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Article *in* Key Engineering Materials · November 2017

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Key Engineering Materials
ISSN: 1662-9795, Vol. 758, pp 273-277
doi:10.4028/www.scientific.net/KEM.758.273

Submitted: 2017-06-12
Revised: 2017-08-03
Accepted: 2017-09-07
Online: 2017-11-15

In Vitro Characterization Perspectives Using Fourier Transform Infrared Photoacoustic Spectroscopy (FTIR PAS) and Diffuse Reflectance Infrared Spectroscopy (DRIFT)

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Keywords: FTIR-PAS, FTIR-DRIFT, *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, *in vitro*, Principal Component Analysis (PCA), Nanocrystalline apatite (NA).

Abstract. The activity of antibacterial material is conventionally estimated by using an indirect method – a bacteria suspension is inoculated onto a surface, and then the bacteria are collected from the surface and examined as to whether they can form colonies on the agar plate [1]. In the present study, the presence of bacteria was examined by direct detection. Our study is based on FTIR PAS with an interferometer cantilever detector [2]. Our work discusses the possibility of identifying and distinguishing the presence of different bacteria (*Staphylococcus epidermidis and Pseudomonas aeruginosa*) and the possibility to evaluate the crystallization processes on the pressed calcium phosphate surface.

Introduction

Infection is a major problem in orthopedics, leading to implant failure [1]. Microorganisms like *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* growing in biofilms typically cause infections [2]. There are various methods for both quantitative and qualitative evaluations of biofilm formation, for example, "live-dead-staining", confocal laser scanning microscopy, fluorescence microscopy, electron microscopy (REM/SEM), or atomic force microscopy (AFM). However, they need a special pre-treatment like staining (live-dead-staining, CFSM) or carbon-sputtering (REM/SEM) [2-6].

FTIR spectroscopy, coupled with a multivariate statistical analysis, offers a wide range of applications for the detection and differentiation of bacteria, and the investigation of calcium phosphate based biomaterials. In previous studies, the spectra of bacteria were collected in the transmission mode from dried films or dried smears of cultures on IR transparent plates (ZnSe, ZnS, etc.) [7,8,9]. The diffuse reflectance spectroscopy (DRIFT) sampling method was used to analyze powder or solid samples, including freeze-dried samples, or a dried single colony mixed with KBr [10], but the photoacoustic spectroscopy (PAS) spectra of colonies was collected in an isotonic solution, or directly on the sample holder [11-12].

The present study will show FTIR spectroscopy sampling methods: DRIFT and cantilever-enhanced photoacoustic spectroscopy to be rapid and reliable techniques, which could be explored as routine diagnostic tools for bacterial and calcium phosphate based implant analysis without special sample preparation. Our study will show that FTIR PAS with a cantilever detector offers great sensitivity and can simultaneously collect spectra from the biomaterial surface as well as bacteria [13] and, when coupled with the Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA), can, identify and discriminate bacteria.

Materials and Methods

Sample preparation

Synthesis. Amorphous calcium phosphate was synthesized by the wet chemical method in a basic environment (reactants $Ca(NO_3)_2 \cdot 4H_2O$, $(NH_4)_2HPO_4$) with Ca/P 2.5:1 and pressed into d=10 mm, h=2mm pellets.

Microbiological testing. Gram-positive *Staphylococcus epidermidis* and gram-negative Pseudomonas *aeruginosa*, were prepared in a sterile saline suspension by the *McFarland* standard with an optical density of 1.0. Suspensions were trickled drop by drop directly on the pellet surface.

Characterization of bacteria and pellet surface

Cantilever-enhanced photoacoustic spectroscopy (FTIR-PAS). PAS spectra were taken with Gasera PA301 at 450 – 4000 cm^{-1}, resolution of 4 cm^{-1}, 10 scans, the cell filled with helium gas (flow 0.5 l/min). A special preparation method was not required for bacteria; 1 cm of pressed calcium phosphate pellet, with or without bacteria suspension, was placed in the PAS cell.
Modified diffuse reflectance spectroscopy (FTIR-DRIFT). DRIFT spectra were taken with PerkinElmer Spectrum One at 450 – 4000 cm^{-1}, resolution of 4 cm^{-1}, 8 scans. A special preparation method was not required for the bacteria and calcium phosphate powder; samples were measured directly on the diamond sampling stick.

Spectral *pre-processing.* The FTIR spectra were viewed, smoothed, and had their baseline correction and normalization performed with the freeware software *SpectraGryph 1.0* and *Grams/AI*. The spectra were normalized to the most intense band. All spectra were smoothed and denoised by Savitzky – Golay filter (polynomial order 4 and points 10).

Statistical Analysis. The Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA) were performed using *SIMCA* software. PCA was used to identify the dominant clusters in the data set [14]. For the hierarchical cluster analysis, Ward's algorithm was used [15]. We performed unsupervised multivariate technique, because this method do not require a dependent variable for modelling, it search for patterns among the independent variables, and groups of samples are formed based on the structure of the variables [16].

Spectra Analysis. The FTIR bands were assigned to chemical functional groups based on domain/assignment interpretations made by earlier studies: bacteria [11], nanocrystaline apatite [17,18] and the simultaneous identification of both bacteria and calcium phosphate [13].

Results and Discussions

Two non-destructive FTIR sampling methods were used to determine and distinguish bacteria: FTIR-DRIFT and cantilever-enhanced FTIR-PAS. The FTIR-DRIFT was performed to rapidly screen for the primary data (minimum concentration for identification and possibility to distinguish) and FTIR-PAS - to simultaneously investigate the tablet surface along with the colonized bacteria.

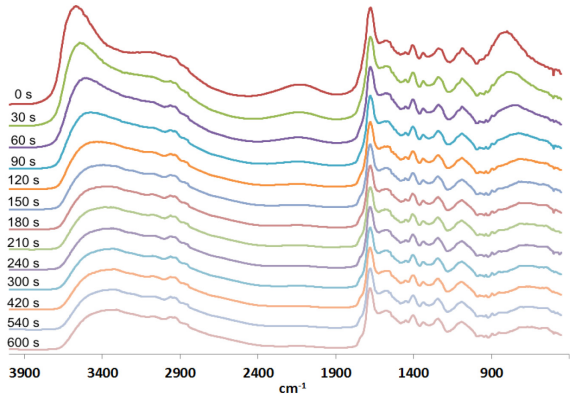

Fig.1. *S.epidermidis* FTIR DRIFT spectra over time

FTIR-DRIFT. During the recording spectra, we observed changes in the spectra region 2500 – 4000 cm^{-1} over time (Fig.1). We assumed it was connected with the drying process on the diamond sampling stick. To confirm our assumptions, we recorded DRIFT spectra over time (0 – 600 s).

Both bacteria *S. epidermidis* and *P. aeruginosa* showed very similar spectra on simple visual examination. To evaluate and compare the obtained spectra, we performed pre-processing (smoothing, baseline correction, and normalization) and 2nd derivative and unsupervised multivariate statistical analysis with PCA and HCA. The principal component analysis with the PC and HCA containing seven PC, with a total variance of 90%, showed two clusters that occur regardless of bacteria drying (Fig.2). The DRIFT method showed the possibility to very rapidly distinguish *S. epidermidis* and *P. aeruginosa* bacteria without prior sample preparation, and that sample drying does not affect the outcome.

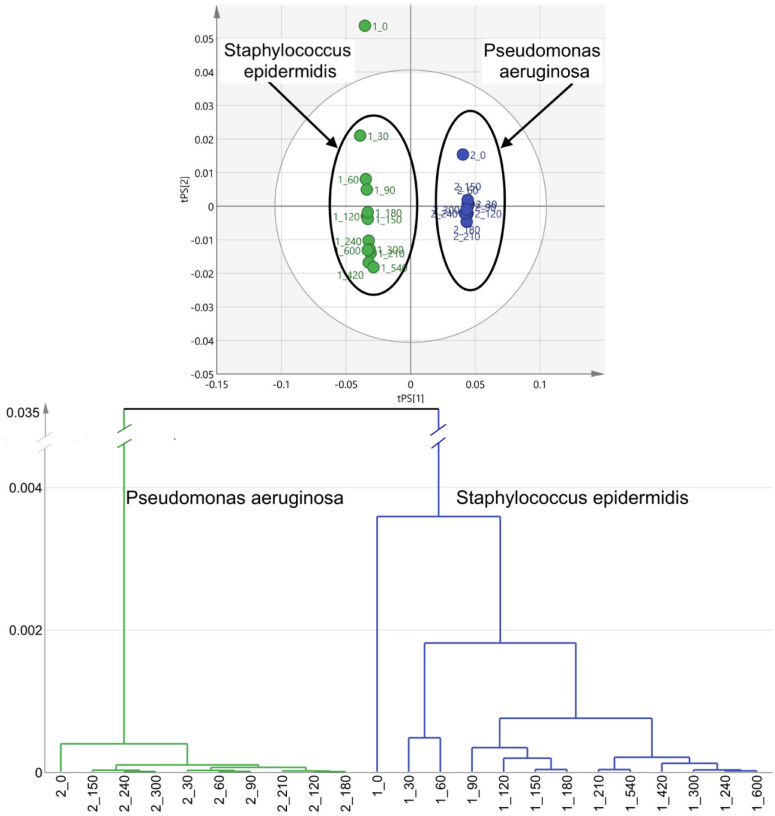

Fig.2. *S.epidermidis* and *P. aeruginosa* PCA and HCA for DRIFT spectra over time

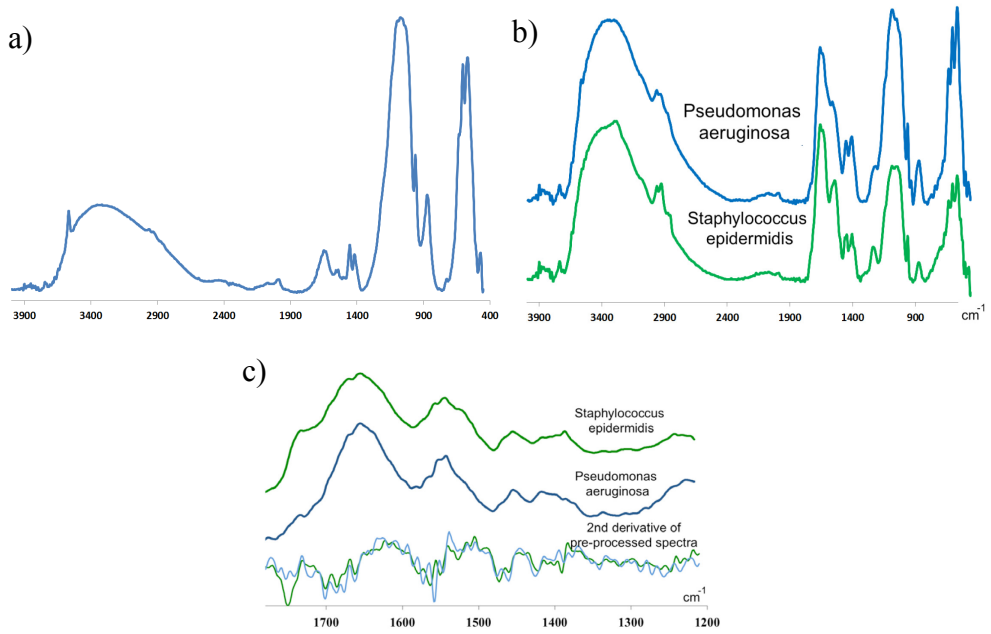

Fig.3. FTIR-PAS spectra: a) NA, b) NA with bacteria and c) 1200 – 1700 cm^{-1} spectra region of overlapping peaks

FTIR-PAS. FTIR PAS spectra were obtained of the pressed nanocrystalline calcium phosphate (NA) surface and the NA surface with *S. epidermidis* and *P. aeruginosa* bacteria (Fig.3), as well as the separately grown bacteria. The recorded NA spectra showed well defined peaks: 460 – 700 cm^{-1} (υ_4 PO_4, υ_L OH), 900 – 1200 cm^{-1} (υ_1, υ_3 PO_4), 1340 – 1600 cm^{-1} ($\upsilon_3CO_3^{2-}$), OH 3750 cm^{-1} and the presence of H_2O at 2500 – 3900 cm^{-1}(Fig.3a).

The separately grown bacteria spectra, upon visual examination, showed overlapping peaks: 2800 and 3000 cm^{-1} (-CH_3, >CH_2, ≡CH), 1200 – 1800 cm^{-1} (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}). The NA surface with *S. epidermidis* and *P. aeruginosa* bacteria appeared for both NA and bacteria characteristic peaks (Fig.3b). The spectra region 1000 – 1900 cm^{-1} showed overlapping amide and >CH_2 peaks (from organic matter) with carbonate and OH bands (from inorganic NA matter) (Fig.3c).

Furthermore, we developed a *S. epidermidis* and *P. aeruginosa* bacteria discrimination model (Fig.4a). To extrapolate the fine data from the obtained spectra for bacteria characterization, we performed pre-processing (smoothing, baseline correction, and normalization) followed by 2nd derivative and multivariate statistical analysis with PCA and HCA. That model was used to simultaneously investigate the NA surface and discriminate bacteria on it (Fig.4b and c). The obtained NA and bacteria spectra showed the biggest difference in the 450 - 1400 cm^{-1} region. Therefore, only the 1400 – 4000 cm^{-1} region of NA + bacteria spectra was included in the model.

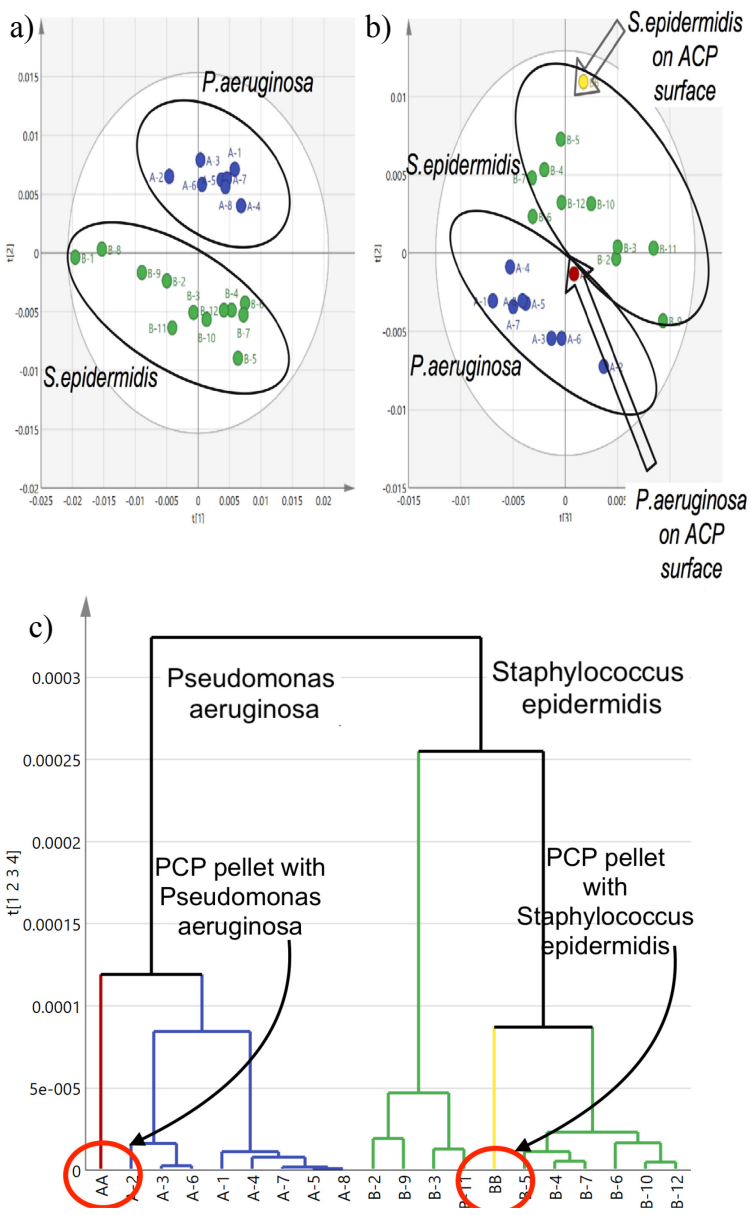

Fig.4. Multivariate statistical analysis: a) PCA 2D-biplot of bacteria, b) 2D-biplot of bacteria on NA surface and c) HCA of bacteria on NA surface

Conclusions

FTIR – DRIFT sampling method coupled with spectra pre-processing and multivariate statistical analysis can successfully be used as a rapid screening method without sample preparation for the identification and discrimination of bacteria.

The cantilever – enhanced FTIR – PAS sampling method, coupled with the developed bacteria discrimination model, can be used for qualitative identification and discrimination of bacteria not only on an NA surface but different apatite based biomaterials as well.

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