Correlative TERS imaging of *B. Subtilis* spores

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***Abstract*— Tip-enhanced Raman Scattering (TERS) has recently emerged as a unique and powerful analytical tool to directly localize and identify proteins and their conformation in a complex environment at the nanoscale. This analytical technique relies on the combination of near-field scanning probe microscopy with plasmonic-enhanced Raman spectroscopy. In TERS, in fact, a metalled tip is used as a sort of optical nano-antenna that gives place to the a plasmonic amplification of the Raman signal only at the tip apex of the scanning probe, which, in turn, allows near field, sub-diffraction spatial resolution of the acquired spectral information. Moreover, TERS allows obtaining simultaneously topographic and chemical information of the analysed surface region, which constitutes an important step for surface analysis in its broadest sense. In this work, we apply TERS spectroscopy for surface analysis of the Bacillus subtilis spore, a very attractive bio-system for a wide range of applications regulated by the spore surface properties. The observed TERS spectra reflect the complex and heterogeneous environment explored by the plasmonic tip, therefore exhibiting significant point-to-point variations at the nanoscale. Herein, we demonstrate that TERS data processing via principal component analysis allows handling such spectral changes, thus enabling an unbiased correlative imaging based on TERS. Our experimental outcomes suggest a denser arrangement of both proteins and carbohydrates on specific spore surface regions simultaneously revealed by AFM phase imaging. Successful TERS analysis of spores' surface is useful for bacterial surface-display systems and drug-delivery.**

Keywords - Tip-Einhanced Raman Scattering (TERS), B. Subtilis spores, Principal Components Analysis (PCA).

I. INTRODUCTION

A deep and detailed understanding of the mechanisms that regulate biological processes requires innovative, sensitive and non invasive techniques able to study the response of biosystems at the level of single cell or even single molecule [1]. Raman-based analytical techniques have recently emerged as suitable tools to match these requests [2,3]. In particular, the development of Surface-Enhanced Raman Scattering (SERS) has overcome the traditional limits of conventional Raman spectroscopy, many related to the intrinsically low cross-section associated to the low Raman scattering process [4-9]. The SERS enhancement can reach to up a factor of 10^{12} providing access to detection limits down to single molecule level. Moreover, the enhancement of Raman signals occurs only for molecules located in close proximity of SERS-active sites (hot spots), therefore surpassing the spatial resolution of spectroscopic measurements inherently limited by the diffraction limit of light. An optimal control of such SERS active sites constitutes the basis idea of tip-enhanced Raman scattering (TERS) [10-12]. TERS relies on the combination of scanning probe microscopy and Raman spectroscopy. The basic idea is the use of a metalled tip as a sort of optical nano-antenna, which gives place to SERS effect close to the tip end with near-field resolution. TERS allows obtaining simultaneously topographic and chemical information of the analysed surface region, which constitutes an important step for surface analysis in its broadest sense. Nowadays, TERS potentialities have been exploited in many research fields, ranging from the development advanced materials to biomedical research.

A recent application of TERS analysis relies on the investigation of the surface of *Bacillus Subtilis* (*B. Subtilis*) spores [12]. Bacterial spores are formed by bacilli and clostridia in response to adverse environmental conditions through a complex process called sporulation. The result of this process is a dormant cell able to endure many forms of environmental stresses (including heating and exposure to UV radiation) for long periods, up to thousands of years [13-15]. Nonetheless, when the environmental conditions ameliorate the spore come back to vegetative life by a process known as germination. This remarkable feature has inspired many spore-based biotechnological applications. In particular, bacterial spores displaying heterologous antigens on their surface have been proposed as mucosal vaccines and tested on animal models [13]. This spore-based delivery system presents several advantages over other approaches, including a high stability and a marked capacity to increase the bioavailability of the displayed antigens. Recently, a non-recombinant method to display heterologous proteins on the surface of bacterial spores has been recently developed [16], based on the adsorption of selected antigens/enzymes on the spore surface. Interestingly, adsorbed molecules are stabilized and protected by the interaction with the spore, suggesting that this system could reduce the rapid degradation of the antigen, often observed with other delivery systems and identified as a major drawback of mucosal vaccines. Although the molecular details of spore adsorption have not been fully addressed, spore-based technology promises to represent a novel surface display system. In particular, being this approach non-recombinant and based on a host with a remarkable safety record, it appears particularly well suited for the biotherapeutic molecules delivery to human mucosal surfaces. A complete understanding of the physicochemical properties

of the spore surface is then essential toward a rational design of the displayed molecules and for optimizing the non-recombinant display system efficiency.

Herein, we demonstrate that TERS analysis of the spore surface, combined with the use of advanced statistical tools such as Principal Component Analysis (PCA) [17] provide precious information on the chemical functionality expressed at the spore surface. This information is essential for the rationalization of proteins/enzymes adsorption at this biological interface, therefore paving the way to more feasible development strategies of spore-based drug-delivery systems.

II. EXPERIMENTAL DETAILS

A. TERS system

The TERS setup used for this investigation has been described in details elsewhere [12]. It is based on the WiTec Alpha 300 system and consists of an inverted Raman microscope combined with an AFM system. The Raman excitation source (linearly polarized, 532 nm) is focused on the sample through a dry 60× objective (PlanApo, Olympus, NA 0.8). For Raman/AFM imaging, the sample was scanned by a closed-loop XY piezo scanning stage (P-734, Physik Instrumente, Germany), allowing sample positioning with an accuracy of 3 nm.

TERS tips were provided by Next-Tip S. L., and consisted in commercial AFM tips with elastic constant of 2.8 N/m and resonance frequency at ca. 75 kHz, on which ~ 14 nm gold nanoparticles (Au-NPs) were deposited upon ultra-high vacuum conditions. In this investigation we analyzed Wild-type *B. Subtilis* spores, which are often considered as a model system for spore-forming bacteria. They exhibit a roughly ellipsoidal shape, with the short axis ~ 0.6 µm and the long axis ~ 1.3 µm (average values). AFM analysis of spores surfaces was performed in AC mode, in order to prevent damage of the relatively soft spore surface caused by the interaction of the tip. Before TERS analysis, AFM images of the spore were acquired in order to select the regions to be investigated. Therefore, the laser probe was switched on for TERS analysis. Sample photo-damage was avoided by reducing the incident power to a few tens of µW. Optimal matching of the laser focus with the tip apex was searched by a piezo-driver.

B. PCA analysis

PCA is a statistical tool, applied in many fields of science, for the analysis of large spectral data sets [17]. It allows the reduction of the number of variables of a dataset retaining, at same time, most of the variation within the data. When applied to Raman spectra, PCA condenses the information contained in each spectrum (which has a number of observables equal to points of each spectrum) in only a few variables. The procedure employed for such data compression involves the diagonalization of the correlation matrix of the initial data; as a result, the new observables (Principal Components, PCs) are uncorrelated data carrying the most relevant information. The order of the PCs denotes their importance in highlighting differences within the spectra dataset, with PC1 describing the highest amount of variation. The coefficients of the combination of PCs in terms of the original variables are referred to as *loadings* and express the weight of each original observable to the global variance of data. The coordinates of the original data sets in the PCs space are instead referred to as *scores*. In this work, PCA was performed by using subroutines available in *Matlab* (The MathWorks, Inc.) after spurious cosmic rays and fourth order polynomial background subtraction.

III. RESULTS AND DISCUSSION

In TERS analysis, spectra observed during a sample scan reflect the complex and heterogeneous environment explored by the plasmonic tip. Therefore, they present significant point-to-point variations at the nanoscale. In particular, this effect is important in the analysis of bio-interfaces, typically exhibiting complex arrangements of proteins, lipid and carbohydrates. In Figure 1, part a, we show an AFM-phase map of several *B. Subtilis* spores, captured in the initial phase of spore-core dehydration. It was obtained with a scan step of 15 nm and a scan rate of 200 points/s. The driving frequency was set in correspondence to 90% of the peak amplitude of the cantilever oscillation. Part b of Fig. 1, instead, shows a typical, quite complex TERS spectra observed in this investigation. It was obtained by a laser power impinging on the sample and an integration time of 50 µW and 2s, respectively. Notably, the relatively broad features are likely due to the overlapping contribution of many spectral features, originated from molecules located in different nanoenvironments. Nevertheless, many bands typically related to amino acids were clearly distinguishable, such as the band around 830 cm^{-1}, ascribable to C-C symmetric ring stretching of Tyrosine, and bands around 1546 and 1574 cm^{-1}, due to Tyrosine and Tryptophan ring vibration, respectively.

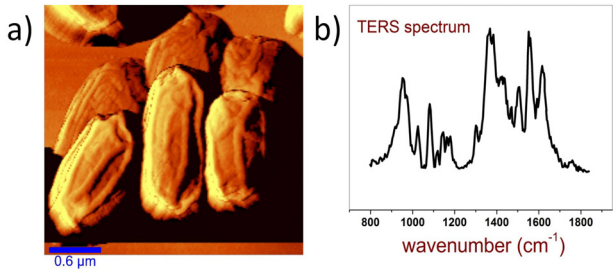

Fig. 1. **a)** AFM-phase map of *B. Subtilis* spores. b) A typical TERS spectrum of spore surface.

Moreover, other bands can be assigned to carbohydrates and/or glycans of glycoproteins, such as bands around 930 and 1205 cm^{-1}, due to C-C and C-O-C stretching, respectively. The same spectral richness has been observed in all the acquired spectra. In such condition, it is quite hard to pick up useful information from TERS data. For instance, as demonstrated in ref. [12], TERS maps reporting the intensity of selected spectral features do not provide useful information to highlight the presence of spore-surface domains clearly identifiable by other nanoscopic observables. In particular, it is reasonable to

expect a strong correlation between AFM-phase maps and TERS maps, being both surface representations sensitive to the surface chemical signatures. Information rich TERS maps can be instead created by taking advantage of advanced statistical tools such as PCA. To test the effectiveness of PCA for unravelling the information provided by TERS spectra of spore surface, we have applied it to the analysis of spectra acquired in a spore-surface zone across a surface ridge clearly visible in the spore AFM-phase map. The results of this analysis are shown in Figure 2. In particular, in panel 1 we report a 120 nm × 120 nm phase map, obtained with a scan step of 20 nm. The brighter pixels (corresponding to a positive phase lag) correspond to points on the ridge. Panel 2, instead, reports TERS maps obtained by reporting the intensity of assigned spectral features (indicated in the labels under each map). Clearly, no apparent correlation can be revealed between these maps and the phase-map. As illustrated in panel 3, this situation dramatically changes when TERS data are analysed by PCA. In particular, both PC2 and PC3 score maps exhibit a clear correlation with the phase map (PC1 components only takes into account of a residual background, so it was not taken into consideration). The aforementioned correlation can be quantified by the Pearson coefficient $P_{1(2)}$ [12], calculated between the phase-maps and the PC1(2) score map. Importantly, as reported in Fig. 2, both values are higher than 0.7. This outcomes clearly demonstrate the effectiveness of PCA for the analysis of TERS data, and, in particular, for obtaining really informative TERS maps.

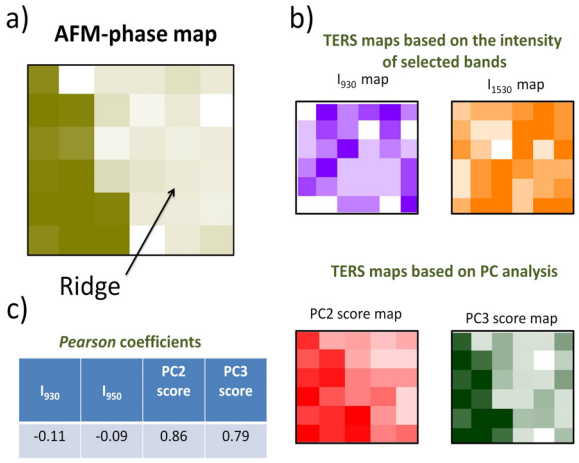

Fig. 2. **a):** AFM-phase map of a spore region across a ridge. b): TERS maps obtained by reporting the intensity of selected features (upper part) or the score values resulting from PCA. c) Pearson coefficient calculated between the AFM-phase map and the TERS maps shown in part b).

IV. CONCLUSIONS

We have demonstrated that, when properly analysed, TERS maps of spore surfaces can be correlated with the corresponding AFM-phase maps, thus allowing one to discriminate the relevant chemical distribution of this complex biological system, otherwise non-intuitively identifiable. The obtained results pave the way for a rational design of spore-based drug-delivery systems.

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