical and vibrational characterization studies of ZnO thin films deposited by spray pyrolysis at 200 °C. (a) FTIR–ATR spectrum in the absorbance mode. Characteristic peaks are related to Zn–O bond, (b) UV–vis spectrum in transmittance mode.

processes were examined using Fourier transform infrared spectroscopy in the attenuated total reflectance mode (FTIR–ATR). For characterization of the ZnO sensor surface, atomic force microscopy (AFM) analysis was developed. Moreover, bacterial detection was validated by the polymerase chain reaction (PCR).

2. RESULTS AND DISCUSSION

2.1. ZnO Film Characterization. Figure 1a shows the FTIR–ATR spectra of the deposited ZnO films. Characteristic peaks at 426 cm^{-1} are related to Zn–O stretching vibration and vibration-phonon mode E_1 (transversal optic) also, a second peak at 490 cm^{-1} is attributed to the Zn–O stretching vibrations that are correlated with previous reports.^{19–21} Moreover, Figure 1b shows the optical transmittance for ZnO films in the expected region. The spectrum showed high transparency in the visible region. Considerable differences between optical constants of the bulk material and thin films of different kinds of depositions are often reported.²² In this study, the optical gap energy was estimated by the Tauc method²³ using the values from the optical transmittance and film thickness (90 nm) by the extrapolation of the linear region of the $h\nu$ versus $(\alpha h\nu)^2$, the obtained value was 3.28 ± 0.02 eV.²⁴ High transparency in the visible light region has been associated with a decrease of film roughness because of the light scattering effect. The effects of the film formation mechanism, precursor concentration, and deposition temperature are directly related to the film morphology and transparency.²⁵ In addition, Figure 2 shows the XRD patterns of ZnO thin films deposited by spray pyrolysis on corning glass substrates. A preferred growth orientation in plane (100) is observed, and the observed peaks (100), (002), (101), (102), and (110) are related to the hexagonal wurtzite-type structure for polycrystalline ZnO thin films; these features are in agreement with previous reports.^{26,27} The average crystal size of the ZnO thin films is 45.9 nm, and it was estimated using Scherrer's equation,²⁸ for the preferential (100) orientation. Based on the above, spray pyrolysis is a suitable technique for good quality deposition of highly transparent ZnO thin films at low temperature, allowing it to be compatible for low-cost electronics applications.^{24,29}

2.2. ZnO Thin Film Functionalization. Surface activation was performed to achieve the generation of hydroxyl radicals on ZnO films. This process is carried out when the film is

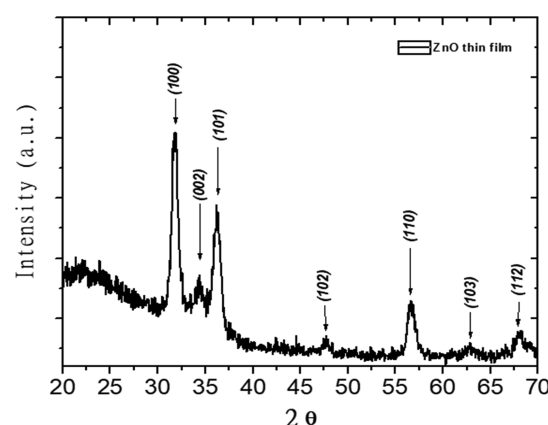
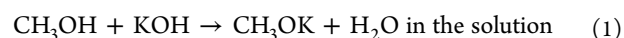


Figure 2. X-ray diffraction pattern of the ZnO thin films deposited by spray pyrolysis on corning glass substrates. It is observed a hexagonal wurtzite-type structure for polycrystalline ZnO thin films.

submerged into the KOH/CH₃OH solution, which can be explained by the following reaction



From this process, the functionalization of thin films with 3-aminopropyltrimethoxysilane (APTMS) was carried out. Figure 3 shows a comparison between two FTIR–ATR spectra in the functionalization step. An APTMS standard spectrum is included as a reference to visualize its conserved regions, while the other spectrum belongs to a functionalized ZnO film after treatment with APTMS. The most outstanding features are located between 1000 and 1200 cm^{-1} , where two peaks are observed at 1031 and 1115 cm^{-1} related to Si–O–Si and Si–OCH₃ bonds, indicating the siloxane formation and the horizontal APTMS polymerization. The absorption peak corresponding to Si–OCH₃ is related to a partial polymerization from APTMS because of the presence of unreacted methyl groups. Likewise, the observation of a low intensity signal between 2880 and 2928 cm^{-1} related to the CH_x stretching modes that are associated to the methyl groups mentioned before. However, this is an expected behavior that has been observed by other authors^{30–32} and does not affect the functionalization process as demonstrated by the antibody immobilization step (further discussed in Section 2.3). The next signal at 1448 cm^{-1} is associated with the symmetric –NH₃⁺ deformation mode while the signal at 1655 cm^{-1}

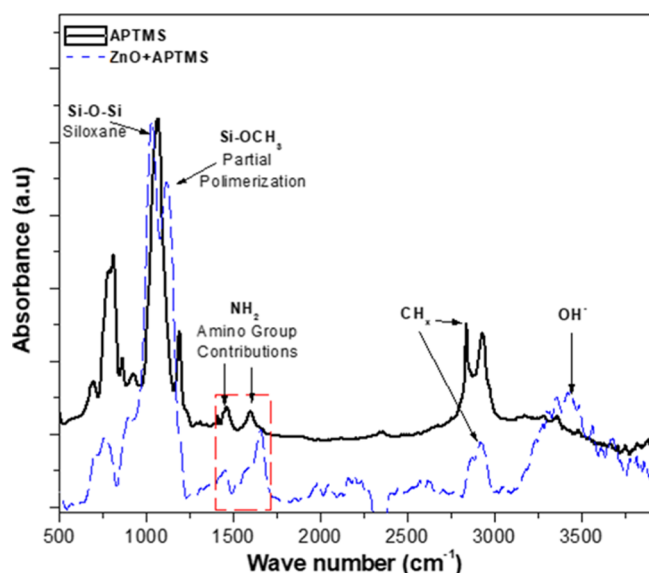


Figure 3. Comparative study of FTIR-ATR spectra after the functionalization process. The siloxane formation signals and the amino group contributions of the APTMS are observed.

corresponds to the low frequency side of the -NH_2 bending mode.³³ The presence of these absorption peaks suggests that when the functionalized films are exposed to air humidity, they interact weakly with NH_2 groups, resulting in the protonation of the amine. These signals confirm the presence of the APTMS terminal group. On the other hand, the generation of reactive OH^- radicals from the surface activation process is associated with the absorption peak at 3389 cm^{-1} , confirming the presence of hydroxyl groups. The general behavior of silanization has been described by Vashist,⁹ and it is mainly focused on the use of 3-aminopropyltriethoxysilane APTES. There are several reports that describe hydrolysis in APTES^{30,33} and the corresponding bands are similar to those observed in the present work. The general reaction is basically the same, the main difference is the loss of the APTMS methyl-group. Although there are three ways of binding to the substrate (surface attachment and condensation, multilayer formation, and hydrogen bonding),⁹ it is expected that the highest percentage corresponds to the surface bond and condensation enabling the polymerization and therefore the siloxane generation. The observed spectra show that the functionalization was properly developed.

2.3. Antibody Immobilization. Antibody immobilization on thin films focuses on the bond between the amino terminal group from silane and the carboxylic acid located in the antibody constant region. Hence, it is suggested that the binding to the heavy chain antibody enables a link to the silane which leads to the attachment of a determined percentage of antibodies on the surface. To verify antibody immobilization, FTIR-ATR characterization was developed. Figure 4 shows the antibody immobilization spectrum, where conserved peaks from the APTMS functionalization process are observed, mainly the Si-O-Si bond corresponding to siloxane. However, the signal related to a partial polymerization is not observed, suggesting that a percentage of Si-OCH₃ bonds that had an available NH_2 group were able to link the antibody, which can be confirmed by the presence of FTIR-ATR characteristic protein signals.¹ The absorption peak at 558 cm^{-1} corresponds to the S-S bond associated with the

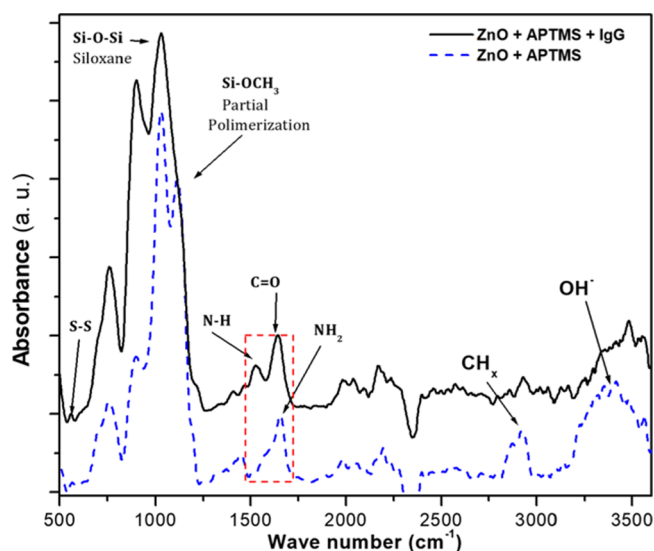


Figure 4. Comparative study of FTIR-ATR spectra after the immobilization process. Signals related to amide I (C=O), amide II (N-H), and disulfide bridges (S-S) are observed, confirming the antibody immobilization.

disulfide bridge that is present in the protein structure. This signal allows to infer that the used immunoglobulins (IgG) were immobilized on ZnO films. Because the antibody is a protein, two main contributions have been observed belonging to amide I and amide II. The signal related to amide I at 1643 cm^{-1} corresponds to C=O stretching vibrations of the peptide bond groups with minor contributions from the out-of-phase C-N stretching vibration and the amide II peak at 1528 cm^{-1} is related to the bending N-H vibration mode which supports the evidence of a correct antibody immobilization.^{1,34} It is worth mentioning that some reports use block nonspecific protein adsorption such as a surface treatment with bovine serum albumin prior to exposure to the target sample for the sensor systems.^{9,35} However, in the present work an important aspect is to ensure that FTIR-ATR observed signals are specific for IgGs, so there are no additional contributions in the vibrational modes related to other blocking proteins. The weak intensity of the CH_x peak is related to the Si-OCH₃ bond disappearance allowing to infer that when antibodies are linked to the available amino groups, the partial polymerization signals are decreased. Once the antibodies were immobilized on the surface, the calculated immobilization efficiency was 64.5% according to the quantification by the Bradford method.

Figure 5 shows the antigen-antibody interaction where an important signal reduction was detected, compared to the immobilization process, mainly associated with *E. coli* detection. Some works on antibody immobilization over sensor surfaces use cross-linking agents as glutaraldehyde which represents additional steps of processing. However, glutaraldehyde shows important signals in diverse regions on the spectrum ($600\text{--}1500, 2500\text{--}3500\text{ cm}^{-1}$)³⁶ that can easily hide signals related to bacteria and silanization, as the signal corresponding to fatty acids at 2935 cm^{-1} , siloxane (1031 cm^{-1}) and amide II (1528 cm^{-1}). Therefore, in the spectrum presented, constructive signals contribution is not observed, the signal decrease corresponding to siloxane and amide I peak at 1643 cm^{-1} indicates a masked signal because of bacterial interaction. The amide II peak becomes different and is not a defined signal, evolving into a shoulder whose contribution

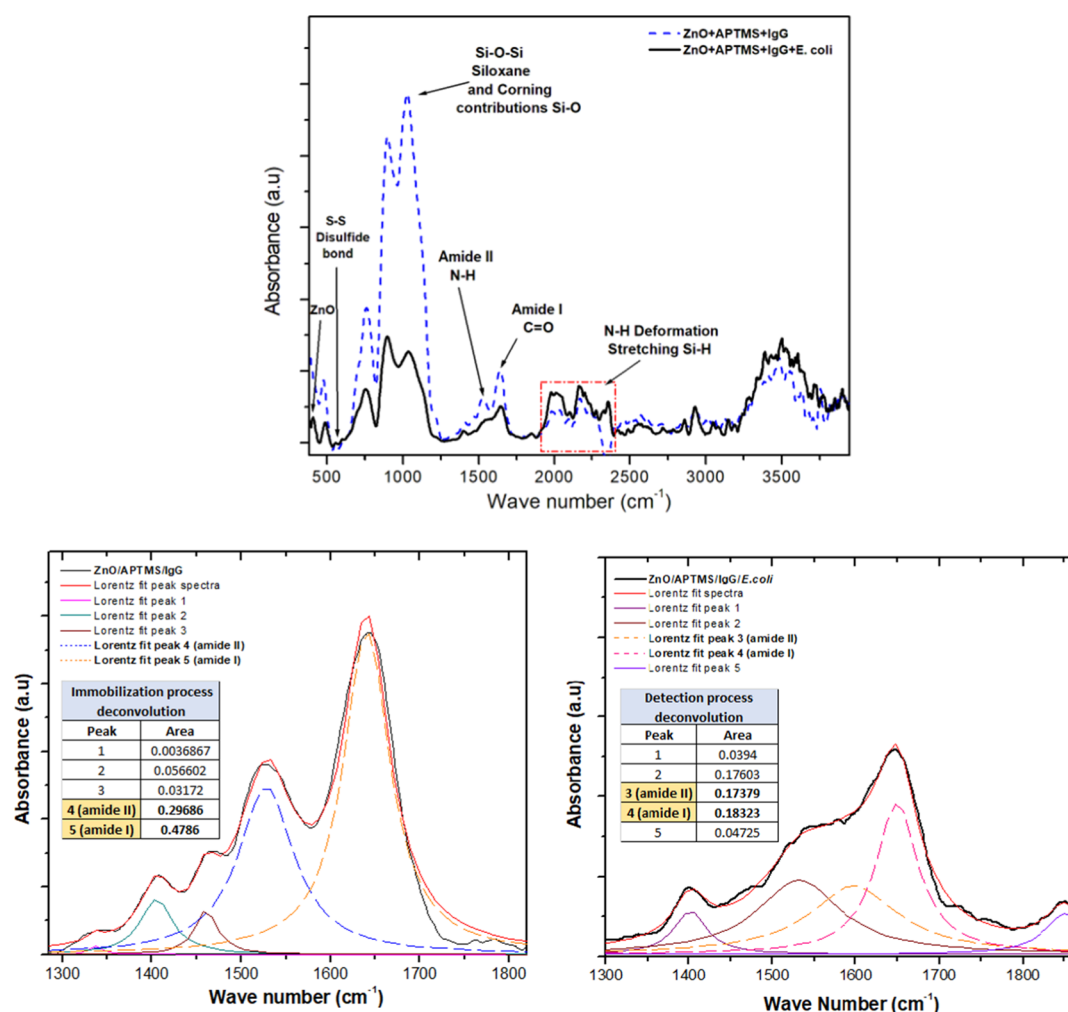


Figure 5. Comparative study of FTIR-ATR spectra after *E. coli* EPEC detection and deconvolution analysis on the amide region (1300–1800 cm^{-1}). The signal of fatty acids (2935 cm^{-1}) is related to the membrane of *E. coli* EPEC confirming the bacterial attachment to the sensor surface.

could only be observed through a deconvolution. It is remarkable that there is an increase in the signals belonging to the deformation of the N–H bond (2002–2371 cm^{-1}) which were not observed in previous processes, this is widely related to the availability of NH_2 groups that were in the films. Once the recognition is done, these signals can be observed more clearly. The other relevant signal is the one corresponding to the fatty acid region at 2935 cm^{-1} , which confirms the presence of bacterial fatty acids as well. The deconvolution analysis from the amide region that corresponds to the immobilization and detection spectra was made with Lorentzian peaks using specialized software. The obtained correlation factors, R^2 , were 0.985 (immobilization) and 0.995 (detection) considering the 5 peaks fitting. The observed signal reduction of peaks at 1643 and 1528 cm^{-1} was correlated to the area represented by each peak. For the case of the amide I signal, the area reduction was 61.7%. This is associated with the decrease in the signal intensity and the fact that there is a lower antibody concentration attributed to this. Hence, it is suggested that bound bacteria on the surface avoid observing a defined signal. The bacterial membrane is composed of several fatty acids, lipopolysaccharides, carbohydrates, and diverse compounds, while proteins represent only the 20% from the membrane.¹ Therefore, the observed FTIR-ATR signal does not have any constructive contribution that

allows a signal intensity increase, this is only associated with the bacterial cells bound to the device. Hence, a decreased signal in the proteic region is observed, and the presence of peaks attributed to the bacterial membrane as well; always maintaining the amide I signal. The amide II region showed a decreased signal ranging from 0.2968 to 0.17379 corresponding to the 41.45% signal reduction from the original area. Table 1 summarizes the active signals found in the FTIR spectra showing the most important FTIR peaks observed along the different stages from the full process because the identification of Zn–O bonds of the thin film until the fatty acid signals from the bacterial detection. In order to evaluate the surface changes that show the ZnO films after each stage of the biofunctionalization process, AFM measurements were used. The Figure 6 shows the set of contact mode AFM images of the (a) ZnO thin film, (b) after functionalization, and (c) after the immobilization process.

For the ZnO thin films the calculated root mean square (rms) roughness is ~ 1.13 nm (15 $\mu\text{m} \times 15 \mu\text{m}$). Adamopolus *et al.*²⁹ report low roughness values for ZnO thin films deposited by the spray pyrolysis that correlate with our measured roughness. In contrast, an important change on the surface roughness was observed after the functionalization process with APTMS that is explained due to the silane polymerization. This polymerization takes place during the

Table 1. Active Signals Found in the FTIR Characterization from the Full Process

FTIR signal peaks and vibration modes		
steps	vibrational mode	wave number (cm ⁻¹)
ZnO thin film	Zn–O stretching	426
	Zn–O stretching	490
functionalization	Si–O–Si	1031
	Si–O–CH ₃	1115
	CH _x (C–H)	2880–2928
	NH ₃ ⁺ (N–H)	1448
	NH ₂ (N–H)	1655
	OH ⁻ (O–H)	3389
immobilization	S–S	558
	amide I (C=O and C–N)	1643
	amide II (N–H)	1528
antigen–antibody interaction	N–H deformation	2002–2371
	fatty acids (C–H)	2935

thermal treatment for the siloxane condensation and formation (Si–O–Si). However, it has been reported that cluster formation increases the roughness as observed by Nagare *et al.*³⁷ This is an expected behavior because of the multilayer formation in our process (Figure 6b), the calculated rms roughness is 2.3 nm which is in agreement with Joshi,³⁸ who reported an increased roughness value of 2.2 nm after functionalization. Furthermore, the antibody immobilization stage shows a new change in the surface morphology that enables the observation of the antibody distribution on the immobilized surface, suggesting that the oriented antibodies attached on the surface are enough for bacterial recognition. According to the observations of Wang, the strategies that are used for antibody immobilization (random or site-directed) show increased roughness because of the absence of cross-

linker agents, such as protein A.³⁹ This is in concordance with our results, the increases in the height of the observed sample (Figure 6c) and its structure allow to infer that different antibody orientations are present. Hence, it is suggested that there are no observable clusters on the film because the cluster formation is related to roughness decrease. These clusters show that antibodies have restrictions on their binding sites that could block the bacterial detection.

2.4. PCR Validation. In order to validate the bacterial detection using a molecular approach, the PCR reaction has been used as a sensitive and reliable alternative for testing antigen–antibody interactions.⁴⁰ Figure 7 shows the PCR



Figure 7. Visualization of PCR products on agarose gel (0.8%) electrophoresis. Initial lane: 10 kb weight marker, lane 1: negative control, lane 2: positive control with 2 primers (bfp and eae), lane 3: sample 1, lane 4: sample 2, lane 5: sample 3, lane 6: control positive *E. coli* EPEC primer bfp, lane 7: sample 1, lane 8: sample 2, lane 9: sample 3, lane 10: positive control *E. coli* EPEC primer eae, lane 11: sample 1, lane 12: sample 2, lane 13: sample 3 and final mane: marker 10 kb.

amplification of exclusive regions of *E. coli* EPEC involved in the pathophysiological adherence mechanism. These genes are:

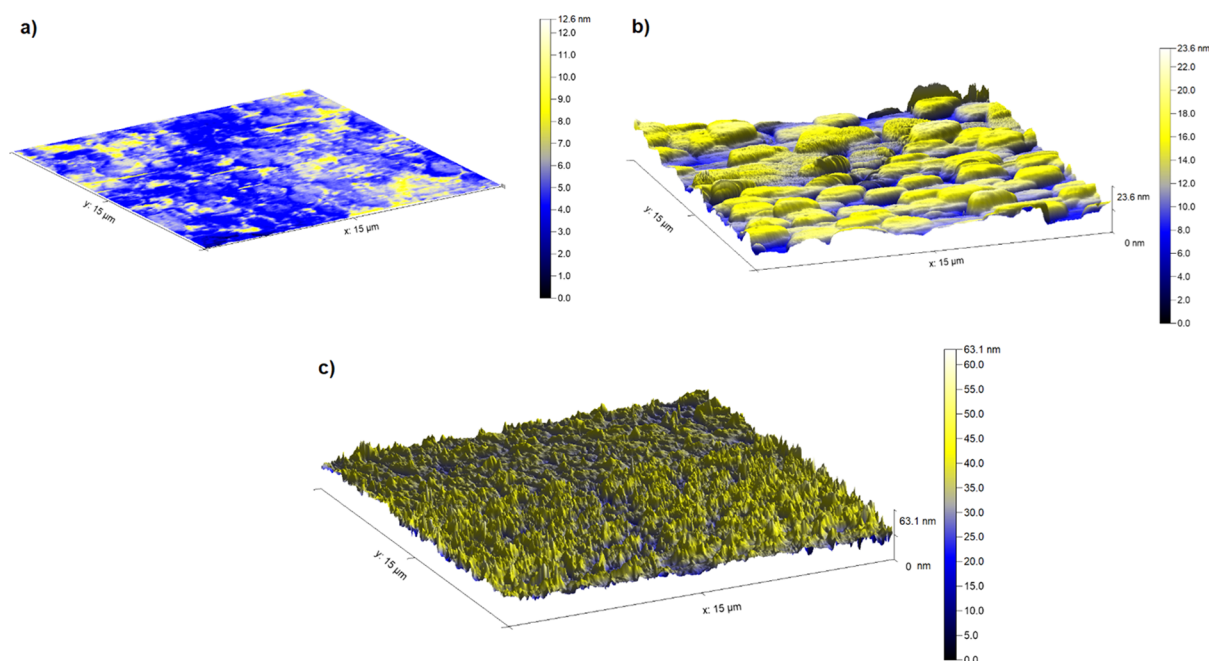


Figure 6. Set of contact mode AFM images of the (a) ZnO thin film, (b) after functionalization, and (c) after the immobilization process. There are observed changes in roughness after each step.

(1) *bfpA* (bundle-forming pilus) that codifies for pili type IV involved in bacterial clustering and formation of tight microcolonies on tissue culture cells as an adherence pattern⁴¹ and (2) *eaeA* that codifies for the bacterial adherent in the translocation mechanism of intracellular signals.^{42,43} In Figure 6, a positive control (lanes: 2, 6 and 10) of gene amplification directly from *E. coli* EPEC genomic DNA was included. Lanes 4, 5, 8, 9, 12, and 13 show PCR amplification from biofunctionalized films using standardized conditions after antigen–antibody interactions (detection). As can be observed, both regions were amplified showing the same amplicon lengths as positive controls. This result proves that the detection time used in this methodology (3–10 s) is enough for bacterial attachment, therefore the *E. coli* EPEC identification is performed in real-time. This suggests that immobilized antibodies are suitably oriented for bacterial detection and, as a result, the bacterial cells detected are enough for its identification by PCR. Lanes 3, 7, and 11 were biofunctionalized samples subjected to a higher antibody immobilization time (out of standardized conditions) and they were negative to PCR validation. Considering that the sensitivity of a conventional PCR ranges from 0.45 to 194 DNA pg,⁴⁴ the obtained genomic DNA was not enough for positive PCR validation. This could have resulted in the blockage of antibody active sites for bacterial epitope recognition confirming that bacteria are unable to bind superficially. It is just accomplished by direct interaction antibody–antigen. This means that unspecific adhesions to the surface are not feasible because of the minimal contact time used during the detection process (3–10 s).

Hence, this study helps to detect notable changes in the general tendency of the use of thin films deposited by specialized techniques for antibody immobilization.⁹ Although several studies have described novel surfaces for antibody adsorption with improved orientations,⁶ they have left aside the study of supports for the optimization of antibody immobilization and its compatibility with electronic devices. Nowadays, one of the main aspects to consider when working on biosensors are the sensor surfaces, materials, and the challenge to provide compatibility between the inorganic material platforms and the biological recognition element. In this sense, biofunctionalized sensor surfaces compatible with low-cost electronics give an opportunity for signal amplification. On the other hand, although there are methodologies based on the use of cross-linking agents, such as glutaraldehyde or protein A, this study provides a methodological strategy that allows the antibody immobilization to silane without the use of intermediaries, providing better compatibility with electronic devices. The perspective of this work is not limited for just one type of microorganism; conversely, a specific identification of other bacterial species is possible using monoclonal antibodies designed for this purpose.

3. CONCLUSIONS

Antibody immobilization has been reported using ZnO thin films deposited by ultrasonic spray pyrolysis at a low temperature. From the analysis of FTIR–ATR, the functionalization with APTMS shows a peak related to siloxane 1031 cm^{-1} , a signal at 1654 cm^{-1} (NH_2) related to the contribution of the amino groups available for binding, methyl groups signals at 2880–2928 cm^{-1} , and hydroxyl groups at 3389 cm^{-1} . The antibody immobilization shows the amide I (1643 cm^{-1}), amide II (1528 cm^{-1}), and disulfide bridge signals (558 cm^{-1})

present in the protein structure, confirming the presence of the antibodies immobilized on ZnO films. Comparing the signals and controls, the specific presence of the protein and bacterial signals was observed, concluding that the antigen–antibody reaction was accomplished. Morphological changes in the sensor surfaces were visualized from the different stages of the process by AFM, enabling to observe the distribution of antibodies on the surface. This was supported by the molecular validation, making it possible to perform the genomic DNA extraction from ZnO biofunctionalized films after real-time contact with *E. coli* EPEC. Although a random immobilization technique was used, a suitable antibody orientation was achieved for bacterial detection that was confirmed by the PCR reaction. Hence, it is suggested that ZnO thin films deposited at low temperatures can be used as a sensor surface compatible with low-cost electronics.

4. MATERIALS AND METHODS

4.1. ZnO Film Deposition and Characterization. ZnO thin films were deposited on corning glass substrates. A precursor solution of ZnO (0.2 M) was prepared by dissolving dehydrated zinc acetate $\text{Zn}(\text{CH}_3\text{COO})_2 \cdot 2\text{H}_2\text{O}$ (99.9%, Aldrich) in methanol. Prior to each deposition, the corning substrates were cleaned using an ultrasonic bath with acetone and then with isopropyl alcohol, during 5 min each. The precursor solution was deposited through a nebulizer CITIZEN CUN 60 at 0.2 mL/min flow rate and 2.5 MHz frequency for 20 min. The samples were deposited at 200 °C on a hot plate under atmospheric pressure. The ZnO thickness was ~90 nm measured by a profilometer. The absorption properties of the ZnO-deposited films were evaluated by FTIR (Vertex 70 Bruker, spectrometer) in the middle region (400–4000 cm^{-1}). The ultraviolet visible (UV–vis) spectroscopy was used to measure the optical transmittance spectra of the films, in an UV–vis spectrophotometer (Evolution 600 Thermo Scientific) from 190 to 900 nm. For the XRD analysis, the pattern of diffraction was obtained using a X-ray diffractometer (XRD) (DISCOVER D8-Bruker axs) at 2θ range between 10 and 90 and 0.002° step. To observe the surface changes from the biofunctionalization process of ZnO films, AFM measurements were taken in AFM Q-Scope 250 equipment (Quesant Instrument Corporation), contact mode, 300 Hz and 4 nm sensitivity. The AFM images were visualized using Gwyddion software (Department of Nanometrology, Czech Metrology Institute) 15 $\mu\text{m} \times 15 \mu\text{m}$.

4.2. ZnO Thin Film Functionalization. The surface activation step was developed from a hydroxylation reaction using a 0.1 M KOH/ CH_3OH solution dissolving 1 mL of KOH (1 M) in 9 mL of CH_3OH (99.8%), in which the ZnO films were submerged during 20–30 min to generate OH^- radicals over the film surface. When the incubation time was concluded, ZnO films were rinsed with methanol and dried with a stream of N_2 . Thereafter, the silanization process was carried out by treatment with APTMS (3-aminopropyltrimethoxysilane) Sigma-Aldrich 97%. For this purpose, ZnO films were submerged in a 2% APTMS solution in dry toluene for 30 min. Samples were then rinsed in dry toluene to remove excessive APTMS, submerged in methanol, and dried with a stream of N_2 . To ensure the correct bond Si–O–Si from APTMS and remnant removal, silanized films were set under thermal treatment in a drying oven for 60–90 min. These functionalization conditions were optimized from several trials

varying the time and concentration of APTMS that provide a suitable silane polymerization on ZnO films.

4.3. Antibody Immobilization. Anti-total *E. coli* EPEC antibodies used were obtained from the total rabbit serum and purified by ionic exchange chromatography. The chromatographic column was packed with 6 mL of diethylaminoethanol–sepharose. A Tris 10 mM (pH 8.5) solution was used to equilibrate the chromatography column. Then, the rabbit serum sample (2 mL) was injected into the system. The fraction elution was carried out with an increasing gradient of NaCl concentrations (50–500 mM) in Tris buffer 10 mM (pH 8.5).

Five fractions were recovered and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis to verify the antibody appearance in each recovered fraction. Once the fraction containing the higher antibody concentration was identified, it was used for the interaction between antibodies and functionalized films. Therefore, ZnO-functionalized films were treated with phosphate-buffered saline (PBS) buffer (pH 7.2) with a final concentration of 0.1 mg/mL *E. coli* EPEC antibodies, for 60 min at 4 °C. Finally, the films were rinsed three times in PBS buffer. Both, the functionalization and immobilization processes were characterized and verified by FTIR–ATR spectroscopy. It is worth mentioning that the immobilization conditions were optimized based on the proper antibody concentration to avoid the clustering of the antibodies that could inhibit the functionality of antibody active sites available for bacterial detection.

4.4. Antigen–Antibody Interaction. The strain *E. coli* EPEC E2348/69 was reactivated by the plate culture in Luria–Bertani (LB) medium. After 24 h growth an individual colony was propagated in a 100 mL LB broth and incubated at 37 °C and orbital agitation of 150 rpm. The optical density at 600 nm (OD_{600}) was measured until the culture reached an $OD_{600} = 0.25 \pm 0.05$ equivalent to 1×10^8 UFC/mL. This value was previously corroborated through growth-plate kinetics using the microserial dilution technique. *E. coli* EPEC culture was transferred to 50 mL Falcon tubes and centrifuged (8000 rpm, 10 min). The obtained pellets were washed twice with deionized water and resuspended in 100 mL of sterile water to maintain the same cell concentration. Then, antibody immobilized films were submerged in this cell suspension during a time interval from 3 to 10 s. After the contact time, ZnO thin films were washed three times and dried to avoid false positives.

4.5. PCR Validation. PCR reaction was used for the amplification of *eaeA* and *bfpA* genes to validate *E. coli* EPEC detection using the protocol and primers developed by Oh *et al.*,⁴³ which are the following:

- *eaeA*220F (5′-CGGCGATTACGCGAAAGA 3′).
- *eaeA*220R (5′-CCTAAATTTGCCGTAAAGCGG-3′).
- EP-*bfpA*(400)-F3 (5′-AGAATGCTATTTTCAGAAG-TAATGAGCG-3′).
- EC-*bfpA*-R (5′-TTACATGCAGTTGCCGCTTC-3′).

For genomic DNA extraction, the thin films were rinsed in the lysis solution and the procedure followed was a traditional alkaline lysis extraction. The reaction started with an initial denaturation at 95 °C for 3 min, followed by the amplification cycle (35 cycles) as follows: 95 °C for 30 s, primer annealing at 58 °C for 1 min, primer extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min. The amplified products

were visualized by DNA gel electrophoresis on 0.8% agarose stained with sybr safe DNA stain.

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Notes

The authors declare no competing financial interest.

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