Application Note #000399

KeyWords
XPS, Bacteria, Cells, Biofilms, Measurements, Surface Analysis

XPS surface analysis of bacterial samples

This application note presents how EnviroESCA can be used to analyze bacterial samples under near ambient pressure conditions in various states of hydration using different levels of humidity. Such investigations of bacterial cell wall surfaces in their hydrated state are essential for studying biological interfaces at work.

Motivation

Bacteria are present in almost every location on Earth. Understanding their complex interactions with their environments requires detailed knowledge of the cell surface. Modern physical techniques as XPS have been used extensively to study the elemental, molecular, and structural composition of various bacterial cell surfaces being very different from the bulk of the cell wall.[1-3] Sample preparation includes several steps of washing and centrifugation, freeze drying and crushing the dried sample into powder. Obviously, such treatment might change the sample properties.

Bacterial properties vary greatly with factors as bacterial type, substrate, growth medium and pH.[3-4] In general bacteria are in a hydrated state and water or salts from buffer solutions influence the surface properties, which can change dramatically while drying. Structural changes on bacterial surfaces due to changes in pH and hydrophobicity may also occur.

The use of innovative near-ambient pressure (NAP-)XPS instrumentation allows the detailed analysis of irregularly-surfaced biofilms. NAP-XPS enables the surface analysis of bacterial samples in their natural hydrated state without complex sample preparation techniques such as freeze-drying or fast-freezing, which are needed for XPS analysis in ultrahigh vacuum.

This note presents NAP-XPS measurements of dry and humid bacterial samples of *Bacillus subtilis* and *Escherichia coli* in near-ambient pressure conditions.

Fig. 1 Studied biofilm of *Bacillus subtilis* on a polycarbonate filter

Method

EnviroESCA utilizes X-ray Photoelectron Spectroscopy (XPS) as analytical technique. Here an electron beam is generated inside the X-ray source and focused on an aluminum X-ray anode. The deceleration of the electrons on the anode generates X-rays. This X-ray beam is monochromated and focused on the sample.

X-ray photons impinging the sample excite electrons in the material which are subsequently emitted with a specific kinetic energy determined by their binding energy and the photon energy of the X-rays. In case of solid samples only electrons from atoms down to a depth of about 10 nm are able to leave the surface.

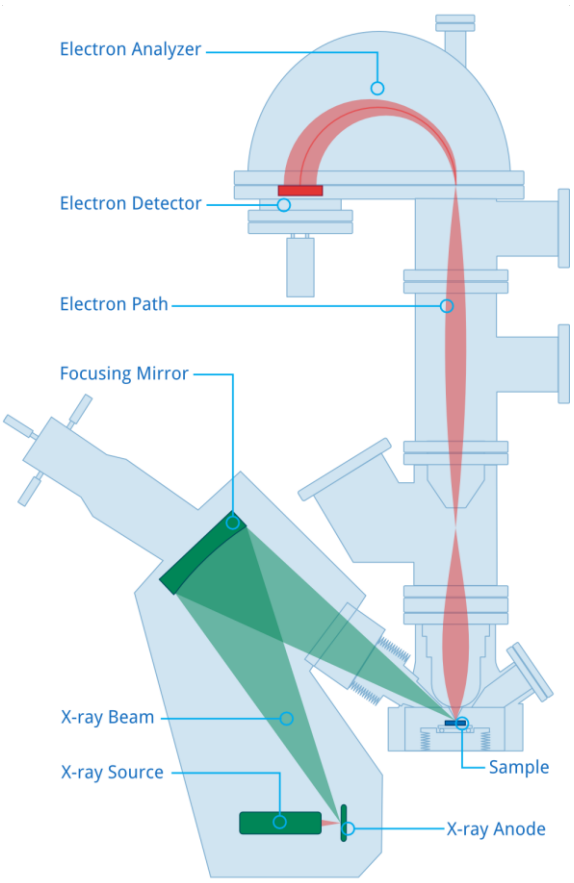

Fig. 2 XPS with EnviroESCA

These electrons propagate through the lens system of the electron analyzer into the hemisphere which acts as a spherical capacitor forcing the electrons onto circular paths with radii depending on their kinetic energy. The path of photoelectrons ends at an electron sensitive detector where the electrons are amplified and measured as intensity in counts per second. A photoelectron spectrum is recorded by sweeping the voltage of the spherical capacitor while measuring the number of electrons per second on the detector. From these spectra a quantitative analysis of the atomic composition of the sample surface can be done.

Experimental Section

EnviroESCA can work under vacuum as well as near ambient pressure (NAP) conditions up to several dozens of mbar. Thus, it is very well suited to investigate surfaces of hydrated biological samples at elevated pressures.

In EnviroESCA an intrinsic charge compensation which we call Environmental Charge Compensation makes additional low energy electron or ion sources unnecessary. As shown schematically in Fig. 3 illumination of the surrounding gas atmosphere with soft X-rays delivers all the free charges that are needed to compensate for surface charging on the sample.

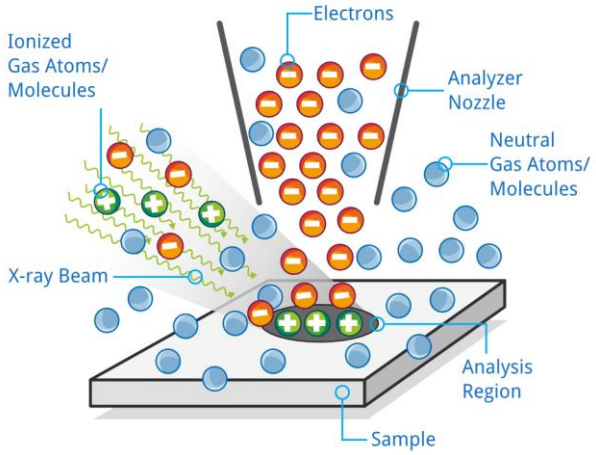

Fig. 3 Environmental Charge Compensation

The NAP-XPS capability of EnviroESCA allows *in situ* surface studies of a multitude of biological samples, e.g., cells, bacteria, biofilms, or viruses, in very different environments. Here we present results of a surface chemical analysis of typical bacterial samples using NAP-XPS (for sample preparation see end of this note).

Dry *Bacillus subtilis* in the form of spores and as biofilm were studied as model systems in argon at 1 mbar. Furthermore, a wet *Escherichia coli* biofilm was analyzed in humid state using a water vapor atmosphere at 12 mbar.

Results

The surface of gram-positive *Bacillus subtilis* samples has been characterized by NAP-XPS in a dry state. Gram-positive bacteria exhibit a rigid and well-defined cell wall located over the cytoplasmic membrane a phospholipid rich bilayer. The cell wall's rigidity is caused by a thick peptidoglycan layer.

According to earlier reports on freeze-dried and fast-frozen bacteria obtained with UHV XPS the information from core-level peaks of oxygen, carbon and nitrogen make it possible to differentiate between major cell wall components related to polysaccharides, lipids and proteins/peptides.[1-4]

High-resolution C 1s, O 1s, and N 1s core-level spectra of a dry *Bacillus subtilis* biofilm are shown in Fig. 4 and are consistent with earlier studies of *B. subtilis*.[1,4]

The C 1s spectrum was fitted with 4 components. Component C-1 is attributed to aliphatic carbon (C-C/C-H) at 285.0 eV, C-2 is attributed to carbon single bonded to oxygen or nitrogen from polysaccharides or peptides/proteins (C-O, C-N), C-3 originates from carbonyl (C=O), amide (O=C-N) or (hemi)acetal (O-C-O) groups, and C-4 is related to carboxylic acids (COOH) or carboxylate esters (COOR).

The O 1s core-level spectrum can be decomposed into two components O-1 at 531.3 eV and O-2 at 532.7 eV, which originate from carbonyl, amide, or carboxylate oxygen (O=C) and oxygen single bonded to carbon (O-C), respectively.

The N 1s peak is dominated by component N-1 at 400.0 eV originating from non-protonated nitrogen atoms located in amide (N-C=O) or amine (C-NH_2) moieties. A second peak component at 402.4 eV can be assigned to protonated nitrogen species typically found on bacterial surfaces.

Drying can have an impact on the bacterial surface. For *Bacillus subtilis* it was reported that sample pretreatments can have significant influence on the obtained surface composition. Contrary to earlier findings from dry samples an increased lipid and decreased protein content could not be observed in the studied sample using NAP-XPS at 1 mbar.[4]

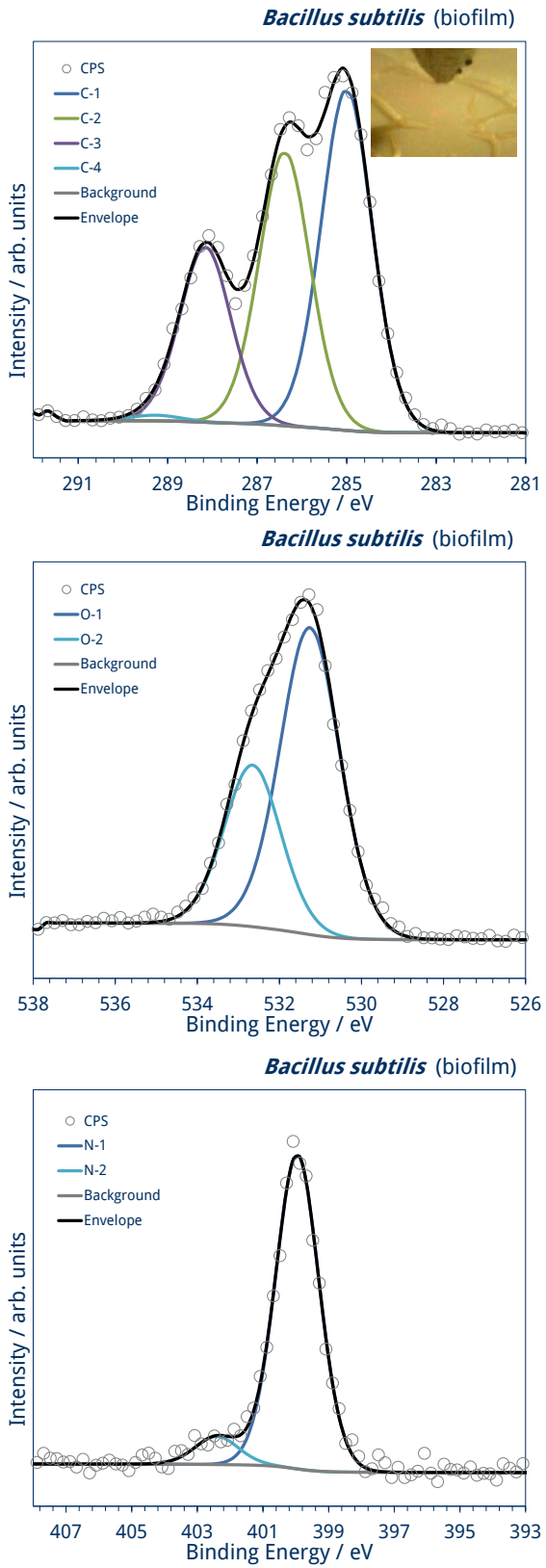

Fig. 4 C 1s (top), O 1s (middle), and N 1s (bottom) core-level spectrum of a dry *Bacillus subtilis* biofilm on polycarbonate at 1 mbar of argon

Fig. 5 shows the high-resolution C 1s core-level spectrum of a second *Bacillus subtilis* sample composed of spores dried on a glass cover slip. The peak shape is very different form the biofilm sample, which is also reflected by the peak areas of components C-1 to C-4 given in Tab. 1. Here the lipid-related component C-1 is dominating the C 1s peak. The difference in chemical and elemental composition (*cf.* Tab. 1) of these two *Bacillus subtilis* samples are most probably a result of different sample forms (spores vs. biofilm), substrates, morphologies, thicknesses, pre-treatments, and preparations.

Biofilms are supported by a matrix of extracellular polymeric substances (EPS) composed mainly of polysaccharides. Thus, much higher amounts of EPS-related components C-2 and C-3 in *B. subtilis* biofilm samples reflect the presence of an extracellular matrix.

In addition a hydrated *E. coli* biofilm on glass was investigated in a humid environment using water vapor at 12 mbar that was evaporated constantly during the measurement from a reservoir of liquid water. The C 1s spectrum is presented in Fig. 6 together with the chemical and elemental composition given in Tab. 1.

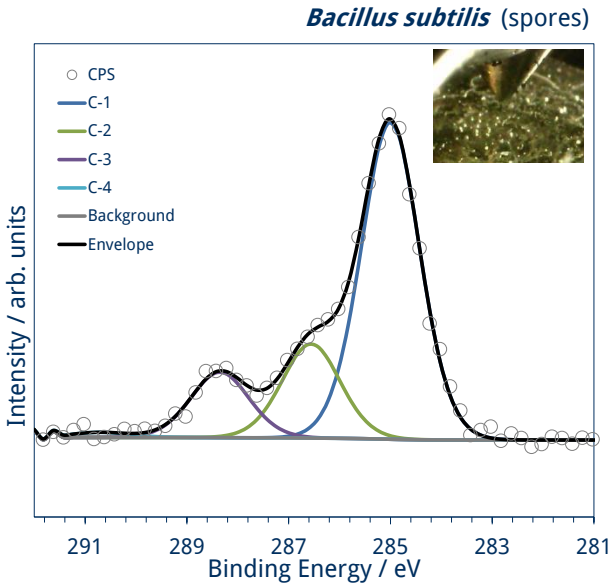

Fig. 5 C 1s core-level spectrum of *Bacillus subtilis* spores dried on glass at 1 mbar of argon

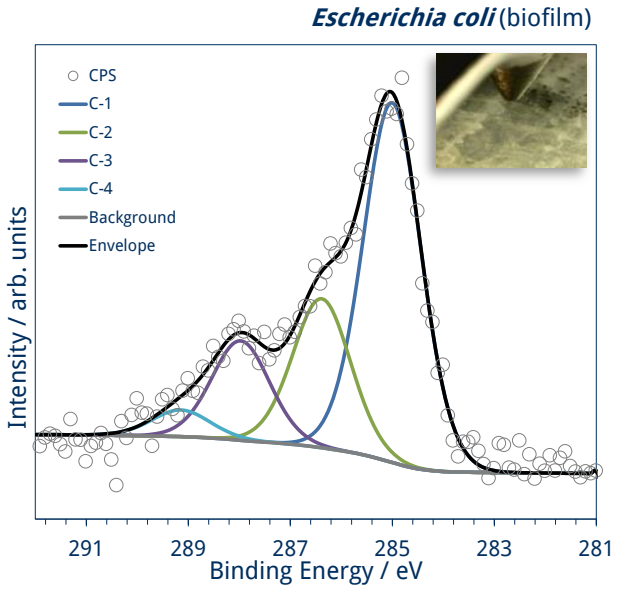

Fig. 6 C 1s core-level spectrum of a hydrated *Escherichia coli* biofilm on glass at 12 mbar of water vapor

Table 1. Elemental composition (atom-%) and relative C 1s peak component area (%) of different bacterial samples

Sample*	O 1s	C 1s	N 1s	C-1 285.0# C-C/C-H	C-2 286.5# C-O/C-N	C-3 288.1# C=O/O-C-O	C-4 289.2# COOR
Bacillus subtilis (biofilm)	22.4	63.5	14.1	42.8	34.4	22.0	0.7
Bacillus subtilis (spores)	55.4	38.2	6.4	65.8	19.5	13.6	1.1
Escherichia coli (biofilm)	50.5	38.3	11.2	56.5	23.5	15.7	4.2

*Additional elements, e.g., Na, Cl, P, Mg, Ca, Sn, and Si were found originating from growth media, buffers, and substrate materials.
#Binding energy (eV) and peak assignment for C 1s.

This last measurement demonstrates the unique capability of EnviroESCA to analyze the surface of hydrated bacterial samples in humid environments with NAP-XPS. Beyond that, investigations of hydrated bacterial samples during drying are possible. Using the same *E. coli* biofilm substantial changes in the C 1s peak attributed to increased signal from polysaccharides could be observed during dehydration.

Beam damage has to be considered for any XPS analysis of biological samples. Therefore, C 1s spectra have been taken at the beginning and at the end of a sample's measurement series. However, no notable changes were observed in the C 1s spectra of all the investigated bacterial samples.

Conclusion

The unique capabilities of EnviroESCA to work in near-ambient pressure conditions using different gas atmospheres allows *in situ* surface characterization of real bio samples as bacteria, cells, biofilms or viruses under biological relevant conditions.

Bacteria and biofilms can be grown directly on the chosen substrate under controlled conditions, and studied in near-ambient conditions with minimal alteration and pre-treatment of the sample.

It is possible to characterize directly the surface of hydrated bacterial samples with NAP-XPS and individual cells wall components, e.g., polysaccharides, peptide/proteins or lipids can be identified and quantified with ease.

NAP-XPS can thereby provide new insights about bacterial surfaces and hydrated bacteria-solid substrate interfaces, e.g., microbial induced biofilm growth or corrosion.

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Sample preparation

Spores

B. subtilis NCIB 3610 dried on glass cover slip, concentration. 1×10^6 spores, originally stored in ddH_2O, first washed in ddH_2O. Sporulated after Fuchs *et al*. 2017. Samples were used with corresponding centrifugation and washing step and dried on glass slide.

Biofilm

B. subtilis NCIB 3 days old, 37 °C on LB-Agar, 1×10^7 spores as in oculum, dried on HTTP filter. Samples were used without centrifugation, washing step but were grown of filter without contact to the medium.

E. coli strain K12 was inoculated overnight in LB-medium. The liquid culture was centrifuged and resuspended in M9 medium to an optical density of 0.05 at 600 nm. For biofilm growth, glass slides were immersed for 24 hours in the bacterial suspension, at 37 °C while shaking at 60 rpm. Immediately before measurements, the biofilm was gently rinsed with phosphate buffered saline (PBS).