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Vibrational monitor of early demineralization in tooth enamel after *in vitro* exposure to phosphoridic liquid

Giuseppe Pezzotti [a,b,*], Tetsuya Adachi [c], Isabella Gasparutti [a,d], Giulio Vincini [a,e], Wenliang Zhu [f], Marco Boffelli [a], Alfredo Rondinella [a,d], Elia Marin [a], Hiroaki Ichioka [c], Toshiro Yamamoto [c], Yoshinori Marunaka [b,g], Narisato Kanamura [c]

[a] Ceramic Physics Laboratory, Kyoto Institute of Technology, Sakyo-ku, Matsugasaki, 606-8585 Kyoto, Japan
[b] Department of Molecular Cell Physiology, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kamigyo-ku, Kyoto 602-8566, Japan
[c] Department of Dental Medicine, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kamigyo-ku, Kyoto 602-8566, Japan
[d] Department of Engineering and Architecture, University of Trieste, Via A. Valerio 6/A, 34127 Trieste, Italy
[e] Chemistry, Material and Chemical Engineering Department "Giulio Natta", Polytechnic of Milan, Via Mancinelli 7, Milan 20131, Italy
[f] Department of Medical Engineering for Treatment of Bone and Joint Disorders, Osaka University, 2-2 Yamadaoka, Suita, Osaka 565-0854, Japan
[g] Department of Bio-Ionomics, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kamigyo-ku, Kyoto 602-8566, Japan

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ABSTRACT

The Raman spectroscopic method has been applied to quantitatively assess the *in vitro* degree of demineralization in healthy human teeth. Based on previous evaluations of Raman selection rules (empowered by an orientation distribution function (ODF) statistical algorithm) and on a newly proposed analysis of phonon density of states (PDOS) for selected vibrational modes of the hexagonal structure of hydroxyapatite, a molecular-scale evaluation of the demineralization process upon *in vitro* exposure to a highly acidic beverage (i.e., CocaCola™ Classic, pH = 2.5) could be obtained. The Raman method proved quite sensitive and spectroscopic features could be directly related to an increase in off-stoichiometry of the enamel surface structure since the very early stage of the demineralization process (i.e., when yet invisible to other conventional analytical techniques). The proposed Raman spectroscopic algorithm might possess some generality for caries risk assessment, allowing a prompt non-contact diagnostic practice in dentistry.

1. Introduction

Abrupt crystallographic discontinuities and disorder in the crystalline arrangement of the contiguous prismatic structures at the surface of dental enamel exacerbate the phenomena of chemical degradation intrinsically associated with surface unsaturation of hydroxyapatite chemical bonds [1–3]. The chemical reactivity might strongly be enhanced at the interface between enamel and biological fluids, with demineralization and remineralization interactions taking place via free surfaces expedited by the non-equilibrium electronic properties of crystal bonds [4,5]. Surface chemistry and crystallographic orientation are indeed intertwining factors in enamel degradation at the molecular scale, and understanding the response of the hydroxyapatite surface to acidic environments is a key concept in rationalizing the physiological behavior of teeth enamel in both health and disease [5,6]. Demineralization of enamel under acidic conditions, which degrades the teeth structure and ultimately leads to caries, takes place at the molecular scale due to dissolution of hydroxyapatite cations [7]. However, although the microscopic origins of the cariogenic process have been clarified [8–10], the complex off-stoichiometry developed at the molecular scale is yet unclear. Moreover, diagnostic purposes and the need for a method of direct and early detection of cation-dissolution phenomena from the hydroxyapatite surface toward acidic environments yet motivate deeper studies. The authors of this paper believe that the combination of purposely designed experiments and accurate theoretical modeling could lead to a new path in dental research, which could improve the present situation in diagnostics and lead to new insight into the early-stage dynamics of the cariogenic behavior of enamel.

In two previous papers [11,12], we have revisited the basics of the vibrational behavior of hydroxyapatite, quantitatively calibrating its Raman behavior through a set of Raman tensor elements in a single-crystal sample, and then confirming their validity for the hydroxyapatite structure of human teeth enamel. An algorithm has also been proposed to statistically describe the statistical population of the crystallographic texture within the Raman microprobe in terms of three Euler angles in space [11]. Application of this newly developed Raman algorithm to the analysis of a series of *in vivo* decayed human teeth has previously demonstrated the suitability of vibrational

* Corresponding author at: Ceramic Physics Laboratory, Kyoto Institute of Technology, Sakyo-ku, Matsugasaki, 606-8585 Kyoto, Japan.
E-mail address: pezzotti@kit.ac.jp (G. Pezzotti).

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spectroscopy in revealing enamel demineralization since its very early stage, and suggested the possibility of quantitatively monitoring such phenomenon for diagnostic purposes [12].

In this paper, we attempted to deepen our previous theoretical achievements and to model *in vitro* the interaction between the vibrational response of demineralized hydroxyapatite and its surface off-stoichiometry. For doing so, we started from modeling the PDOS of healthy hydroxyapatite and reached, as a final output, the morphological simulation of its Raman spectrum in an early-demineralized state. Fits of our predictive calculations of off-stoichiometric spectra were made to the outputs of *ex vivo* experiments performed on healthy human teeth embedded, for increasing periods of time, into acidic (phosphoridic) liquid. Among a number of possible choices for the aggressive environment, we selected the classic CocaCola™ beverage. This choice was made not only because this beverage is a long and well-known acidic beverage drunk worldwide, but also, as shown by other authors [13], because it has a quick and direct effect on demineralization of human teeth enamel structures. However, this study was not conceived as a probe for the effect of CocaCola™ beverage on oral health. We simply assumed here that, even whether different severities should be expected with different acidic agents (e.g., orange juice, coffee, and artificial chemical sweeteners), the demineralization process, namely the effect that they induce on the altered stoichiometry of hydroxyapatite, could be a common one for all of them [7,14–18]. In its ultimate meaning, this experimental and theoretical study would represent a predictive approach for judging about the level of demineralization at its early stage. Accordingly, the concept of "degree of healthiness" of tooth enamel could be put forward as the divergence of its surface hydroxyapatite structure from a stoichiometric state, before and independent of caries actually taking place.

2. Experimental and Computational Procedures

A series of 20 healthy molar teeth were obtained from donors (human patients upon clearance of ethical procedures) at the Department of Dental Medicine of the Graduate School of Medical Science of Kyoto Prefectural University of Medicine (KPUM). The human teeth were collected under the approval of the Institutional Review Board of KPUM (ERB-C-136). Among those 20 samples, 8 samples were selected and thoroughly investigated, while the remaining 12 were only used for separated confirmation of the obtained results. All the *ex vivo* studied samples were free of caries and healthy upon clinical parameters, as shown by the X-ray photographs shown in Fig. 1 for four selected teeth belonging to the examined series. Table 1 lists 8 of the investigated samples and gives their clinical specifications. The studied samples were soaked for increasing periods of time (i.e., between 0 and 30 min) in phosphoridic beverage (CocaCola™ Classic, pH = 2.5; simply referred to as "phosphoridic (or acidic) fluid" henceforth) at constant (room) temperature and under a partial pressure of carbon dioxide. Laser microscopy (Laser Microscope 3D & Profile measurements, Keyence, VK-x200 series, Osaka, Japan) was applied to characterize the morphology of the teeth surface before and after treatment in phosphoridic liquid.

All the Raman spectroscopic experiments described in this paper were carried out in backscattering configuration of the optical probe by means of a triple monochromator (T-64000, Horiba/Jobin-Yvon, Kyoto – Japan) equipped with liquid nitrogen-cooled charge coupled device (CCD), a confocal pinhole, and (cross/parallel) polarization filters. The excitation source in the present experiments consisted of a 532 nm Nd:YVO_4 diode-pumped solid-state laser (SOC JUNO, Showa Optronics Co. Ltd., Tokyo, Japan) operating at an emission power of 200 mW. An objective lens with a numerical aperture of 0.5 was used both to focus the laser beam on the sample surface and to collect the scattered Raman light. All the confocal experiments described in this paper were conducted with a pinhole aperture of 100 μm and with employing an objective lens with a magnification of 100×. The dimensions of the confocal probe were evaluated according to previously established procedures using the probe response function approach [19,20]. The diameter of the probe waist in the focal plane and the penetration depth to which emission of the Raman signal reached 90% of the total Raman intensity were calibrated as ~2 and 104 μm, respectively, with focusing on the free surface of the healthy teeth.

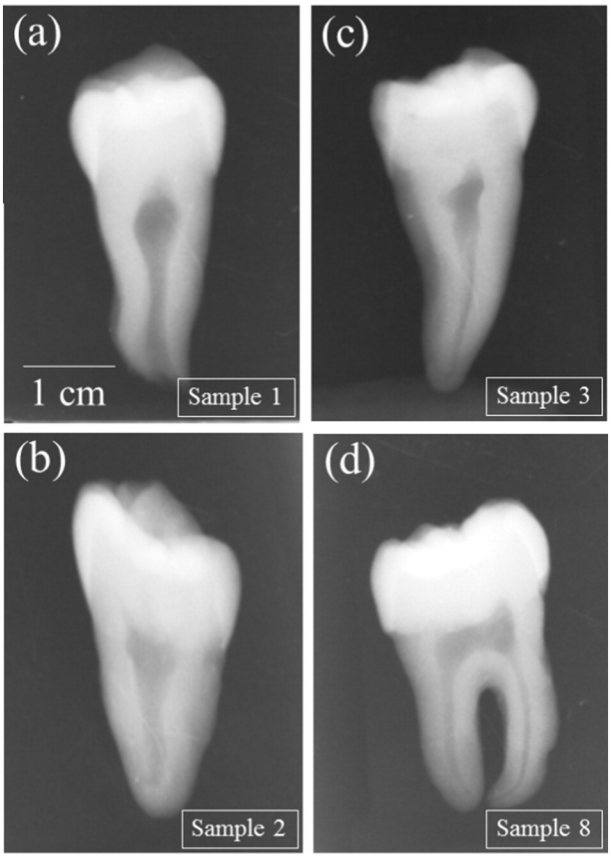

Fig. 1. Radiographic analysis of 4 selected samples among the 20 samples studied. No external or internal caries were detected.

Spectral positions and intensities of Raman bands were also monitored under polarized light either in parallel or in cross probe configuration. A goniometer jig was used for facilitating the quantitative assessment of in-plane angular displacements. The polarization directions of the Raman probe have been labeled according to the Porto notations [21]. According to these notations, specification of, for example, $x(zz)y$ means that the incoming light enters the x-axis of the crystal polarized in the z direction and the scattered light, polarized parallel to the z-axis, is collected along the y-axis. A bar on top of the first axis notation then indicates that the axis possesses an opposite orientation with respect to the specific *versus* of the incoming light. In our experiments, the z-axis of the laboratory frame was taken perpendicular to the sample free surface and the y-axis parallel to the facial-to-lingual direction. Accordingly, parallel (∥) and (⊥) cross configurations corresponded to $\bar{z}(yy)z$ and $\bar{z}(xy)z$ in Porto notations. Each spectrum at a measurement location was collected for 3 scans with the accumulation time of each scan being 60 s. Spectral Raman lines were analyzed using a commercially available software package (Origin 9.1, OriginLab Co., Northampton, MA, USA). Spectral fitting was performed according to Gaussian-Lorentzian functions after subtracting a linear baseline. To reduce as much as possible the error involved in the fitting, in this study we tried to set a limit of the band position and the FWHM within a small range ($\pm 1\ cm^{-1}$) for the fitting, based on the reported values in literature. All mathematical procedures were carried out with the aid of commercially available computational software (MATHEMATICA 7.0, Wolfram Research, Inc., Champaign, IL, USA).