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Simultaneous Identification of Amorphous Calcium Phosphate and *S.epidermidis* Bacteria by Photoacoustic Spectroscopy

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Abstract. The incorporation of biomaterials in human tissue requires methods to study the interface of the implant with the biological setting. We set out to study whether Photoacoustic spectroscopy with a higher level of sensitivity from the cantilever detector could simultaneously detect amorphous calcium phosphate and the bacteria. The calcium phosphate was synthesized, pressed into tablets, and then immersed in a solution containing *S.epidermidis* bacteria. Spectra were recorded after 1, 2, 3, 4 and 5 days. Deconvolution of the spectra at different time periods was able to separate bands belonging to the bacteria and carbonate bands arising from the calcium phosphate. This allowed the simultaneous identification of the biomaterial and bacteria. It was found that the PAS spectra could not identify the bacterial adhesion process due to the low concentration, but the amide peaks at 3 days inferred colonization of bacteria. This was confirmed by SEM that shoed an increase in the bacteria concentration. This is the first step in showing the simultaneous detection of calcium phosphate and bacteria by Photoacoustic spectroscopy, a method that required more research to show changes on the surface of the implant.

Introduction

Amorphous calcium phosphate (ACP) plays an important role in the formation of natural biomaterials and so it is necessary to have the appropriate tools for tracking the transition of unstable ACP to a crystalline apatite. This transformation has been studied more on wet samples [1 – 3], where crystallization occurs more readily than dry heated ACP [4]. Following the state of ACP is a necesary materials characterization capability as well as providing insight into transitions that may results after contact with cells.

Fourier transform infrared (FTIR) can identify functional groups in ACP and carbonated calcium phosphates (ACCP) [5,6] as well as detect bacteria [7,8,9]. A major advantage of FTIR spectroscopy is that spectra can be obtained from almost any physical state of sample (solutions, suspensions, powders). In previous studies, spectra of bacteria have been collected in the transmission mode from dried films or dried smears of cultures on IR transparent plates (ZnSe, ZnS, et.al.) [9], or in the reflection mode on aluminium plate or dried single colony mixed with KBr [10]. Photoacoustic (PA) spectra of colonies have been collected in isotonic solution or directly on the sample holder [11]. FTIR Photoacoustic (PA) spectroscopy with a cantilever detector offers greater sensitivity and can simulataneously collect spectra from the biomaterial surface and the bacteria, thereby providing a glimpse of the events at the interface.

Our study will show that FTIR PA spectroscopy can be used to investigate ACCP (an opaque surface) through the analysis of $\upsilon_3CO_3^{2-}$, $\upsilon_2CO_3^{2-}$, υOH bands [12,13] and simultaneously detect bacteria bands from amide, fatty acid, polysaccharide and phosphate-carrying region [11].

The objective is to evaluate ACCP pellets and bacteria interacting with the biomaterial surface. We have chosen microbial analysis as a model, in which we wanted to simultaneously see two

processes: functional groups of ACCP and the presence of bacteria on the surface. This investigation model includes the following stages: synthesis of powder, formation of pellets, sterilization of pellets, adhesion of bacteria, colonization of bacteria and analysis with FTIR PAS and scanning electron microscopy (SEM).

Methods

Sample preparation. ACCP powder were prepared by mixing a solution A [calcium nitrate $Ca(NO_3)_2$ solution, 30% ammonia] with a solution B [dibasic ammonia hydrogen phosphate $(NH_4)_2HPO_4$ and ammonium carbonate $(NH_4)_2CO_3$ solution], filtered, washed several times with deionized water containing ammonia and dried at room temperature until constant mass. Powder was uniaxially pressed (N=10kN) to 10 mm diameter and 2 mm high pellets. Two methods were selected for sterilization: a) in an autoclave at 121°C and 15psi for 20 min, b) a hot air in an oven at 160 °C for 2h.

Microbiological testing. The process includes the following steps: a) adhesion of bacteria and b) colonization of bacteria.

Adhesion of bacteria. In the study, *S.epidermidis* ATCC 12228 was used as reference culture. Suspensions of bacteria were made from the microbiological cultures in 1 ml of trypticase soy broth (Oxoid, UK) with the concentration of 10^2 colony forming units (CFU)/mL. The samples were cultivated at 37°C for 24 hours.

Modification of this method was used to record PAS spectra and SEM images directly on the ACCP surface. Some ACCP pellets were stored at 37°C in suspension of bacteria for a longer period: 72h – 168h. This method was used only for qualitative detection of bacteria.

Colonization of bacteria. The quantitative sonication-plate count method was used to determine the presence of bacteria and the degree of colonization.

Modified roll plate method [14] was used, to record PAS spectra directly on the ACCP surface. This method was used only for qualitative detection of bacteria. The method was performing by rolling the external surface of a pellet on the surface of an agar plate at least three times, then one pellets surface was placed directly on the agar plate and then incubating the plate for 24h, after which the bacteria were detected by PAS and SEM.

Table 1. Domains, assignments and for *S.epidermidis* bacteria and ACCP.

Wavenumber [cm^{-1}]	**Bacteria**	**ACCP**
2800 – 3000	-CH_3, >CH_2, ≡CH 3000 and 2800 cm^{-1}	-OH ~3750 cm^{-1}
1200 – 1800	-amide I & II bands of proteins, peptides 1800 and 1500 cm^{-1}; -amide III bands 1310 and 1220 cm^{-1}; -CH_3, >CH_2 1500 and 1400 cm^{-1}	-υ_3CO_3 (A, B, non-apatitic) 1400 – 1540 cm^{-1} -OH 1640 cm^{-1}
900 – 1200	-polysaccharide region	-υ_1PO_4 962 – 964 cm^{-1} -υ_3PO_4 bands 1000 – 1140 cm^{-1}
600 – 900	-“fingerprint region”	-υ_3CO_3 (A, B, non-apatitic) 800 – 905 cm^{-1}
600 – 400	-	-υ_4 PO_4, υ_L OH 460 – 700 cm^{-1};

Characterization of the surface. PAS spectra were taken at 450–4000 cm^{-1}, at a resolution of 4cm^{-1}, and an average made from 10 scans. For PAS, the pressed pellets were placed in the PAS cell (Gasera PA301), filled with helium gas (flow 0,5 *l*/min). SEM images were taken were by FEI Quanta 450, 2keV; 3keV, SE detector and distance 10 mm.

Analysis of spectra. The FTIR spectra were viewed and smoothed with freeware software *Specwin32*. Baseline correction and curve fitting analysis was performed using *MagicPlotStudent* software. Deconvolution and convolution was performed involving Lorenzian and Gaussian curve fitting.

The FTIR bands have been assigned to chemical functional groups and all 3 spectra has been divided into several spectral regions on the basis of interpretations made by earlier studies: a) bacteria FTIR domains/assignments [7,8,9] and b) ACCP domains/assignments on [12,13] (Table 1).

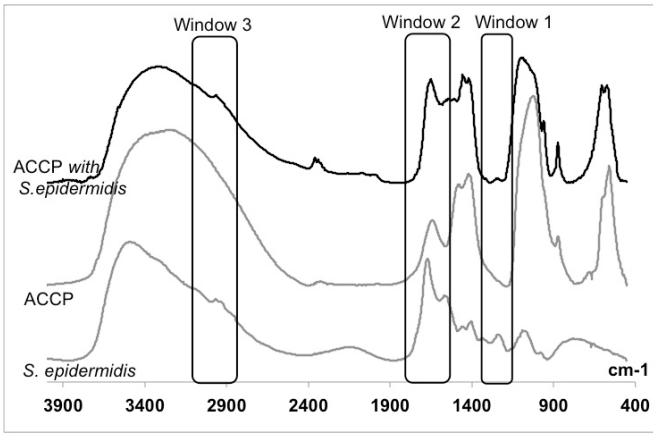

Fig.1. FTIR PAS spectra of ACCP surface, *S.epidermidis* bacteria and ACCP surface with bacteria.

Results and Discussions

FTIR PAS spectra of separately grown bacteria *S.epidermidis*, the ACCP surface and the ACCP surface with *S.epidermidis* are shown in Fig.1. Carbonate bands (1400 – 1540 cm^{-1}; υ_3CO_3 A, B), OH bands (1640 cm^{-1}) were identified on the ACCP surface. Amide bands (I, II, III) in the polysaccharide region suggested the presence of *S.epidermidis* bacteria. By reference to Table 1, we can infer that υ_3CO_3 A, B bands and OH bands of ACCP with overlaping amide (I, II) bands from *S.epidermidis identify both sides of the interface*.

The PAS spectra of ACCP, *S.epidermidis*, and ACCP with bacteria occur in 3 spectral regions showing organic matter and bonds in ACCP:

Window 1 – undisturbed region: 1310 – 1200 cm^{-1} amide III bands; components of proteins.

Window3 – undisturbed region: the $-CH_3$, $>CH_2$, ≡CH functional groups.

Window2 – overlapping amide and ACP bands.

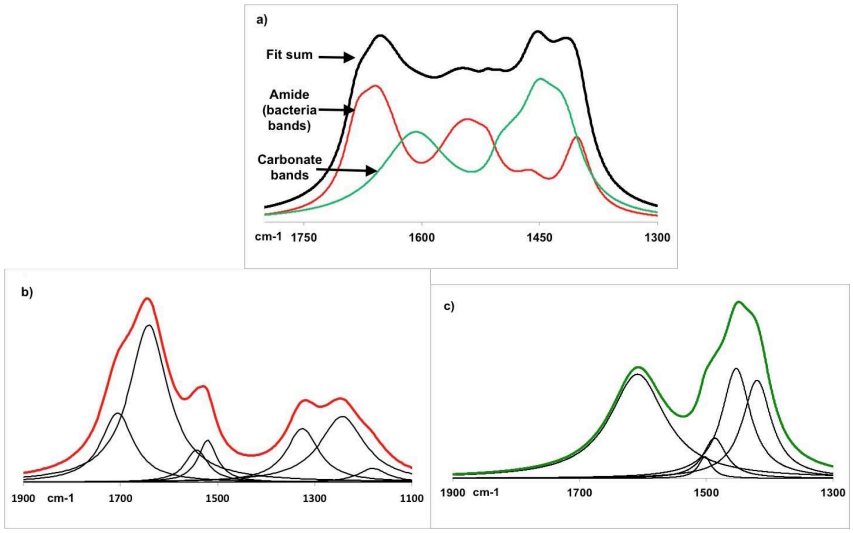

Fig. 2. a) The Deconvolution of ACCP surface with S.epidermidis showing deconvoluted and convoluted b) Amide band and c) Carbonate band.

The main information about bacteria on the ACCP surface can be taken from Window2. Determination of an organic matter is limited by overlapping amide, carbonate and OH bands in 1500 – 1800 cm^{-1} region. Detailed information of the overlapping bands can be obtained by the deconvolution of the PAS spectra (Fig.2). The deconvolution shows organic amide bands (Fig. 2a), and carbonate bands and OH bands from ACCP (Fig. 2b).

Colonization of bacteria could be identified from more intense Amide I and amide II bands, and a greater bacteria concentration in the SEM images (Fig.3.b). Storage of the ACCP pellet in a solution containing bacteria showed amide peaks after 3 days at 37 °C. (Fig 4.). The

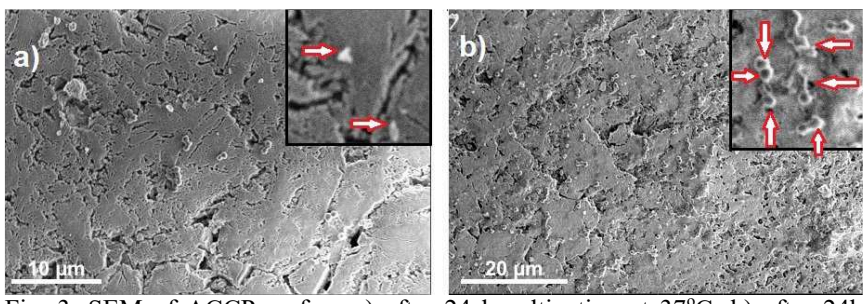

Fig. 3. SEM of ACCP surface a) after 24 h cultivation at 37°C; b) after 24h colonization on the ACCP surface.

first stage of bacterial adhesion could not be detected. SEM images showed a small bacterial concentration on ACCP (Fig.3.a). Further studies will need to determine the minimum number of bacteria that can be identified by the PAS.

Window1: 1310 and 1220 cm^{-1} amide III bands were clearly detected in pure *S.epidermidis*, but barely detected on the ACCP surface..

Window3: $-CH_3$, $>CH_2$, ≡CH functional groups were detected after 24 h from the time of ACCP immersion in bacteria and after the colonization process.

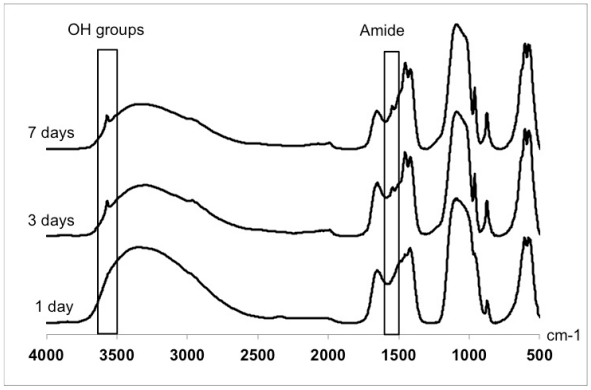

Fig.4. FTIR PAS spectra of ACCP in bacteria suspension after 1, 3 and 7 days.

In order to investigate amide peaks, ACCP pellets were stored in bacteria solution 24h – 7 days. After 3 day was possible to detect formation of OH peak at ~3750 cm^{-1} (Fig.4).

Conclusions

Analysis of FTIR PAS spectra taken from ACCP immersed in bacteria showed bands characteristic of ACCP and *S.epidermidis* bacteria. The adhesion process of bacteria could not be identified due to a low concentration, but colonization could be detected, and was confirmed by SEM.

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