Photon migration of Raman signal in bone as measured with spatially offset Raman spectroscopy

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Spatially offset Raman spectroscopy (SORS) is currently being developed as an in vivo tool for bone disease detection, but to date, information about the interrogated volume as influenced by the light propagation and scattering characteristics of the bone matrix is still limited. This paper seeks to develop our general understanding of the sampling depths of SORS in bone specimens as a function of the applied spatial offset. Equine metacarpal bone was selected as a suitable specimen of compact cortical bone large enough to allow several thin slices (600 μm) to be cut from the dorsal surface. Photon migration at 830-nm excitation was studied with five bone slices and a 380-μm-thin polytetrafluoroethylene (PTFE) slice placed consecutively between the layers. To optimize Raman signal recovery of the PTFE with increasing depth within the bone stack required a corresponding increase in spatial offset. For example, to sample effectively at 2.2-mm depth within the bone required an optimal SORS offset of 7 mm. However, with a 7-mm offset, the maximum accessible penetration depth from which the PTFE signal could be still recovered was 3.7 mm. These results provide essential basic information for developing SORS technology for medical diagnostics in general and optimizing sampling through bone tissue, permitting a better understanding of the relationship between the offset and depth of bone assessed, in particular. Potential applications include the detection of chemically specific markers for changes in bone matrix chemistry localized within the tissue and not present in healthy bone. © 2015 The Authors. Journal of Raman Spectroscopy published by John Wiley & Sons, Ltd.

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Introduction

Raman spectroscopy is a valuable tool with many analytical applications that require chemically specific information. However, conventional Raman spectroscopy is limited to gathering information directly from sample surfaces or depths of only about 100–200 μm from diffusely scattering (turbid) samples.1,2

To allow deeper sub-surface probing, several extensions of the basic Raman methodology have been demonstrated. One possibility is the application of time-resolved Raman spectroscopy using ultrafast (picosecond) Kerr gating.3 This technique relies on the fact that photons emerging from deeper layers inside the sample require longer times to reach the surface and therefore arrive at the detector later than the photons coming from near the surface. By varying the time delay between the excitation laser pulse and switching on the detector, the collection depth of the Raman signals can be controlled. The time-gating method also permits rejection of fluorescence from surface layers. As an example, this method has been successfully demonstrated for transcutaneous investigation of bone.4,5 However, Kerr-gated time-resolved Raman spectroscopy requires expensive and complex instrumentation and suffers from inherently low signal quality owing to the limited number of detected photons within the detection time window. In addition, it requires high laser peak intensities that may cause photo-induced damage, and this limits in vivo applications.

An alternative method for obtaining Raman spectra deep within turbid media is by applying a spatial approach, namely spatially offset Raman spectroscopy (SORS). Shortly after the first demonstration of SORS,6,7 an improved version called inverse SORS was proposed and demonstrated.8,9 As with Kerr gating, SORS permits Raman spectra to be collected from diffusely scattering media far beyond depths accessible using confocal Raman microscopy; depths of the order of several millimeters can be achieved. The key advantages of SORS are the simpler instrumentation and the requirement for much lower and safety compliant illumination intensities compared with those necessary for temporal approaches. This allows the use of continuous wave laser sources that provide superior signal quality compared with the temporal approach as many more photons are collected in total. SORS relies on spatially separating the point of incidence (where the excitation laser beam interacts with the sample surface) from the point that the
Photons at a distance of 12 m has been reported. With respect to stand-off investigation of solids and liquids in opaque plastic botanical composition of drugs through opaque plastic containers for non-invasive diagnosis of bone disease and degeneration. Research of high societal relevance is also the development of SORS enhanced SORS when high Raman band intensities are present. Destructive sub-surface analysis. Areas of intensive research samples paved the way for numerous applications in non-destructive sub-surface analysis. Areas of intensive research include security and forensics for the identification of liquids as well as solids in powder form through the walls of plastic and glass containers. Furthermore, the use of 1064-nm excitation for improved fluorescence reduction as well as the stand-off investigation of solids and liquids in opaque plastic bottles at a distance of 12 m has been reported. With respect to process control, the authentication of pharmaceutical tablets inside their packaging and the quantitative analysis of chemical composition of drugs through opaque plastic containers were demonstrated. Further applications comprise sub-layer discrimination in paintings composed of multiple thin layers. The determination of salmon-quality parameters through their skin and the acquisition of sub-surface chemical information from tomatoes for assessment of ripening.

In the biomedical field, SORS is being developed as a tool for tissue analysis with a particular focus on the detection of tumors and the identification of breast calcifications again in relation to cancer diagnosis. An area of particularly intense research of high societal relevance is also the development of SORS for non-invasive diagnosis of bone disease and degeneration. Numerous investigations have been performed using SORS, inverse SORS, and in advanced illumination and collection geometries permitting the Raman spectra of bones to be retrieved successfully from depths of 3–5 mm beneath soft tissue (skin). Recently, SORS has demonstrated its potential for the in vivo detection of bone disorders such as the genetic condition, osteogenesis imperfecta.

Large penetration depths can be realized using surface-enhanced SORS when high Raman band intensities are present. Under such optimum sample conditions, depths of up to several centimeters in soft tissue and up to about 8 mm in bone have been demonstrated. Previously, numerous investigations have been conducted to determine the optical properties of bone: the absorption and scattering coefficients of different bone types have been reported in the literature. To date, however, there is still a fundamental lack of understanding of the precise range of depths within specific bone tissues from which data are generated in relation to the applied spatial offset. Such knowledge is vital from analytical and diagnostic viewpoints where it is essential to know the depth of origin of the SORS signals to correctly assign any observable changes to the surface or sub-surface sample components, which can be between several hundred micrometers and several millimeters. For example, sampling of exposed bone in the operating theatre that is limited to the surface would be more affected by bone remodeling. Development of SORS technology for sampling during surgical procedures that retrieves information through a whole bone cortex could enable the study of localized bone infection or the presence of bone cancer. As an example, chemically specific Raman bands not present in healthy bone tissue and associated with the presence of pathological minerals due to early stage bone infection of diabetic patients have been identified. This study addresses this fundamental issue by quantifying the depth origin of SORS signals in bone as a function of spatial offset, and these results provide a ‘deeper’ understanding of photon migration within long bone.

**Materials and methods**

A cannon bone (third metacarpal bone) from a mature thoroughbred racehorse (obtained from an equine abattoir) was excised, wrapped in Clingfilm, and fresh frozen in a –20 °C freezer. Before collection of the Raman data, the bone was allowed to thaw at ambient temperature (20 °C), and all soft tissue was removed with a scalp. A 4-cm section was then cut from the mid-shaft of the metacarpal bone and sliced using a diamond bladed band saw (Exact 300). The first slice (approximate thickness: 2 mm) was discarded owing to its natural curvature. Subsequently, five frontal plane slices of cortical bone with a thickness of 604 ± 18 μm (given as average ± standard deviation) were cut from the dorsal surface. After slicing, the individual slices and the remaining piece of the metacarpal bone section (hereafter named as ‘bone segment’, Fig. 1) were thoroughly rinsed with tap water. Prior to the Raman experiments, the specimens were held in a plastic container filled with tap water to prevent dehydration and stored in a refrigerator at 5 °C. During investigations, the samples were removed from the fridge for 1 h prior to making measurements to bring them up to ambient temperature.

The Raman measurements were performed using an inverse SORS custom-built Raman system (Cobalt Light Systems Ltd, Oxfordshire, UK). An NIR diode laser emitting at 830 nm served as the excitation light source delivering an optical power of 320 mW at the sample; it delivers the light as an annular laser illumination zone with a selectable radius (spatial offset), and the signal identified. This study addresses this fundamental issue by quantifying the depth origin of SORS signals in bone as a function of spatial offset, and these results provide a ‘deeper’ understanding of photon migration within long bone.

**Figure 1.** Photograph of the horse metacarpal mid-shaft bone specimens used; ruler shows scale in millimeters. For the measurements, the five slices shown in the front were placed on the bone segment shown in the rear.
collection zone is always located at the center of the illumination ring.\[27\] The instrument is capable of recording multiple spatially offset Raman spectra; in these experiments, the spatial offsets between excitation and collection areas ranged from 0 to 7.5 mm in increments of 0.5 mm. The ring width of the annular illumination zones was 1 mm.\[11\]

The scattered Raman radiation from the collection zone (~1 mm diameter) was focused into a low-loss Optran WF fiber bundle (CeramOptec, East Longmeadow, MA) and transferred into a spectrograph (Raman Explorer, Headwall, MA) equipped with a CCD detector (Andor iDus 420 BR-DD; Andor, Belfast, Northern Ireland). The detection unit had a spectral resolution of 8 cm\(^{-1}\), and the software automatically removed any spurious signals arising from cosmic rays. To ensure high collection efficiency, the fiber-optic bundle used a round configuration of 33 fibers at the sample side, while the other side pointing towards the spectrograph was configured in a linear arrangement to optimally fill the spectrograph input slit as well as the available vertical extent of the CCD.

For the photon migration studies, the bone slices were stacked together (in the order they originally had within the bone before cutting), forming a five-layer system. To establish the photon migration characteristics and measure the depths that the Raman signals were being generated, a slice of polytetrafluoroethylene (PTFE) with a thickness of 380 \(\mu\)m was placed in between each of the layers in turn. PTFE was chosen as it provides a well-separated metric stretch at 734 cm\(^{-1}\) with a thickness of 380 \(\mu\)m was placed in between each of the layers in turn. PTFE was chosen as it provides a well-separated metric stretch at 734 cm\(^{-1}\) well resolved from the strong bone phosphate signal at 961 cm\(^{-1}\). For each PTFE depth position, three randomly selected positions on the sample surface were probed, and at each spot, 20-s spectra (200 \(\times\) 0.1 s accumulations) were collected. During the experiments, the stack was surrounded by air; i.e., no other material or sample holder elements were in the path of the migrating photons. Consequently, photons escaping from the bottom of the sample remain undetected. Measurements were repeated with the bone segment placed below the stack of five bone slices. This was to investigate the influence of additional bone volume beneath the slices in terms of photon migration.

Before data analysis, all the Raman spectra were corrected for spectral variation of the CCD sensitivity by multiplying them individually with a corresponding correction function obtained using a luminescent green glass standard to eliminate any artifacts due to CCD etaloning and filter effects. To determine the Raman intensity ratios, the most prominent bands of PTFE (C=O and C=C symmetric stretch at 734 cm\(^{-1}\)) as well as of bone (phosphate symmetric stretch at 961 cm\(^{-1}\)) were considered. The band intensities and ratios were calculated using Microsoft Excel (Microsoft, Redmond, WA). For each peak, three points to the left and the right, representing the signal background, were selected. Subsequently, a straight line was drawn through the average of the points left and right to approximate the background intensity in the spectral region of the peak. Finally, the peak intensity was calculated as the average of the intensity of these points at the peak center above the previously determined baseline at the wavenumber of the peak position.

Results

Initial investigations were performed using a 380-\(\mu\)m-thick PTFE slice on top of four bone slices with a total bone stack thickness of 2.4 mm. The red curve in Fig. 2 displays the PTFE to bone intensity ratios as the offset is increased. For small spatial offsets, below 1 mm, the intensity ratio is approximately 6.5, but this then rapidly decreases to 0.9 for 5-mm spatial offset. The green curve in Fig. 2 illustrates the PTFE band intensity of the PTFE slice on top of the bone layer stack divided by the PTFE band intensity of the single PTFE slice with nothing but air below it, showing an increase in ratio from 1.6 up to 5.6 with increasing spatial offset from 0 to 5 mm. The blue curve in Fig. 2 gives the intensity ratio with SORS offset of the phosphate band from the stack of bone layers with and without the PTFE slice on top. The intensity ratio slightly increases from 0.4 up to 0.6 when increasing the spatial offset from 0 to 5 mm.

The next set of experiments involved moving the PTFE slice from the top to in between each successive bone layer and monitoring the strength of the PTFE Raman band at 734 cm\(^{-1}\) at each offset. Intensity ratios displayed in Fig. 3 were calculated by dividing the band intensity of the single PTFE slice by the band intensity of the PTFE slice at selected positions on top of, or within, the bone layer stack. All the curves in Fig. 3 show a similar trend, a lowering of the band intensity ratios with increasing spatial offset.

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Selected Raman intensity ratios demonstrating PTFE Raman signal enhancement and bone signal shielding effects: red curve (squares) shows PTFE signal intensity (PTFE slice on top of four bone slices) divided by bone signal intensity (PTFE slice on top of four bone slices); green curve (diamonds) depicts PTFE signal intensity (PTFE slice on top of four bone slices) divided by PTFE signal intensity (single PTFE slice only); and blue curve (triangles) shows bone signal intensity (four bone slices under PTFE slice) divided by bone signal intensity (stack of four bone slices only).

![Figure 3](https://example.com/figure3.png)

**Figure 3.** Intensity of single PTFE layer divided by intensity of PTFE layer at different positions on top and within the bone stack probed at various SORS offsets (logarithmic scale); inset shows selected PTFE layer location within bone layer stack.
Dependence of PTFE signal recovery on spatial offset – fixed PTFE depth

A major question to address is how deep the PTFE layer can be placed inside the bone, and one is still able to detect its characteristic Raman spectral signature. In fact, even at the largest depth investigated (3 mm), with the bone segment below the stack, the prominent PTFE band at 734 cm\(^{-1}\) can be identified by visual inspection (see dashed rectangle in Fig. 4a). Measurements show the PTFE to bone intensity ratio increases from about 0.01 for zero offset to 0.065 for a SORS offset of 7.5 mm. Note: in the figure, the spectra have had the broad fluorescence background removed using a fifth-order polynomial fitting algorithm\(^{[42]}\) (Microsoft Excel 2010), and the spectra have been offset vertically (by 2000 counts each), but for the calculations, the spectra were left untreated in order to avoid any spectral distortions.

Figure 4b illustrates the signal to noise (S/N) ratio of PTFE at the depth of 3 mm as a function of spatial offset. The background-noise value was estimated from a wavenumber range between 660 and 710 cm\(^{-1}\) that contains no Raman signal from bone or PTFE. It is evident that the S/N ratio increases for larger spatial offsets with values ranging from about 2 for zero offset up to about 6 for a 7.5-mm offset; i.e. an increase by a factor of 3 is observed. Interestingly, S/N ratios reach a plateau for SORS offsets larger than 3 mm, subsequently showing only minor increases with increasing offset on average.

Dependence of PTFE signal recovery on spatial offset – variable PTFE depth

In the next step, PTFE to bone intensity ratios at each probed PTFE depth were calculated for all investigated SORS offsets. In each case, the experiments were repeated with and without the bone segment underneath the stack so that the effect of optical losses from the back of the system could be quantified. The results for three selected depth positions of the PTFE layer inside the bone are displayed in Fig. 5. Generally, the curves representing the bone

Figure 4. (a) Raman spectra of horse metacarpal cortical bone with a PTFE layer located below 3 mm of bone and with the bone segment placed beneath the stack. The spectra are displayed for selected spatial offsets and are vertically offset for clarity; (b) signal to background noise ratio of PTFE band at 734 cm\(^{-1}\) as a function of spatial offset.

Figure 5. PTFE to bone intensity ratios dependent on SORS offset for horse metacarpal mid-shaft using bone slices only as well as slices above the bone segment; (a) PTFE at depth of 0.6 mm, (b) PTFE at depth of 1.8 mm, and (c) PTFE at depth of 3.0 mm.
layer stack with and without the bone segment beneath show a similar trend. However, the maxima of PTFE to bone ratio depends on the depth of the PTFE slice inside the bone material and the spatial offset applied. Figure 5a shows that the maximum PTFE to bone intensity ratio varies between 45% and 48% at a 2-mm offset for the PTFE at 0.6-mm depth. This maximum changes to approximately 16% at roughly 5–7 mm for a depth of 1.8 mm (Fig. 5b) and about 5–8% at ~7 mm for PTFE at 3-mm depth (Fig. 5c).

To obtain the SORS offset at which the maximum PTFE to bone intensity ratio was located, the top sections of the curves presented in Fig. 5 were fitted with a Gaussian distribution (Ezyfit Toolbox used in conjunction with MATLAB R2013a). Figure 6 (red curve) shows the monotonic increase in SORS offset required to obtain the maximum PTFE to bone ratio with increasing PTFE depth when investigating the bone slices with the bone segment beneath. In contrast, when using the bone slices only, for PTFE depths larger than about 1.8 mm, the spatial offset required to obtain the maximum PTFE to bone intensity ratio reaches a plateau at about 6 mm (green curve).

In order to estimate the maximum accessible penetration depth from which the PTFE signals could still be recovered from the bone material, the S/N ratios of the PTFE signal at 734 cm\(^{-1}\) have been calculated. Figure 7 shows a plot of the S/N ratios as a function of the PTFE depth below the bone surface using bone slices with the bone segment placed below them. We observe that the decrease in the intensity ratio is more pronounced for small SORS offsets than for large offsets.

Based on Fig. 7, the penetration depth for a given SORS offset can be determined taking into account the 3-sigma criterion. In this case, the PTFE band is considered to be detectable if its intensity equals three times the noise level. The parts of the curves representing the three largest depths of the PTFE slice inside the bone, i.e. 1.8, 2.4, and 3.0 mm, underwent linear fitting to calculate the depth where the S/N ratio equals three. The results are presented in Fig. 8. With the bone segment beneath the slices, the curve shows a monotonic increase with increasing spatial offset. The PTFE signals could still be recovered from penetration depths up to 3.7 mm using large SORS offsets, for example, 7 mm. In contrast, for the stack of bone slices without the bone segment in place, only minor variations in penetration depth can be observed with increasing spatial offset. Here, maximum accessible depth is limited to 3.1 mm.

**Discussion**

The presented SORS experiments provided a comprehensive description of photon migration effects inside diffusely scattering media, here, bone. In a two-layer system, i.e. a thin PTFE slice on top of 2.4 mm of bone, using larger spatial offsets, preferentially interrogates the deeper layers (bone), and the relative contribution from the top layer (PTFE) is significantly decreased.

**Diffuse scattering and ‘photon reversal’ effect**

The aforementioned results show that the recovery of Raman photons from a specific depth depends on the nature of the material below the probed volume. This is illustrated by the investigation using the 380-μm-thin PTFE layer with and without bone material beneath and clearly highlights the importance of diffuse scattering within the bone tissue. We note that for small spatial offsets, probing mainly the surface of the PTFE slice, there is a noticeable signal enhancement of PTFE amounting to roughly 60% when bone is put underneath the sample (Fig. 2). This signal enhancement can be interpreted by photon migration within the bone which, as PTFE, is a turbid medium.

![Figure 6. SORS offsets giving maximum PTFE to bone ratio for selected PTFE depths below the bone surface using horse metacarpal mid-shaft.](image)

![Figure 7. PTFE S/N ratios as a function of bone layer thickness above the PTFE slice for selected spatial offsets using bone slices of horse metacarpal mid-shaft with the bone segment beneath.](image)

![Figure 8. Calculated penetration depths based on the 3-sigma criterion for horse metacarpal mid-shaft.](image)
With the bone underneath the PTFE layer, laser photons that have already traveled through the thin PTFE sample have a probability to reverse their propagation direction by means of multiple diffuse scattering events inside the underlying bone. This diffuse scattering partly induces a ‘photon reversal’ redirecting at least some photons back towards the overlying PTFE where they can undergo Raman scattering, enhancing the corresponding signal intensity. However, using the PTFE slice alone, i.e. without a diffusely scattering medium underneath, photons passing through it will retain their propagation direction and therefore cannot contribute to the Raman signal intensity. The same ‘photon reversal’ effect is also responsible for returning Raman photons generated within the PTFE slice and leaving the bottom PTFE interface. That is, placing bone underneath the PTFE slice returns Raman photons, which would otherwise be lost to the air, back into the PTFE slice increasing the likelihood of them migrating to the top surface of PTFE and being detected.

We also observe Raman signal attenuation of the lower layers caused by the upper layer. For example, when probing a 2.4-mm-thick stack of bone with and without a thin PTFE layer on top, a shielding effect of the bone signal could be observed. With the PTFE slice on top of the bone stack, the bone signal intensity is reduced by roughly 60% for spatial offsets up to 1 mm (Fig. 2). This shielding of bone signal by the top (PTFE) layer is reduced with increased spatial offset leading to a signal decrease of about 40% above the 4-mm offset. With increasing SORS offset, as expected, the probed volume is moving deeper into the sample. Consequently, the probed bone volume increases while the investigated PTFE volume decreases.

Comparison of isolated and buried PTFE layers

It has been shown that for all spatial offsets, the PTFE signal intensity is higher when the PTFE slice is placed on top of the bone stack as compared with the isolated PTFE slice. However, when putting the PTFE slice inside the bone layer stack, there is a spatial offset at which the intensities are equal (i.e. the ratio one becomes zero (see dashed lines in Fig. 3). If the PTFE is located at a depth of 0.6 mm, equal band intensities occur at an offset of ~2.1 mm. For all curves in Fig. 3, the signal intensity from the single PTFE slice is significantly reduced as the collection volume is extended deeper inside the PTFE slice with increasing offset. However, at a spatial offset of 2.1 mm and the PTFE at 0.6-mm depth, the contribution from the buried PTFE equals that of the isolated PTFE layer probed at the same spatial offset. Putting the PTFE layer at depths of 1.2 and 1.8 mm inside the bone stack, equal intensities to the isolated PTFE layer are found at SORS offsets of 3.4 and 4.2 mm, respectively. The absolute intensities of the PTFE layers located within the bone stack are of course rapidly decreasing with increasing depth. Nevertheless, the results show that using 4.5 mm or greater spatial offsets, even the intensity of the PTFE layer buried below 1.8 mm of bone exceeds that of the isolated PTFE layer measured with the same offset illustrating the dominant role of photon migration in SORS measurements and its ability to provide detailed information on chemical composition beneath surfaces. These results demonstrate that when assessing the photon migration characteristics for optimizing SORS measurements, any reference material must be used within the sample volume, or on top or below it, and isolated measurements do not give realistic results.

PTFE signal recovery from selected depths

We now consider the role of the sample zones that may lie beyond the SORS sampled volume. Considering a PTFE layer depth of 0.6 mm inside a bone layer stack of five slices (total thickness: 3 mm), the PTFE to bone intensity ratios are higher without the bone segment placed below the bone slices. At first sight, this might be unexpected as there should be a PTFE signal enhancement due to the previously described ‘photon reversal’ effect. That is, while the PTFE signal is enhanced, the bone signal increases as well. With the PTFE placed 0.6 mm below the bone surface, the bone signal enhancement is up to 30% higher than that for PTFE, thus leading to a reduced PTFE to bone intensity ratio. It turns out that the underlying four bone slices, total thickness of 2.4 mm, and without placing the bone segment below, already provide a sufficiently large diffuse scattering volume that effectively returns Rayleigh photons to the PTFE layer where they in turn generate Raman scattered signals. Moving the PTFE layer deeper to a depth of 1.8 mm, the difference between placing the bone segment underneath and using the 5 bone slices only is much smaller. In this case, the PTFE and bone signals experience virtually the same enhancement, resulting in nearly equal intensity ratios. However, at spatial offsets larger than 4 mm, the highest PTFE to bone ratio can be realized when using the bone slices only. Beside the ‘photon reversal’ effect, putting the bone segment beneath this additional bone volume means that extra bone material is sampled and in turn provides an additional contribution to the intensity of Raman bone signals. The results show that underlying of two bone slices (thickness: 1.2 mm) supplies a sufficiently large diffuse scattering volume to enable effective backscattering of laser photons that will have passed through the PTFE layer.

Putting the PTFE slice at the bottom of the bone layer stack, i.e. below 3 mm of bone material, a large difference can be observed using the bone slices with and without the bone segment underneath. In this case, the ‘photon reversal’ effect of the additional bone volume is essential to detect a high amount of Raman photons from the PTFE layer. Using the slices only, any Raman or laser photons passing through the PTFE leave the sample at the PTFE–air interface, and therefore, backscattering does not occur. This explains why the maximum achievable intensity ratio is only about 5% compared with more than 8% when having the bone segment placed below the slices.

Our results also demonstrate that the prominent PTFE signal at 734 cm\(^{-1}\) can still be identified by visual inspection even if the PTFE layer is located below 3 mm of bone tissue, on the condition that the bone segment is put beneath the PTFE and acts as a diffusely scattering medium. In this case, large spatial offsets allow for better visibility of the small PTFE signal by providing a higher PTFE to bone signal ratio in combination with a better S/N ratio. The results show a usual feature observed in SORS data sets of differing offsets in that the surface spectra, while generally dominating at each offset, exhibit a decreased relative intensity contribution using larger spatial offset providing the ability to recover sub-surface signals.

Major sampling depth inside bone

Generally, in line with expectations, the deeper the PTFE is placed inside the bone sample, the larger the spatial offset required to obtain the maximum PTFE to bone ratio. The plots presented in Fig. 6 indicate at what SORS offset one obtains the maximum intensity from a specified depth. Importantly, this means that for a given offset, it is now possible to state the approximate depth from which the major Raman signal contribution arises.

If the bone segment is put below the stack of bone layers, i.e. mimicking a real bone, the ‘photon reversal’ effect of the underlying bone material enables effective backscattering and thus
encompasses the number of Raman photons originating from the PTFE layer. In contrast, when using the bone slices only, for PTFE depths larger than about 1.8 mm, the spatial offset required to obtain the maximal PTFE intensity ratio does not change significantly as the PTFE is moved to greater depths. This is due to the loss of photons caused by the missing photonic reversal effect. For small PTFE depths, there is still sufficient bone material below the PTFE to enable quite effective backscattering of photons. Hence, the shape of the curves representing bone slices with and without the bone segment beneath is similar up to PTFE depths of ~1.8 mm. If the collection volume is moved further into the sample at a certain point, it will start to cross the sample–air interface, thus reducing the effective probed volume within the sample. It should be noted that the given values do not represent the maximum depth at which ‘foreign’ signals can still be detected within the bone tissue as the maximum accessible depth is determined by the S/N ratio of the particular Raman band of the target material, in our case PTFE.

Maximum penetration depth for signal recovery

Small spatial offsets give a higher S/N ratio of PTFE for smaller depths as the PTFE volume and the probed volume have a maximum overlap in such cases. Moving the PTFE deeper inside the bone reduces the spatial overlap between the collection volume and the PTFE slice, resulting in a dramatic decrease of S/N ratio of the PTFE signals. In contrast, using a large spatial offset, i.e. probing greater depths, results in a small S/N ratio when the PTFE sample is located at a small depth. Here, increasing the PTFE depth inside the bone will consequently increase the spatial overlap between the collection volume and the PTFE slice, leading to larger values for the S/N ratio of the PTFE signals. However, in all cases, this effect is accompanied by an overall signal decrease of PTFE signals with increasing PTFE depth, resulting in the observed curve shapes in Fig. 7.

In the applied configuration involving unfocussed 830-nm laser radiation with 1-mm illuminating beam diameter even for zero spatial offset, depths of about 2.7 mm can be probed. This can be particularly advantageous when investigating biological samples as probing a larger volume helps to reduce sample heterogeneities. Maximum accessible penetration depths of 3.7 mm were achieved using the slices with the bone segment underneath. Decreased values of 3.1 mm using no bone segment in place can be understood as the lack of the photonic reversal effect, which significantly reduces the amount of backscattered photons.

It should be stressed that the determined penetration depths for zero spatial offset are not equivalent to values achievable using a confocal Raman microscope where much smaller laser and Raman collection spot sizes are deployed. This has been confirmed experimentally here using a confocal Raman microscope (Renishaw inVia) with a 20× objective. Five slices of horse metacarpal bone were used, and the PTFE slice was placed below 0.6 mm of bone material (one single slice). Laser power and integration time were selected to match the values applied for the corresponding SORS measurements described earlier. Focusing at the surface, the confocal Raman microscope failed to detect the PTFE band as the signal was masked completely by the background noise with S/N value of about 0.2. In contrast, the PTFE signal was clearly observable using zero spatial offset with the SORS system exhibiting an S/N ratio of roughly 68, i.e. more than two orders of magnitude larger.

Conclusion

This investigation provides insight into photon migration in bone material. An important issue we have addressed is being able to identify the location of the probed volume within the bone specimen for a given SORS offset and quantify the relationship between larger spatial offsets and at what depth Raman signals are generated from. This is important when considering the detection of specific bone alterations. We have further demonstrated a subtle but important effect on SORS signals and how they are influenced by the nature of the upper and lower layers required to be interrogated. It turns out that a sufficient amount of diffuse scattering material must be present underneath the investigated depth within the sample to enable effective signal recovery. In this way, a new type of signal not present in the native bone spectrum, e.g. PTFE, could be detected up to depths of 3.7 mm through cortical compact bone. This highlights the potential future development of SORS and SORS probes for use in the operating theatre, i.e. having exposed bone surfaces, for diagnosis of conditions such as cancer or infections deep within the bone volume characterized by chemically specific Raman bands different from those of healthy bone tissue.

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References

[1] C. A. Froud, I. P. Hayward, J. Laven, Appl. Spectrosc. 2003, 57, 1468.
[2] N. J. Everall, Analyst 2010, 135, 2512.
[3] P. Matousek, N. Everall, M. Towie, A. W. Parker, Appl. Spectrosc. 2005, 59, 200.
[4] M. D. Morris, P. Matousek, M. Towie, A. W. Parker, A. E. Goodship, E. R. C. Draper, J. Biomed. Opt. 2005, 10, 014014–1.
[5] E. R. C. Draper, M. D. Morris, N. P. Camacho, P. Matousek, M. Towie, A. W. Parker, A. E. Goodship, J. Bone Miner. Res. 2005, 20, 1668.
[6] P. Matousek, I. P. Clark, E. R. C. Draper, M. D. Morris, A. E. Goodship, N. Everall, M. Towie, W. F. Finney, A. W. Parker, Appl. Spectrosc. 2005, 59, 393.
[7] P. Matousek, Appl. Spectrosc. 2006, 60, 1341.
[8] M. V. Schulerich, K. A. Dooley, M. D. Morris, T. M. Vanasse, S. A. Goldstein, J. Biomed. Opt. 2006, 11, 060502–1.
[9] S. L. Jacques, Phys. Med. Biol. 2013, 58, R37.
[10] A. N. Bashkatov, E. A. Genina, V. I. Kochubev, V. V. Tuchin, J. Phys. D Appl. Phys. 2005, 38, 2543.
[11] K. Buckley, P. Matousek, Analyst 2011, 136, 3039.
[12] C. Eliasson, N. A. Macleod, P. Matousek, Anal. Chem. 2007, 79, 8185.
[13] C. Eliasson, N. A. Macleod, P. Matousek, Vib. Spectrosc. 2008, 48, 8.
[14] P. W. Loeffen, G. Maskall, S. Bonthron, M. Bloomfield, C. Tombling, P. Matousek, Proc. SPIE 2011, 8018, 80181E-1.
[15] R. J. Hopkins, S. H. Pelfrey, N. C. Shand, Analyst 2012, 137, 4408.
[16] B. Zachhuber, C. Gasser, E. t. H. Chrysostom, B. Lendl, Anal. Chem. 2011, 83, 9438.
[17] C. Eliasson, P. Matousek, Anal. Chem. 2007, 79, 1696.
[18] W. J. Olds, E. Jaatinen, P. Fredericks, B. Cletus, H. Panayiotou, E. L. Izake, Forensic Sci. Int. 2011, 212, 69.
[19] W. J. Olds, S. Sundarajoo, M. Selby, B. Cletus, P. M. Fredericks, E. L. Izake, Appl. Spectrosc. 2012, 66, 530.
[20] C. Conti, C. Colombo, M. Realini, G. Zerbi, P. Matousek, Appl. Spectrosc. 2014, 68, 686.
[21] N. K. Afseth, M. Bloomfield, J. P. Wold, P. Matousek, Appl. Spectrosc. 2014, 68, 255.
[22] J. Qin, K. Chao, M. S. Kim, Postharvest Biol. Technol. 2012, 71, 21.
[23] P. Matousek, N. Stone, J. Biophotonics 2013, 6, 7.
[24] M. D. Keller, S. K. Majumder, A. Mahadevan-Jansen, Opt. Lett. 2009, 34, 926.
M. D. Keller, E. Vargis, N. de Matos Granja, R. H. Wilson, M.-A. Mycek, M. C. Kelley, A. Mahadevan-Jansen, J. Biomed. Opt. 2011, 16, 077006–1.

N. Stone, R. Baker, K. Rogers, A. W. Parker, P. Matousek, Analyst 2007, 132, 899.

P. Matousek, E. R. C. Draper, A. E. Goodship, I. P. Clark, K. L. Ronayne, A. W. Parker, Appl. Spectrosc. 2006, 60, 758.

M. V. Schulmerich, K. A. Dooley, T. M. Vanasse, S. A. Goldstein, M. D. Morris, Appl. Spectrosc. 2007, 61, 671.

M. V. Schulmerich, J. H. Cole, J. M. Kreider, F. Esmonde-White, K. A. Dooley, S. A. Goldstein, M. D. Morris, Appl. Spectrosc. 2009, 63, 286.

P. I. Okagbare, D. Begun, M. Tecklenburg, A. Awonusi, S. A. Goldstein, M. D. Morris, J. Biomed. Opt. 2012, 17, 090502–1.

K. Buckley, J. G. Kerns, A. W. Parker, A. E. Goodship, P. Matousek, J. Raman Spectrosc. 2014, 45, 188.

K. Buckley, J. G. Kerns, P. D. Gikas, H. L. Birch, J. Vinton, R. Keen, A. W. Parker, P. Matousek, A. E. Goodship, IBMS BoneKEy 2014, 11, 1 Article no. 602. doi: 10.1038/bonekey.2014.97

N. Stone, K. Faulds, D. Graham, P. Matousek, Anal. Chem. 2010, 82, 3969.

H. Xie, R. Stevenson, N. Stone, A. Hernandez-Santana, K. Faulds, D. Graham, Angew. Chem. Int. Ed. 2012, 51, 8509.

B. Sharma, K. Ma, M. R. Glucksberg, R. P. Van Duyne, J. Am. Chem. Soc. 2013, 135, 17290.

M. Firbank, M. Hiraoka, M. Essenpreis, D. T. Delpy, Phys. Med. Biol. 1993, 38, 503.

Y. Liu, Y. Wang, Z. Qian, J. Zhao, X. Cao, W. Li, J. Biomed. Opt. 2014, 19, 117002–1.

J. D. Currey, Bones: Structure and Mechanics, Princeton University Press, Princeton, 2006.

K. A. Esmonde-White, F. W. Esmonde-White, C. Holmes, M. D. Morris, B. Roessler, Trans. Orth. Res. Soc. 2012, 37, 909.

J. Mihály, S. Sterkel, H. M. Ortnner, L. Kocsis, L. Hajba, É. Furdyga, J. Mink, Croat. Chem. Acta 2006, 79, 497.

C. A. Lieber, A. Mahadevan-Jansen, Appl. Spectrosc. 2003, 57, 1363.