Mapping Bone Surface Composition Using Real-Time Surface Tracked Micro-Raman Spectroscopy

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Keywords

Bone · Bone surface · Biomineralization · Raman spectroscopy

Abstract

The surface of bone tells a story – one that is worth a thousand words – of how it is built and how it is repaired. Chemical (i.e., composition) and physical (i.e., morphology) characteristics of the bone surface are analogous to a historical record of osteogenesis and provide key insights into bone quality. Analysis of bone chemistry is of particular relevance to the advancement of human health, cell biology, anthropology/archaeology, and biomedical engineering. Although scanning electron microscopy remains a popular and versatile technique to image bone across multiple length scales, limited chemical information can be obtained. Micro-Raman spectroscopy is a valuable tool for nondestructive chemical/compositional analysis of bone. However, signal integrity losses occur frequently during wide-field mapping of non-planar surfaces. Samples for conventional Raman imaging are, therefore, rendered planar through polishing or sectioning to ensure uniform signal quality. Here, we demonstrate ν_1 PO_4^{3-} and ν_1 CO_3^{2-} peak intensity losses where the sample surface and the plane of focus are offset by over 1–2 μm when underfocused and 2–3 μm when overfocused at 0.5–1 s integration time (15 mW, 633 nm laser). A technique is described for mapping the composition of the inherently ir-

regular/non-planar surface of bone. The challenge posed by the native topology characteristic of this unique biological system is circumvented via real-time focus-tracking based on laser focus optimization by continuous closed-loop feedback. At the surface of deproteinized and decellularized/de-fatted sheep tibial cortical bone, regions of interest up to 1 mm^2 were scanned at micrometer and submicrometer resolution. Despite surface height deviations exceeding 100 μm , it is possible to seamlessly probe local gradients in organic and inorganic constituents of the extracellular matrix as markers of bone metabolism and bone turnover, blood vessels and osteocyte lacunae, and the rope-like mineralized bundles that comprise the mineral phase at the bone surface.

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Introduction

Morphology of the bone surface, for example, endosteal and periosteal surfaces of cortical bone, is a spatial representation of local metabolism [Marks et al., 1996] and age [Boskey and Coleman, 2010] and therefore of key significance to bone physiology. The bone surface exhibits an inhomogeneous geometry across several distinct length scales that swiftly transition from one to the next. Given the complex topography, scanning electron microscopy (SEM) is highly versatile and therefore among

the most frequently used methods to image bone over wide fields of view, large height (*z*-direction) deviations, and across multiple length scales [Shah et al., 2019].

Spatially-resolved, 2D (*x*-*y*, *x*-*z*, or *y*-*z*) chemical information may be obtained using a diverse range of techniques. These include energy dispersive X-ray spectroscopy, Fourier transform infrared spectroscopy, Raman spectroscopy, Time-of-Flight Secondary Ion Mass Spectrometry, and small-angle X-ray scattering, where flat/planar surfaces (or thin sections) are desirable [Kazanci et al., 2007; Li et al., 2010; Brüel et al., 2011; Paschalis et al., 2011; Henss et al., 2013]. A third dimension is typically accessed via volumetric imaging, i.e., tomography [Binkley and Grandfield, 2017].

X-ray micro-computed tomography is a popular, non-destructive 3D analytical tool for morphometry and biomechanical imaging [Müller, 2009], however, limited chemical and nearly no molecular information can be obtained. Atom probe tomography yields 3D elemental distribution and mass spectrometric information [Gordon et al., 2012; Langelier et al., 2017; Wang et al., 2018]. Employing synchrotron radiation, more recently developed technologies such as X-ray diffraction computed tomography [Mürer et al., 2018], and small-angle scattering tensor tomography [Liebi et al., 2015; Grünwald et al., 2020] enable bone mineral and collagen orientation analysis in 3D.

Early pathological alterations can be observed at the bone surface (i.e., the mineralization front) [Hoffmann et al., 2017; Lind et al., 2018]. Therefore, the chemical composition of the bone surface can provide further clues toward better understanding the effects of certain diseases or drugs, visualizing the ability of osteocytes to remodel their perilacunar matrix, making hypotheses about ancient populations (diet, climate, etc.), developing a more accurate chemical signature for bone that can be mimicked by implant biomaterials, and so forth. However, the bone surface is not readily accessible using traditional sample manipulation methods for obtaining a cross-sectional view, for example, polishing. However, owing to undulations and rapidly changing topography, the possibility of directly mapping chemical information from the bone surface has thus far remained a challenge. In conventional micro-Raman spectroscopy/imaging, where measurements are made across a fixed focal plane, height deviations at the surface of a non-planar specimen give rise to underfocused or overfocused areas, resulting in severe losses of Raman signal integrity. Here, we tackle the challenge posed by the native topology and height deviations of the bone surface and overcome Raman sig-

nal deterioration that occurs during wide-field mapping using micro-Raman spectroscopy in combination with real-time surface tracking.

Methods/Design

Bone Sample Processing

Cortical bone from sheep tibia (*n* = 1) was fixed in 10% neutral buffered formalin and cut into 41 × 1 cm² pieces, each spanning the entire thickness of the cortex (i.e., one surface being endosteal and the other being periosteal). Deproteinized bone: Two pieces were immersed in 5% sodium hypochlorite (NaOCl) at room temperature for 14 d in order to remove cells and the organic matrix and dehydrated in a graded ethanol series (50–100%). Decellularized/defatted bone: Two pieces were exposed to 20 mM NH₄OH in deionized water for 15 min to remove the adherent lining cells without affecting the extracellular matrix [Taylor et al., 2015] and defatted in acetone for 30 min [Kerns et al., 2014]. Sheep cortical bone was obtained as part of an unrelated study [Shah et al., 2016a], approved by the Ministry of National Education, Higher Education and Research (NAMSA, Chasse-sur-Rhône, France).

Scanning Electron Microscopy

To exemplify the various length scales of organization at the bone surface, deproteinized bone was visualized by scanning electron microscopy using a Quanta 200 environmental SEM (FEI Co., The Netherlands) operated in backscattered electron (BSE) and secondary electron (SE) modes at low vacuum (1 Torr water vapor pressure) and 15–25 kV accelerating voltage.

Micro-Raman Spectroscopy

Micro-Raman spectroscopy was performed using a confocal Raman microscope (Renishaw inVia™ Qontor®) equipped with a 633 nm laser and LiveTrack™ focus-tracking technology that works through a continuous closed-loop feedback of focus. The laser was focused down on to the endosteal surface using ×50 and ×100 objectives, and spectra were collected in the 300–1,800 cm⁻¹ spectral range using a Peltier-cooled charge-coupled device deep depletion near-infrared enhanced detector behind an 1,800 g mm⁻¹ grating. Background fluorescence subtraction and cosmic ray removal were performed in Renishaw WiRE 5.2 software.

To illustrate loss of signal integrity owing to the non-planar surface, multiple depth series were acquired from -10 μm (overfocus; below the sample surface) to +10 μm (underfocus; above the sample surface) at 1 μm intervals. Raman spectra were collected using an ×100 objective at 500 ms and 1,000 ms integration times (*n* = 3). At each plane of focus, background subtracted ν_1 PO₄³⁻ and ν_1 CO₃²⁻ peak intensities and signal-to-noise ratios (taken as the ratio between the background subtracted ν_1 PO₄³⁻ or ν_1 CO₃²⁻ peak intensity and the intensity of the subtracted fluorescence signal at the corresponding wavenumbers) were obtained. One-way analysis of variance (ANOVA) with post-hoc Bonferroni correction was used for statistical analysis between planes of focus, where *p* values <0.05 were considered statistically significant. Mean values ± standard deviations are presented. Surface topography-dependent loss of signal integrity is further exemplified by Raman mapping of the deproteinized bone surface with and without the real-time surface tracking (LiveTrack™).

Fig. 1. Surface of deproteinized bone visualized using backscattered electron (BSE) and secondary electron (SE) imaging. Overview: Blood vessels (broken circles) and osteocyte lacunae at the bone surface, where undulations are seen as elevations (high contrast) and depressions (low contrast) in the SE mode. Rope-like mineralized bundles: The extracellular matrix is assembled into a

continuous interwoven mesh. BSE and SE overlay reveals local variations in mineral density. Osteocyte lacuna: The floor of the osteocyte lacuna is made up of anisotropically aligned, mineralized bundles with submicrometer-sized porosities in between. The extralacunar matrix comprises high density (mineralized bundles) and low density (voids and porosities) regions.

Using LiveTrack™, Raman maps were acquired from the surface of deproteinized and decellularized/defatted bone at 200–3,000 ms integration time per pixel and isotropic pixel sizes of 5 µm, 1 µm, 300 nm, and 200 nm. 2D maps of bone surface height deviation (change in the z axis) were obtained at each region of interest (ROI), and 3D visualizations were generated using Gwyddion (<http://gwyddion.net>). Maps of the ν_1 PO_4^{3-} (930–990 cm^{-1}) band intensity and either the ν_1 CO_3^{2-} (1,060–1,085 cm^{-1}) band intensity or the Amide III band (1,220–1,300 cm^{-1}) integral area are presented. Additionally, data segmentation was performed using k -means filtering cluster analysis with correlation coefficient as the measure of distance (WiRE 5.2, Renishaw). From the average Raman spectrum of each cluster, the mineral-to-matrix ratio (MMR) was taken as the integral area ratio between ν_2 PO_4^{3-} (420–470 cm^{-1}) and Amide III (1,220–1,300 cm^{-1}).

Deproteinized Bone Surface as Viewed by Scanning Electron Microscopy

Being sensitive to local variations in composition (Z - or atomic number) and topography, respectively, correlative BSE and SE imaging reveals the multiscale architecture of the bone surface (Fig. 1). Micrometer-scale features include blood vessels (50–70 µm) and osteocyte lacunae (8–15 µm). The extracellular matrix is assembled into an interwoven mesh of rope-like mineralized bundles comprising nanoscale apatite platelets that tangentially surround individual collagen fibrils [Shah et al., 2016c, 2020]. Submicrometer-sized openings for dendritic extensions of osteocytes are found between anisotropically arranged mineralized bundles at the floor of the osteocyte lacuna.

Conventional Micro-Raman Spectroscopy and Surface Topography Dependent Signal Deterioration

When in-focus (i.e., $z = 0$ µm), comparison of integration times reveals decreases in ν_1 PO_4^{3-} (45%; $p = 0.005$) and ν_1 CO_3^{2-} (43%; $p = 0.022$) peak intensities between 1,000 and 500 ms (Fig. 2). The ν_1 PO_4^{3-} and ν_1 CO_3^{2-} peak intensities decrease dramatically when the offset between the sample surface and the plane of focus is more than a few micrometers. Particularly when overfocused, the signal loss tolerance is superior at longer integration times. Here, significant decreases ($p < 0.05$) in peak intensities are observed when the laser is focused ≥ 3 µm and ≥ 4 µm below the sample surface at 500 ms and 1,000 ms, respectively. When underfocused, ν_1 PO_4^{3-} and ν_1 CO_3^{2-} peak intensities decrease rapidly from 2 to 3 µm above the sample surface. Deterioration in the signal-to-noise ratios with increasing distance of the sample surface from the plane of focus also reveals a trend toward integration time dependence. At 500 ms, ν_1 PO_4^{3-} and ν_1 CO_3^{2-} signal-to-noise ratios deteriorate significantly ($p > 0.05$) when the laser is focused ≥ 3 µm above and/or ≥ 6 µm below the sample surface, that is, the background fluorescence makes a stronger contribution to the overall signal. In comparison, ν_1 PO_4^{3-} and ν_1 CO_3^{2-} signal-to-noise ratios display better stability at 1,000 ms and remain consistent through ± 10 µm discrepancy between the sample surface and the plane of focus.

During wide-field mapping of non-planar surfaces, conventional micro-Raman spectroscopy does not offer a means to compensate for variations in height deviation. Therefore, while certain parts of the ROI remain in-focus other parts may be underfocused or overfocused, resulting in loss of signal integrity. Real-time focus-tracking (i.e., with LiveTrack™) allows to overcome such surface topography-dependent signal deterioration (Fig. 3).

Fig. 2. Surface topography-dependent deterioration in Raman intensity and signal-to-noise ratio at 500 ms and 1,000 ms integration time. **a** $v_1 \text{ PO}_4^{3-}$ at 500 ms. **b** $v_1 \text{ PO}_4^{3-}$ at 1,000 ms. **c** $v_1 \text{ CO}_3^{2-}$ at 500 ms. **d** $v_1 \text{ CO}_3^{2-}$ at 1,000 ms. Asterisks denote statistically significant difference between in-focus (0 μm) and out-of-focus (underfocused: +1 to +10 μm; overfocused: -1 to -10 μm) planes. Counts = Number of Raman scattering events detected.

Raman Mapping the Deproteinized Bone Surface

The compositional characteristics as well as topological variations of the deproteinized (i.e., anorganic) bone surface, and thus the most recently laid down mineral at any given point/location, are obtained simultaneously. The spatial distribution and concentration gradients of bone mineral can be mapped at different length scales and level of detail, depending on pixel size and field width.

The general trends in tissue dynamics can be probed at micrometer pixel sizes (Fig. 4). Regions exhibiting weaker PO_4^{3-} and CO_3^{2-} signals (or low PO_4^{3-} and/or CO_3^{2-} density) represent more recently formed bone. The CO_3^{2-} content, in particular, is considered a marker of bone turnover [Deymier et al., 2020]. Blood vessel openings at the bone surface and lacunae of incompletely embedded osteocytes, also referred to as osteoblastic-osteocytes [Franz-Odenaal et al., 2006], can also be resolved.

Cellular and subcellular length scales become accessible at sub-micrometer pixel sizes, including rope-like mineralized bundles that comprise the bone surface and the mineral phase at the osteocyte lacuna floor (Fig. 5).

Raman Mapping the Decellularized/Defatted Bone Surface

The distribution of inorganic (PO_4^{3-} and CO_3^{2-}) and organic (Amide III, Pro, Hyp, Phe, Tyr, Ala) extracellular matrix components at the surface of decellularized/defatted bone can also be probed following removal of surface adherent cells (Fig. 6). Cluster analysis reveals progressive increases in $v_1 \text{ PO}_4^{3-}$ and $v_1 \text{ CO}_3^{2-}$ peak intensities and the mineral-to-matrix ratio (from 0.07 to 0.33) simultaneously with a shift in the relative contributions of $\delta(\text{N-H})$ and $\nu(\text{C-N})$ vibrations to the Amide III band profile. Confined to poorly mineralized areas, cluster analysis reveals a broad feature at 945 cm^{-1} assigned to amorphous calcium phosphate [Crane et al., 2006] and another feature at 995 cm^{-1} that is likely attributable to amino acid alanine (Ala) [Rolfe et al., 2016]. Ala enters osteoblasts through a sodium-dependent transport mechanism and is involved in subsequent protein synthesis [Veldman and Schmid, 1998]. Various amino acids, including Ala, impose an indirect anabolic effect on bone [Jennings et al., 2016; MacDonell et al., 2016], and disturbances in plasma and callus amino acid levels are noted in impaired fracture healing [Wijnands et al., 2012]. Ala and Phe content decrease with progressive mineralization, while the Ala/Phe ratio changes from 1.0 to < 0.01 .

Fig. 3. Wide-field Raman mapping of non-planar ROIs with (+) and without (–) signal stabilization via real-time focus-tracking. Pixels in the ROI that remain out of focus without LiveTrack™ are brought into focus with LiveTrack™. **a** Example #1: $500 \times 1,000 \mu\text{m}^2$ ROI. $\times 50$ objective. $5 \mu\text{m}$ pixel size. 200 ms integration time per pixel. **b** Example #2: $200 \times 200 \mu\text{m}^2$ ROI. $\times 100$ objective. $1 \mu\text{m}$ pixel size. 200 ms integration time per pixel. **c** Illustration of Raman signal stabilization using LiveTrack™. Arrows indicate direction of laser propagation. Broken lines indicate plane of focus. WL, white light image.

Fig. 4. Deproteinized bone at the micrometer level; blood vessels (large, low z structures; broken circles) and osteocyte lacunae (smaller, low z structures). Raman maps of the $\nu_1 \text{PO}_4^{3-}$ band are shown using Raman intensity-based segmentation. **a** $1,000 \times 1,000 \mu\text{m}^2$ ROI. $\times 50$ objective. $5 \mu\text{m}$ pixel size. 200 ms integration time per pixel. **b** $500 \times 500 \mu\text{m}^2$ ROI. $\times 100$ objective. $1 \mu\text{m}$ pixel size. 200 ms integration time per pixel. WL, white light image.

Fig. 5. Deproteinized bone at the submicrometer level; rope-like mineralized bundles (arrows) and osteocyte lacunae (demarcated with broken lines). **a** $70 \times 60 \mu\text{m}^2$ ROI. $\times 100$ objective. 300 nm pixel size. 300 ms integration time per pixel. **b** $24 \times 24 \mu\text{m}^2$ ROI. $\times 100$ objective. 200 nm pixel size. 300 ms integration time per pixel.

Fig. 6. Decellularized/defatted bone at the micrometer level. **a** $250 \times 200 \mu\text{m}^2$ ROI. $\times 100$ objective. 1 μm pixel size. 2,000 ms integration time per pixel. Raman maps of $\nu_1 \text{PO}_4^{3-}$ and Amide III are shown. **b** Cluster analysis (50,000 spectra). Three clusters, denoted by 1 (green; 3,200 spectra), 2 (blue; 6,432 spectra), and 3 (magenta; 40,368 spectra) represent poor-, low-, and high mineral density zones, respectively. Cluster 1 is outlined in yellow on $\nu_1 \text{PO}_4^{3-}$ and Amide III maps in **(a)**. **c** Average Raman spectra of clusters 1–3.

Integral areas $420\text{--}470 \text{ cm}^{-1}$ and $1,220\text{--}1,300 \text{ cm}^{-1}$ are used to calculate mineral-to-matrix ratios (MMR), as shown in the bar chart. CO_3^{2-} content ($\nu_1 \text{CO}_3^{2-}$ at $1,072 \text{ cm}^{-1}$) increases simultaneously with mineral density, as shown in right inset. Presence of amorphous calcium phosphate (ACP) is noted within cluster 1. The Ala/Phe ratio decreases from 1.04 at $\text{MMR} = 0.07$, to 0.56 at $\text{MMR} = 0.13$, and 0.004 at $\text{MMR} = 0.33$. The $\delta(\text{N-H})$ signal at $1,274 \text{ cm}^{-1}$ is more intense within clusters 1 and 2.

Fig. 7. Decellularized/defatted bone at the submicrometer level. **a** $70 \times 35 \mu\text{m}^2$ ROI. $\times 100$ objective. 500 nm pixel size. 1,000 ms integration time per pixel. Raman maps of $\nu_1 \text{PO}_4^{3-}$ and Amide III are shown. **b** Cluster analysis (9,800 spectra). Three clusters, denoted by 1 (green; 1,699 spectra), 2 (blue; 3,807 spectra), and 3 (magenta; 4,294 spectra) represent low MMR, high MMR + high void, and high MMR + low void zones, respectively. **c** MMR calculated from integral areas $420\text{--}470 \text{ cm}^{-1}$ and $1,220\text{--}1,300 \text{ cm}^{-1}$ of average Raman spectra of clusters 1–3. **d** $26 \times 18 \mu\text{m}^2$ ROI. $\times 100$ objective. 200 nm pixel size. 2,000 ms integration time per pixel. Raman maps of $\nu_1 \text{PO}_4^{3-}$ and Amide III are shown. **e** Cluster analysis (11,700 spectra). Three clusters, denoted by 1 (green; 2,805 spectra), 2 (blue; 4,687 spectra), and 3 (magenta; 4,208 spectra) represent low MMR, high MMR + high void, and high MMR + low void zones, respectively. **f** Average Raman spectra of clusters 1–3. All spectra normalized to the intensity of the 430 cm^{-1} $\nu_2 \text{PO}_4^{3-}$ subcomponent (inset) and y axis shifted. Amide III, Phe, and $\delta(\text{CH}_3)$, $\delta(\text{CH}_2)$ signals are strongest within cluster 1.

On the submicrometer level (Fig. 7), cluster analysis reveals two spectrally identical subregions of the extralacunar matrix, confirmed using linear regression analysis ($R^2 > 0.999$, $p < 0.0001$), that differ only in total Raman intensity in the $350\text{--}1,500 \text{ cm}^{-1}$ range (21 and 28% difference at 300 and 200 nm pixel size, respectively). Of these, the lower intensity regions appear to originate from a network of subsurface voids attributable to channel-like canalicular spaces.

Relative to the extralacunar matrix, while a sudden height deviation is not apparent at the osteocyte/lacuna (as opposed to deproteinized bone where the floor of the osteocyte lacuna is readily accessible), spectral contributions of the organic components are stronger and the mineral-to-matrix ratio is comparatively lower, indicating the presence of more recently deposited extracellular matrix.

Discussion/Conclusion

The surface of bone is a representation of the local metabolism at any given point in time. The organic and inorganic phases of bone form an interpenetrating composite [Hamed et al., 2012; Schwarcz et al., 2014]. On the micrometer level, bone is made up of $1\text{--}2 \mu\text{m}$ diameter bundles of collagen fibrils arranged into a continuous in-

terwoven network [Reznikov et al., 2014]. Similar architecture is observed at the surface of deproteinized bone [Shah et al., 2020]. At the floor of the osteocyte lacuna, these collagen fibril bundles are preferentially aligned parallel to the osteocyte lacuna [Shah et al., 2016c]. High resolution Raman imaging also finds applications in biomineralization processes such as intralacunar mineralization following osteocyte apoptosis, which has previously been undertaken in resin embedded, plane parallel, polished samples [Shah et al., 2017].

Micro-Raman spectroscopy enables studying the hierarchical architecture of complex biological surfaces. However, signal integrity is lost rapidly when the discrepancy between the sample surface and the plane of focus is more than a few micrometers. Here, the combination of Raman spectroscopy and real-time surface tracking opens new possibilities for evaluating interfaces and surfaces that exhibit a naturally occurring multiscale topography, for example, the bone-implant interface, cranial sutures, native and fractured bone surfaces of bone, and conditions characterized by defective mineralization fronts [Boyde et al., 2017]. A potential application of the technique is to detect changes or disturbances in bone me-

tabolism following short-term exposure to stimuli, such as anabolic/therapeutic agents or mechanical loading, directly at the mineralization front.

In addition to eliminating spectral contributions of the organic components that overlap the PO_4^{3-} bands, deproteinization maximizes the topological challenge for the surface tracking technology (LiveTrack™) by creating micrometer-sized spaces (i.e., vascular channels and osteocyte lacunae), submicrometer irregularities, and nanoporosities. Though the hierarchical architecture of bone remains intact after prolonged exposure to NaOCl [Chen et al., 2011], deproteinization may remove the most immature, nanometer-sized, amorphous mineral deposits from the mineralization front, also known as calcospherulites [Midura et al., 2007]. Indeed, amorphous calcium phosphate is detectable at the surface of decellularized/defatted bone. The use of NH_4OH , however, has been shown as a highly effective method for decellularization of cell-secreted matrices without affecting the structural integrity of the extracellular matrix [Taylor et al., 2015]. The Amide III band profile reveals that the secondary structure of collagen is retained in a near-native state following decellularization, while the relative contributions of $\nu(\text{C-N})$ and $\delta(\text{N-H})$ vibrations and the Ala/Phe ratio change with progressive mineralization.

From a practical standpoint, consideration must be given to the working distance of the objective and the geometry of the specimen, so that curvatures and protrusions do not cause physical obstruction. For this reason, another limitation of this technique is that very large ROIs (e.g., several micrometers across) cannot be mapped using short working distance objectives. Low power objectives (e.g., $\times 10$, $\times 20$, $\times 50$) offer longer working distances at the expense of lateral resolution, and therefore larger step/pixel sizes are advisable. The numerical aperture is smaller than high power objectives (e.g., $\times 100$) and the signal-to-noise ratio is comparatively worse. Though not considered in the present work, a confocal aperture (or pinhole) may be introduced into the optical path in order to improve depth resolution and generate less subsurface information by limiting the beam diameter and the in-focus volume, since this reduces the number of Raman scattered photons (referred to as “counts”). An obvious implication for both conventional and surface tracked micro-Raman spectroscopy is that signal integrity (peak intensity and/or signal-to-noise ratio) is considerably diminished, necessitating longer integration time and/or number of accumulations.

The examples presented here show the symmetric stretching modes of $\nu_1 \text{PO}_4^{3-}$ and $\nu_1 \text{CO}_3^{2-}$, which are considered sensitive to polarization (and/or sample orienta-

tion). But without a polarization analyzer, the collected signal comprises all polarized and depolarized Raman scattered photons. The $\nu_1 \text{PO}_4^{3-}$ band, however, is very intense and therefore valuable when the signal-to-noise ratio may be low, and often the only detectable apatitic PO_4^{3-} band in certain experimental setups [McElderry et al., 2013]. Additionally, since the CO_3^{2-} content of bone increases with tissue age [Burnell et al., 1980; Legros et al., 1987], and increasing B-type CO_3^{2-} substitution is implicated in bone embrittlement [Akkus et al., 2004], many studies derive information about the chemical and physical characteristics of the mineral phase from the $\nu_1 \text{CO}_3^{2-}$ band [Awonusi et al., 2007], for example, Raman metrics such as $\nu_1 \text{CO}_3^{2-}/\nu_1 \text{PO}_4^{3-}$ and $\nu_1 \text{CO}_3^{2-}/\nu_2 \text{PO}_4^{3-}$ [Nyman et al., 2011; Shah et al., 2016b; Shah et al., 2018].

Need is eliminated for an independent surface pre-scan, which is liable to alter the sample (e.g., drying, contamination). A potential limitation of the technique is that sites of ongoing resorption may be more challenging to detect, while regions of recent osteogenic activity are easier to identify. Although the height deviation maps are inferior in lateral resolution compared to atomic force microscopy, both technologies remain limited by line-of-sight principles.

In conclusion, we demonstrate, for the first time, the possibility to spatially map the chemical composition of the surface of bone, which reveals early changes and trends in extracellular matrix composition. Excitingly, detection of lower mineral content (or mineral-to-matrix ratio) in the extracellular matrix associated with the osteocyte lacuno-canalicular network may point toward metabolic processes such as perilacunar remodeling [Dole et al., 2017]. During micro-Raman spectroscopy of non-planar surfaces such as that of bone, real-time surface tracking facilitates signal stabilization, enabling seamless acquisition of chemical information across wide fields of view and multiple distinct length scales that characterize this unique and complex biological system.

Statement of Ethics

Sheep cortical bone used in this work was obtained as part of an unrelated study approved by the Ministry of National Education, Higher Education and Research (NAMESA, Chasse-sur-Rhône, France).

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

F.A.S. made the conception and design of the study, did data acquisition, data analysis and interpretation, and wrote the manuscript. K.R. did data acquisition, data analysis and interpretation, and wrote the manuscript. A.P. made the conception and design, wrote the manuscript, and did financial support. All authors read and approved the final version of the manuscript and agreed to be accountable for all aspects of the work.

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