Detection of adsorbed cytochrome c on hydroxyapatite thick films using a microwave sensor

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Abstract— **An interdigitated microwave sensor was used to detect the adsorption of cytochrome c on hydroxyapatite thick films. Changes in the microwave spectral response were indicative of the presence of adsorbed cytochrome c. The sensitivity of the system was also evaluated using different protein loadings on the e films. The results suggest that the microwave sensor can be utilized to detect protein.**

Keywords-Protein detection, hydroxyapatite, microwave sensing, interdigitated electroe

I. INTRODUCTION

Hydroxyapatite (HA) is a calcium phosphate which is the main inorganic component found in vertebrate bone, dentin and enamel [1] HA can be synthetized and possesses characteristics such as biocompatibility, non-toxicity, chemical stability and bioactivity as well as osteoconduction which make the material suitable for medical use. Synthetic HA has long been used as a scaffold for studying biological activity, especially in relation to calcification process. Recently, electrical properties *e.g.* piezoelectricity [2] and pyroelectricity [3, 4] of nanocrystalline films of HA have been reported. Furthermore, nanocrystalline thin films of hydroxyapatite have shown potential to host cells and proteins [5, 6] at their surface as a verifiable means for studying electrical and electromechanical interactions between the biological system and the biomaterial. The low roughness of these sol-gel deposited thin films can however be problematic for cellular interaction studies as cells typically do not preferentially grow on surfaces that are too smooth [7]. In practice, biomedical implant surfaces are often deliberately roughened or textured to facilitate cellular ingrowth [8]. Considering this and the fact that thick hydroxyapatite films have shown pressure induced capacitance changes [9], thick films of HA can potentially serve as model surfaces for the investigation of the dielectric behavior of HA over the GHz range.

Microwave sensing measures the dielectric response of a material at GHz frequencies [10, 11]. Microwave sensing has previously been used for applications including real time monitoring of different vegetable oils [12], the detection of glucose using a flow system [13] and the evaluation of activated carbon. The technique presents advantages of being nondestructive and requiring minimum to zero sample preparation. When a material is placed in contact with electromagnetic waves it will interact with these waves in a unique manner. This interaction could arise from a frequency change, attenuation or reflection of the electromagnetic signal. The permittivity of a material is related to its ability to transmit or store energy with changing frequency [14]. Microwave sensing measures the magnitude of reflection and transmission changes. This reflection signal depends on the structure of the material under examination. Depending on the nature and the permittivity of the sample, different spectra is obtained [15]. The permittivity (1) of a material is defined as a measure of how an electric field is affected by a dielectric medium. Thus microwave spectra are complex functions and arise from two properties: stored energy (ε') and loss of energy (ε'') which are related to the relative permittivity as:.

$$\varepsilon_r = \varepsilon' + j\varepsilon'' \quad (1)$$

Different microwave sensing structures such as cavities or antennas are used for the measurement of dielectric response in the GHz frequency [16]. Microwave interdigitated electrode (IDE) type sensors are sensitive to dielectric changes; their sensitivity can change close to the sensor surface [17] and their impedance can be controlled by the geometry of their design [18].

Dielectric properties of HA powders and thick films have been previously demonstrated using a microwave cavity [19]. Cytochrome c is a well characterized heme protein with large patches of positive charge [20] that can facilitate interaction with negatively charged surfaces such as those obtained on HA. In this paper we present the response of adsorbed cytochrome c on HA thick films in the GHz frequency range using an IDE microwave sensor.

II. Experimental Procedure

A. Materials

The following chemicals were obtained from Sigma-Aldrich: Polyvinyl butyral (Butvar B-98, Solutia Inc.), diethyelene glycol butyl ether (BDG ≥ 99 %,) cytochrome c, from equine heart (≥ 95%), potassium phosphate monobasic (99%), and potassium phosphate dibasic (Riedel-de Haën). Hydroxyapatite powders with particle sizes < 25 µm were obtained from Cambioceramics. All solutions were prepared using water that was purified with an Elga Maxima system an exhibited a resistivity of 18.2 MΩ.

B. Hydroxyapatite films

Borosilicate cover glass slides pieces were washed with isopropanol and dried. Squares (1.8 cm^2) were marked out on the glass using sellotape as a spacer. HA powders were ground using a high energy Gyro-Mill (Glen Creston Ltd, Stanmore, UK). HA powders were mixed with polyvinyl butyral (PVB) used as a binder and diethylenglycolmonobutylether added as a solvent. The obtained HA paste was smeared over borosilicate cover glass slides using a glass microscope slide as a squeegee. The obtained films were dried at 200°C for 15 min and then annealed (ramp rate of 5°C/min and for 1 hour at 700°C).

C. Fim modification

On removal from the furnace the glass slides with 1.8 cm^2 HA films were cooled and then immersed in different concentrations of cytochrome c in 10 mM KH_2PO_4 buffer, pH 7.0 for 90 min. Protein concentrations were determined spectrophotometrically using a molar adsorption coefficient of 100 mM-1cm-1 at 409 nm. Prior to all measurements the films with cytochrome c were rinsed in protein-free buffer solution to remove all non-immobilized protein.

D. Scanning Electron Microscopy

A Hitachi SU-70 Scanning Electron Microscope (SEM) was used to capture micrographs for morphological and thickness evaluation of the films. Thin films of gold were deposited on HA films to reduce charging effects.

E. Microwave measurements

Microwave measurements were performed using an interdigitated electrode (IDE) attached to a vector network analyzer (Rohde and Schwarz ZVA24) as previously reported [17]. All measurements were carried out in air. The reflected signal was recorded for all samples for all samples (60,000 points for each measurement) over the range 0.01-15 GHz.

III. Results and Discussions

Hydroxyapatite (HA) is a leading biocompatible material extensively used as grafts and grafts, coatings and scaffolds, thus the understanding of protein interaction with HA is of interest. HA thick films were deposited on borosilicate glass . SEM images of the obtained HA thick films can be seen in figure 1. Glass substrates were fully covered with porous thick films of HA. The SEM images show a spherical morphology of the films (Figure 1 (a)). Figure 1 (b) shows the cross section micrograph of the HA thick films that reveals a thickness of approx. 60 µm. It is worth noting that surface roughness can be advantageous for cellular interaction studies since it is more similar to bone itself. High magnification images of these thick films showed the nano-crystalline nature of HA as can be observed in Figure 1 (c). In addition no cracks or other defects were found.

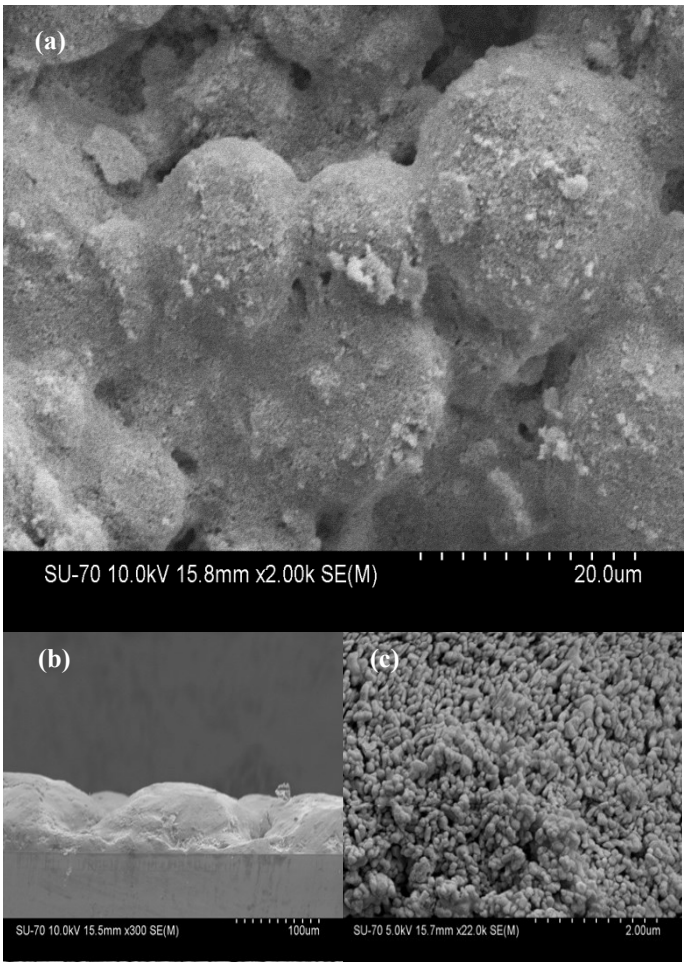

Figure 1. SEM micrographs of HA thick film: (a) surface morphology, (b) cross section and (c) high resolution image of the film.

Dielectric properties of HA and its response in the GHz frequency range has been previously analyzed using a microwave cavity [19]. Figure 2 illustrates the spectra recorded for HA and air using and IDE microwave sensor in the 0.01-15 GHz frequency region. The obtained microwave spectra were unique for HA with different frequency peaks than air. The spectra were identical for all 30 measurements and identical response for two different samples was also obtained. Thus confirming the repeatability and reproducibility of HA samples.

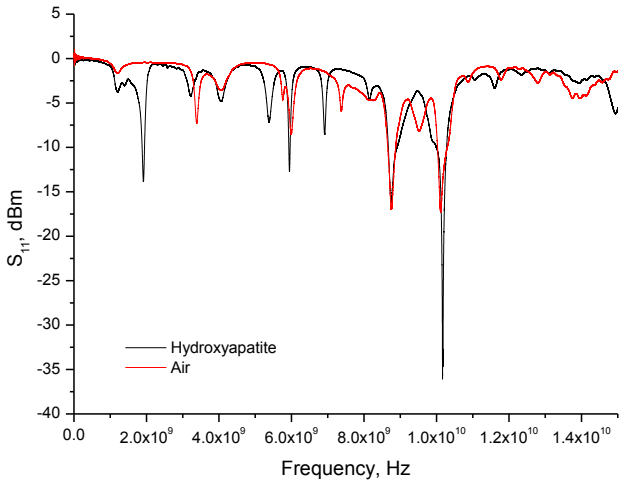

Figure 2. Microwave response of IDE antenna in 1-15 GHz range for air and HA.

HA thick films were modified with cytochrome c to analyze protein detection using an IDE microwave sensor. Two different concentrations were used for this and the protein loading on the films was calculated using a molar adsorption coefficient of 100 $mM^{-1}cm^{-1}$ at 409 nm [20]. The protein loadings at low and highs solution concentrations were .24 and 1.74 nmol cm^{-2}, respectively. The modified HA films were placed in contact with an IDE type microwave sensor and spectra in the 0.01-15 GHz frequency range were recorded. Figure 3 illustrates the spectra of the modified HA films, HA and air within the 2.5-4 GHz frequency range. The obtained spectra show different patterns for each sample thus demonstrating the presence of cytochrome c on the HA layer. The response was similar for all samples, demonstrating the reproducibility of the response.

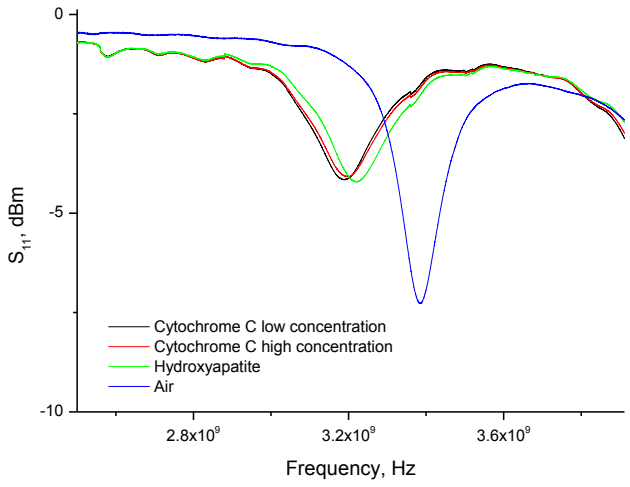

Figure 3. Reflected signal (S_{11}) of the microwave sensor for HA samples modified with cytochrome c, 2.5-4.0 GHz range.

The thickness of the cytochrome c layer is small in comparison with that of the HA films; this could explain why the signal difference is so small. To further analyze the obtained spectra of the modified films; HA spectra were subtracted (Figure 4). The difference on the concentration is more noticeable confirming the sensitivity of the technique.

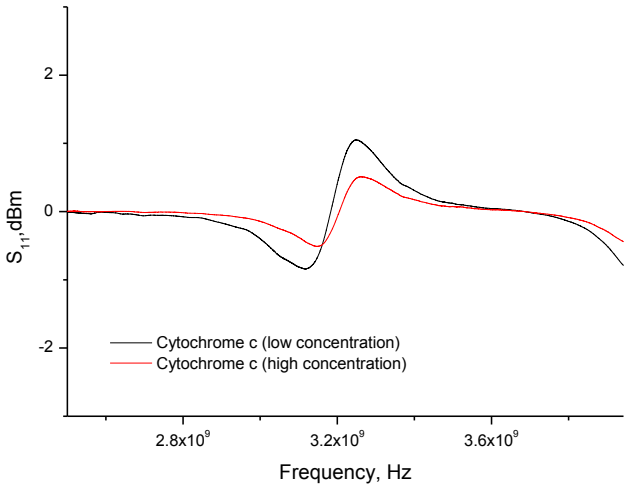

Figure 4 Reflected signal of the IDE sensor in 2.5-4.0 GHz range with HA subtracted.

CONCLUSION

The detection of cytochrome c on HA thick films using a microwave sensor has been confirmed. The sensor response in the microwave frequency range revealed that the resonant frequency changed when in contact with different materials. The sensitivity of the sensor was demonstrated at different protein concentrations. The slight difference in signal may be attributed to the small thickness of the protein layers adsorbed. This approach has the potential to provide a response to protein adsorption, however to detect specific binding will require systems with a recognition element. Work is currently in progress to develop such systems.

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