Original Article

Development of AlN thin films for breast cancer acoustic biosensors

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\textbf{A B S T R A C T}

Although the development of biosensors has become popular in the recent past, there are still many opportunities to develop specific designs that address public health problems of third world countries. This paper presents the initial efforts toward the development of an affordable acoustic biosensor for breast cancer detection. The core of the sensor consists of a piezoelectric AlN thin film that requires specific crystallographic and morphological features. In this study, the processing-structure relationship of our radio-frequency magnetron sputtering (r.f. MS) system was established. Al/AlN films were produced via r.f. MS varying the applied power and atmosphere composition. The films were analyzed by glancing angle X-ray diffraction, scanning electron microscopy + energy dispersive analysis, X-ray photoelectron spectroscopy, and transmission electron microscopy. The results indicate that applied power had a much stronger influence on the phase selection, orientation and morphology of the films and was attributed to the effect of power on ad-atom mobility on the substrate. Higher power values resulted in films better suited for biosensor applications. The presence of the Al adhesion layer favored the formation of undesirable metastable c-AlN. Preliminary results on the biofunctionalization of the films were encouraging, but require further work both in the protocol and on the effect of the film surface on this process.

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1. Introduction

Statistics on breast cancer are alarming. This disease is the most common type of cancer among women worldwide and it is increasingly affecting women in underdeveloped countries [1]. It is widely accepted that early detection is one of the
main factors to reduce the mortality rate [2]. However, typical detection methods such as self-examination and mammography are not as widespread in poorer countries. Self-examination requires careful training, often not the case- and even if performed correctly does not have the resolution required for early detection. The more accurate mammography is out of reach, both economically and geographically, for a large sector of poorer populations [3]. Thus, it is clear that any efforts in the development of affordable, portable, accurate technologies for early breast cancer detection would be highly desirable for underdeveloped countries, although the benefits would be worldwide.

One of the alternatives to develop such an affordable detection method is the use of biosensors. These devices basically consist of a biological recognition element and a transducer. The biological recognition element captures the biomarker of the disease of interest from a biological media. The transducer takes the biological signal and transforms it into an optical, thermal or acoustic one. Upon further processing this signal generates a read-out indicating the presence of the biomarker. In particular, acoustic sensors, which were originally developed for telecommunications, have been adapted for biosensing in recent years due to their high sensitivity, portability, and handling of small amounts of biofluids. More importantly, they have the potential to become affordable [4]. In this technology, acoustic waves travel through a piezoelectric material, whose surface has been treated to capture a biomarker of interest. If this occurs, the additional mass of the biomarker will alter the characteristics of the waves and produce a signal. Almost universally, the piezoelectric material utilized is either quartz, AlN, ZnO, or PbZrTiO₃. The sensitivity of the sensor can be increased if the thickness of the piezoelectric material is reduced, for it leads to an increase in its resonant frequency [5]. Thus, nowadays these materials are typically incorporated in the form of thin films. In this regard, most of the research has concentrated on AlN or ZnO films deposited through a variety of techniques, however, magnetron sputtering (MS) stands out as one of the better suited methods for the fabrication of biosensors [6–8]. Furthermore, in terms of the recognition of the biological signal, a biosensor should be highly selective for a specific biomarker associated to breast cancer. The biofunctionalization of the surface by interactions between materials and organic compounds depends on the chemical composition, stiffness, surface energy, topography and roughness of the biomaterial surface in contact [9], features that can be tailored in MS thin film deposition.

Since the role of the piezoelectric thin film is central for the development of an effective biosensor and its properties are largely determined by the structure developed during deposition, the present study is part of an investigation into determining the optimal deposition method and parameters to obtain the best piezoelectric films. In particular, the aim of this study was to establish the effect of applied power and reactive atmosphere during deposition of AlN thin films by r.f. reactive magnetron sputtering through an extensive structural characterization. The potential performance of these films was investigated, not only based on their microstructural features, but also on their response to biofunctionalization experiments specific for breast cancer detection. The self-assembled monolayer (SAM) formation for biomarker capture was applied by induction of chemisorption, through affinity interaction of receptor protein-antibody [10].

2. Experimental procedure

2.1. Deposition experiments

The Al/AlN films were produced by reactive magnetron sputtering of an Al target in a commercial PVD reactor with an r.f. power source, model CHA600 Industries. Twelve films were obtained by varying the atmosphere composition and applied power as indicated in Table 1. The substrates were 304L stainless steel disks, 1.5 mm thick and 25.4 mm in diameter. The steel composition was 0.019% C, 1.570% Mn, 0.32% Si, 18% Cr, 8.05% Ni and 0.033% P, 0.028%S, Fe balance. Before each deposition, the surface of the disks was prepared with abrasive SiC paper up to a 1200 grit, followed by polishing to mirror finish and ultrasonic cleaning for 5 min in ethanol. A high purity Al target was used and the substrate to target distance was approximately 7 cm.

A typical deposition run started after achieving the vacuum pressure of 0.66 Pa, which was generated after 3 h by a cryogenic pump. A flow of 60 sccm of argon was then introduced, increasing the pressure to 1 Pa. In these conditions, the r.f. source was turned on at the selected power value and the deposition of an Al adhesion layer was carried out for 10 min. This step was followed by the deposition of the AlN layer by introducing the selected flow of nitrogen, resulting in a pressure value of around 1.33 Pa. The AlN deposition step was carried out for 3 h for all samples.

2.2. Characterization of films

The films were characterized by scanning electron microscopy (SEM), energy dispersive microanalysis (EDS), glancing angle X-ray diffraction (GAXRD), optical profilometry (OP) and X-ray photoelectron spectroscopy (XPS). SEM and EDS where performed in a Jeol 6360LV equipment. GAXRD was performed at 40 kV and 25 mA with Cu-Kα radiation in a Bruker D8 ECO-ADVANCE apparatus for the phase identification. Two times steps were used: 0.5 s and 1 s, and X-ray incidence angles (θ) from 0.3° to 1.0°. A Bruker Contour GT-K non-contact optical surface profilometer was used for the surface roughness measurements with a 5× magnification objective lens and white light. The profilometer is equipped with a fully automated turret, programmable x, y and z movement and a NanoLens™ AFM module. For each sample, five measurements were taken and the mean and standard deviation where calculated. For XPS, an ultra-high vacuum (UHV) Scanning XPS microprobe PHI 5000 VersaProbe II from Physical Electronics was used. An Al K X-ray source (hν = 1486.6 eV) was applied at 25 W and with an MCD analyzer. At 45° to the normal surface the spectra were captured in a constant pass energy mode (CAE) for survey surface of E₀ = 117.40 eV and high-resolution narrow scan of 11.75 eV. A reference to the background silver 3d₃/₂ photopeak at 368.20 eV was done for the peak positions obtaining a FWHM of 0.56 eV, C 1s hydrocarbon groups and Au 4f₅/₂ at 285.00 eV and 84.0 eV, respectively.
Table 1 – Summary of the deposition conditions for the Al and AlN films.

<table>
<thead>
<tr>
<th>Atmospere composition (sccm)</th>
<th>60 Ar/45 N₂</th>
<th>60 Ar/30 N₂</th>
<th>60 Ar/21 N₂</th>
<th>60 Ar/12 N₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Applied power (W)</td>
<td>150</td>
<td>250</td>
<td>350</td>
<td>150</td>
</tr>
<tr>
<td>Sample number</td>
<td>S1</td>
<td>S2</td>
<td>S3</td>
<td>S4</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>250</td>
<td>350</td>
<td>150</td>
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<tr>
<td></td>
<td>S5</td>
<td>S6</td>
<td>S7</td>
<td>S8</td>
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<td></td>
<td>150</td>
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<td>350</td>
<td>150</td>
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<tr>
<td></td>
<td>S9</td>
<td>S10</td>
<td>S11</td>
<td>S12</td>
</tr>
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</table>

2.3. Biofunctionalization experiments

This section includes the immobilization procedure for producing the scaffold required to capture the biomarker of interest (human HER2) in the selected films (S7, S8, and S9), as well as the protocol to produce the reference HER2 concentration curve (ELISA assay) required to interpret the results from spectrophotometry.

2.4. Immobilization of anti-HER2

Protein G was coupled to the films by an overnight incubation (1 µg/mL in phosphate buffered saline) at 4 °C. The films were washed with TBS-Tween, and then blocked with PBS – 10% bovine serum albumin for 30 min at 37 °C. After blocking, the films were washed with TBS-Tween to remove the remaining albumin and rinsed with PBS to take out the tween residues. Anti-HER2 antibody HER2 (c-erbB-2) Mouse Monclonal Antibody (clone TAB250) Concentrate (#2800032) was incubated 1.5 h at 37 °C, followed by rinsing with TBS-Tween and PBS. Blocking was then carried out as above, and the films were washed with TBS-Tween and PBS.

2.5. Enzyme-linked immunosorbent assay (ELISA) for human HER2

The standard curve was obtained according to the kit standards (human HER2 Total #KHO0701; Invitrogen Corporation, Carlsbad, CA, USA). HuHER2 protein was reconstituted with 1 mL of standard diluent buffer. Serial dilutions were produced with the HuHER2 protein, in order to obtain several concentrations for the curve (0, 0.39, 0.78, 1.56, 3.13, 6.25, and 12.5 ng/mL of HER2). These were incubated in the wells of the coated (with Anti-HER2 capture antibody) ELISA plates and in the films for 1 h at 37 °C to facilitate the antigen-antibody reaction. After this procedure, the supernatant was discarded and washed with the kit wash buffer (10 mM phosphate buffer (pH 7.4, 150 mM NaCl, 0.05% Tween 20) to remove the unbound protein. Detection antibody was added to each well and to the films, and incubated for 1 h at 37 °C. The supernatant was discarded and washed in order to remove the unbounded antibodies. A solution of anti-rabbit IgG horseradish peroxidase-conjugated antibodies (HRP) was added to all the ELISA plate wells and films and incubated for 30 min at room temperature. In this step, the secondary anti body recognizes all the conjugate (capture antibody-HER2-detection antibody) and reveals the bonding via the (HRP) enzymatic reaction. Once again, the supernatant was discarded and the wells and films were washed as before. Finally, the chromogen stabilizer solution was added and left for 30 min at room temperature to produce a characteristic color. The results were analyzed in a spectrophotometer (SmartSpec Plus Spectrophotometer, Bio-Rad Laboratories Inc. US) at 450 nm. The intensity of the color analyzed by absorbance is proportional to concentration of HER2.

3. Results and discussion

3.1. Deposition experiments: effect of applied power vs. atmosphere composition

Fig. 1 presents the typical phases encountered in the films, using the diffractogram of sample S3 as an example. The GAXRD analysis indicated that all the samples showed the presence of hexagonal aluminum nitride (h-AlN) and a cubic phase, which could be associated to either cubic aluminum nitride (c-AlN), Al from the adhesion layer or unreacted Al within the AlN. In some instances peaks from the 304L substrate were also present. Fig. 2 is a compilation of the GAXRD information (time step: 0.5 s, incidence angle α = 0.5°) for the 12 samples produced. Here, the intensity of the peaks for the various phases encountered was plotted as a function of atmosphere composition for the three applied power values. Prior to analyzing this figure, it is important to indicate that the thickness of the films increased with applied power. The thickness range observed at each power were approximately 0.55–1.17 μm, 1.6–2.7 μm, and 2–3.7 μm, for 150 W, 250 W, and 350 W, respectively. Also, a rough estimate of the X-ray penetration depth was obtained following the criteria described in Ref. [11] giving a value of 0.75 μm. Thus, for the samples obtained at 150 W, most of the film was illuminated by the X-rays and the results may have some contribution from the substrate. On the other hand, for the samples obtained at 250 W and 350 W, the results
mostly correspond to the upper portion of the films. Despite this difference, it is clear from this figure that the applied power had a much stronger effect than composition of the atmosphere on phase formation. In terms of atmosphere composition, as the \%N\textsubscript{2} decreased there was a slight decrease in the amount of c-AlN for all power values. At constant power, as the \%N\textsubscript{2} decreased the number of reflections for h-AlN also decreased. There was no strong effect of the N\textsubscript{2} content on the presence of the h-AlN (002) peak.

The strong effect of the applied power can be seen in both, the phase selection and the orientation of the h-AlN. As power increased, more hexagonal AlN reflections appeared, and their intensity grew. More importantly, the presence of the h-AlN (002) peak was only clear at the highest power of 350 W, although not at the highest \%N\textsubscript{2} at this power value, but at 21 sccm of N\textsubscript{2}. This is, a (002) preferred orientation was not necessarily favored by a high N\textsubscript{2} content in the atmosphere, but rather by a higher applied power. This suggests that the formation of the (002) texture is more related to the energy of the depositing species, than to the abundance of nitrogen. Accompanying the changes in the GAXRD information, there were also clear differences in the structure of the films with applied power. These features were analyzed in detail in the samples obtained at 21 sccm N\textsubscript{2} for the three applied power values (S7 = 150 W, S8 = 250 W, and S9 = 350 W) by SEM, optical profilometry, XPS, additional GAXRD, and TEM below.

First, to help clarify the origin of the cubic reflections, Fig. 3 shows the survey spectra and details for the Al2p, N1s, and O1s peaks corresponding to S9. The position of Al2p peak was not useful to determine which phase, Al or AlN, existed since the published values for these phases overlap. However, the N1s peak is clearly closer to the position that indicates it was bound to Al in AlN, thus it was inferred that the cubic phase observed in the diffractograms was indeed c-AlN, at least in a sizable region close to the surface. Cubic AlN is not the most stable phase at room temperature and atmospheric pressure and should transform into h-AlN after annealing [12]. During deposition, it may be stabilized if it forms over a surface with similar crystalline structure, such as MgO [13], which crystallizes in a cubic structure. In the present films, it forms on the Al of the interlayer, which has a lattice parameter very close to that of cubic AlN. However, as deposition progresses, it eventually transitions to mostly hexagonal AlN, as discussed below. This result points to the selection of an alternative non-cubic material for the adhesion layer in future depositions. An additional important result revealed by the XPS analysis was that the films contained a significant amount of oxygen, which is also important and discussed ahead.

The general surface features of samples S7, S8, and S9 are presented in Fig. 4, which includes SE (secondary electron) images and the results from optical profilometry. The SE micrographs show that the film thickness, i.e. the deposition rate, and the surface roughness increased with applied power. However, the last effect may also be associated to a general increase of roughness with film thickness [14]. Roughness may become a critical feature of the AlN films, as it can affect their response to biofunctionalization [15,16], which will be further investigated.

Additional GAXRD analyses for S7, S8, and S9 at a slower scan rate (time step: 1 s) and different X-ray grazing angles (0.3°–1.0°) were carried out. The results are presented in the left column of Fig. 5 as the variation of the intensity of the h-AlN and c-AlN reflections with grazing angle, i.e., information depth. The values in these plots were corrected for absorption following the procedure suggested by [11] and only the peaks that had a clear value above the background were included.
On the right column of Fig. 5, TEM bright field images of the samples are also included. An estimate of the depth analyzed by GAXRD, based on the expression by M. Birkholz [11], have been marked on the TEM images. Analysis of Fig. 5 indicates that an outermost fine columnar region in the films is mostly composed of polycrystalline h-AlN, which only displayed a strong (002) texture for the highest applied power. The growth rate of this region increased with applied power. Although
the morphology of this layer was similar among the samples, the surface roughness values indicate that a higher applied power led to the formation of thicker columns. Deeper into the films, the presence of c-AlN increased, this is initially the formation of c-AlN was favored, although co-existing with h-AlN. The stronger h-AlN peaks throughout the thickness for...
the higher applied power values suggest that the transition from c-AlN + h-AlN to mostly h-AlN occurs at an earlier deposition stage in this case. The featureless region below has been attributed to a transition amorphous layer required to initiate the nucleation of crystalline AlN [14]. It is clear that this transition occurred more rapidly as power increased. Incorporation of oxygen within the AlN region did not affect the crystallinity of the film, but may have an effect on the piezoelectricity and perhaps the biofunctionalization of the films. Also, the deposition rate of the Al adhesion layer increased significantly with applied power.

It is important to discuss the oxygen level within the AlN layer. As can be seen in Fig. 3, this level is quite significant, and Fig. 6 shows that it is maintained for some depth below the surface. This is not surprising as oxygen-free AlN films are very difficult to produce, even in ultra-high vacuum conditions [17]. However, these authors indicate that the introduction of oxygen does not alter the structure of the AlN, even for contents up to 30%, and that the O replaces N in the h-AlN structure. This explains the absence of Al₂O₃ or oxynitride phases in the diffractograms, despite the large amount of O present. Although in the present films the presence of oxygen did not alter the crystal structure of the h-AlN, it can have an effect on its morphology and properties, particularly the piezoelectric effect [17–20]. However, the oxygen content in the AlN can be controlled to tailor some properties [18], but it would require a close control of the composition of the gases utilized, specifically Ar, as it seems to have a joint influence with O on the AlN properties [20]. Furthermore, the effect of oxygen in the AlN lattice on the biofunctionalization process remains to be determined. Currently, additional experiments are being carried out to minimize the presence of oxygen.

Considering the stronger effect of the applied power on the film structure, the results above can be explained in terms of an increase in the sputtered particle energy and flux as power increases, which results in higher ad-atom mobility on the substrate surface. This is further aided by a higher substrate temperature resulting from more energetic bombardment. As power increases, the higher ad-atom mobility leads to higher deposition rates for both the Al and the AlN films.

Additionally, for the case of AlN growth rate, it has been observed that transition to target poisoning during reactive MS, and the associated lower sputtering rates, is accelerated with decreasing power [21,22]. The formation of the stable h-AlN over other metastable phases is also to be expected to arise from higher ad-atom mobility with power. Accordingly, as power increases, a thinner amorphous transition layer formed and the metastable c-AlN was less abundant. More importantly, the formation of a h-AlN (002) can only be achieved if the atoms have enough energy to produce the close packing of these planes, while in the case of the lowest applied power, resulted in a more loosely crystal surface (101) predominating. The formation of similar layered structures during AlN deposition by magnetron sputtering has been observed [14,21,23]. The important conclusion is that in our setting, orientation of the film can indeed be manipulated to produce a specific texture.

### 3.2. Biofunctionalization assays

A central feature of the deposited AlN films for biosensors is its ability to undergo a successful biofunctionalization. After the microstructural characterization of films 7, 8, and 9, they were subjected to the biofunctionalization assays described in the experimental procedure. Fig. 7 shows the results of these experiments of the HER2 recombinant protein on the surface for the three samples. Although no clear trends were observed among the films, the results demonstrate that their surface was successfully coated with antibodies and proved by the recognition of HER2 recombinant. This is, the surface of the films was adequate to immobilize the protein G that acts as the anchor for the recognition complex-antibodies structure for the HER2 breast cancer biomarker. The parameters were obtained by interpolation in the concentration curve proportionated by the kit analysis. The HER2 recombinant protein adds to the anti-HER2 antibody with high avidity as we expected, so the analysis showed that all the samples expressed a concentration around 15 ng/mL of HER2. These concentrations were higher than expected. A more detailed analysis of the surface of the films is necessary to determine if some structural features at this location are causing some antibodies add to the surface in a non-specific alienation. The
protocol for the blocking process needs to be modified; therefore, further analysis with variation in some of the process parameters will be performed.

4. Conclusions

Al/AlN thin films intended for acoustic biosensors were deposited on SS304L substrates by r.f. reactive magnetron sputtering. In the conditions studied, the effect of applied power was stronger than that of atmosphere composition on the phase selection, orientation and morphology of the films and was attributed to the effect of power on ad-atom mobility on the substrate. It was inferred, by comparison to other studies, that the presence of the Al adhesion layer favored the formation of undesirable metastable c-AlN due to similarity in crystal structures and thus future work must include other adhesion materials. A considerable amount of oxygen was incorporated into the films. The control and effect of this element is imperative for future work. Biofunctionalization of the films was successful, however further analysis of the effects of the surface characteristics of the adhesion film on the AlN film structure and in turn of the AlN surface on the biofunctionalization process is required.

Conflicts of interest

The authors declare no conflicts of interest

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