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Detecting mineral content in turbid medium using nonlinear Raman imaging: feasibility study

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Abstract

Osteoporosis is a bone disease characterized by reduced mineral content with resulting changes in bone architecture, which in turn increases the risk of bone fracture. Raman spectroscopy has an intrinsic sensitivity to the chemical content of the bone, but its application to study bones *in vivo* is limited due to strong optical scattering in tissue. It has been proposed that Raman excitation with photoacoustic detection can successfully address the problem of chemically specific imaging in deep tissue. In this report, the principal possibility of photoacoustic imaging for detecting mineral content is evaluated.

Keywords

Raman scattering; stimulated Raman scattering; photoacoustics; osteoporosis

Introduction

Osteoporosis is a wide spread disease which affects a vast majority of the elderly population [1]. Aging can result in reduced mineral content of bones, making them brittle and more susceptible to damage and deformation [2]. While X-ray diagnostics provides a good way of assessing a bones' mass density [3], which is an indirect measurement of mineral content, repeated exposure to X-ray radiation is not desirable and can lead to ambiguous interpretation of the data.

Conversely, Raman spectroscopy can provide good assessment of the chemical composition within bone, and therefore has potential as a powerful tool for evaluating a bones' structural integrity and to monitor the progression of osteoporosis [4–7]. However, the major obstacle to successful implementation of any optical method for diagnostic imaging is the significant optical scattering within the tissues themselves, which makes the inversion problem of image recovery complicated, or even impossible.

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Over the past two decades, several successful solutions have been implemented to reduce the negative effects of scatter in tissues during optical imaging. The first is diffuse optical tomography [8], which is based on analysis of the signals collected by multiple detectors placed at positions outside the sample of interest. While the solution to this inverse problem requires sophisticated mathematical algorithms, the object reconstruction has been successfully achieved using a number of systems. Another approach has been to utilize time-gated optical imaging, in which only non-scattered photons are collected to provide information about a selected tissue [9]. While both approaches have shown a moderate success, the major drawback is that, in a highly scattered tissue the fraction of photons that do not experience scattering is extremely low. This small fraction of photons useful for imaging results in a substantial reduction of optical signal and a huge background from scattered photons.

An alternative approach is to use photoacoustic imaging. Here, the imaging contrast is provided by a selective optical excitation, while the solution to the inverse problem is assisted through the detection of ultrasonic waves [10]. Ultrasonic waves are scattered much less than photons in soft tissues, and a complete image reconstruction becomes possible either by means of photoacoustic tomography through a set of ultrasound transducers [11–12], or by photoacoustic microscopy utilizing a specially designed confocal ultrasound detector [13–14]. Both systems have demonstrated spectacular results for imaging objects in tissues.

To allow characterization of the chemical content of a bone, which is hidden beneath a layer of scattering tissue, one should also use some sort of vibrational spectroscopy. Infrared spectroscopy is hardly applicable because of a strong absorption in the mid-IR spectral region by most soft tissues. Raman spectroscopy is typically considered to provide weak signal, but given a high density of identical molecules in a volume under investigation, signal detection is not as challenging as that for chemicals in low abundance in tissues containing substantial water content, such as blood. In fact, Morris *et al.*, have recently demonstrated the feasibility of combining the chemical specificity of Raman spectroscopy with object reconstruction in diffuse optical tomography [15], and of a feasibility of time-gated Raman spectroscopy for chemically specific deep-tissue imaging [16].

However, Raman spectroscopy can be also successfully implemented together with photoacoustic imaging [17–18] to achieve both deep-tissue imaging and chemical specificity. In this case, rather than relying on spontaneous Raman scattering, two laser pulses are introduced to excite a specific vibrational level (Fig. 1). The excess of the input energy is transferred to a molecular system. As a result, the local volume is heated and, through a rapid thermal extension, acoustic waves can be generated. In the assumption that all the excess energy is transferred into the energy of ultrasound waves, one can write an energy balance equation and directly evaluate the power of those waves induced by the stimulated Raman excitation process. However, it is often useful to weigh up Raman excitation against more traditional linear photoacoustic. Thus, we will assess the effective nonlinear absorption coefficient to make a direct comparison with linear tissue absorption.

In this work, we first describe the theoretical background of photoacoustic imaging based on the stimulated Raman excitation, and estimate the strength of the signal under typical excitation conditions in non-scattered medium. To understand the bone-specific Raman vibrations, which are important for accessing the progression of osteoporosis, we evaluate the spontaneous Raman signal and its variation due to the change of the chemical content of a bone affected by an acidic solution. Then we study the effect of light scattering on the strength of the nonlinear Raman signal, and propose and demonstrate a novel way to substantially reduce a non-specific background using a time-delayed excitation scheme.

Materials and Methods

Theory

As a result of simultaneous propagation of two frequencies, ω_{pump} and ω_{Stokes} , such that their difference, $\Omega = \omega_{pump} - \omega_{Stokes}$, resonates with a pre-selected Raman transition, the amount of energy absorbed by a molecular system as the result of stimulated Raman excitation is given by [18–19]

$$\Delta E = A \cdot L \cdot \tau_{Stokes} \cdot I_{Stokes}(z, t) \cdot \frac{\Omega}{\omega_{Stokes}} g, \quad (1)$$

where A is the beam's cross-section; L is the propagation distance; τ_{Stokes} , $I_{Stokes}(z, t)$ are the pulse duration and the intensity of the Stokes beam, respectively. g is the Raman gain coefficient [19] given by

$$g = \frac{8\pi^2 N c^2}{\hbar \omega_{pump}^3 \Gamma} \frac{d\sigma}{d\Omega} I_{pump}(z, t), \quad (2)$$

where N is the concentration of molecular species of interest; Γ is the line-width (FWHM) of the Raman line; and $d\sigma/d\Omega$ is a differential Raman cross-section.

Combining Eqs. (1) and (2) results in the effective nonlinear absorption coefficient due to stimulated Raman scattering, β_{SRS} , which has the same units as the linear absorption coefficient but has a physical measure of the efficiency of the energy conversion into vibrational energy:

$$\beta_{SRS} = \frac{\Omega}{\omega_{Stokes}} g. \quad (3)$$

Considering that $\omega_{Stokes} \cong \omega_{pump}$ and the differential Raman cross-section is scaled as the fourth power of the incident frequency, one can clearly see that the efficiency of the energy conversion does not really depend on the excitation wavelength. Conversely, the efficiency of the energy coupling strongly depends on the strength of the Raman transition, which is characterized by its differential cross-section and inverse value of the Raman line-width.

We take the example of the 960 cm^{-1} Raman line of hydroxyapatite [5], which is rather strong ($d\sigma/d\Omega = 10^{-30} \text{ cm}^2 / \text{sr}$ for $\lambda_{pump, Stokes} \cong 1000 \text{ nm}$, and $\Gamma \cong 10 \text{ cm}^{-1}$). Assuming a dense bone structure ($N \cong 1.8 \cdot 10^{21} \text{ cm}^{-3}$), we can evaluate the maximum value of β_{SRS} , which is determined by the maximum allowed incident light intensity (energy density $E \cong 50 \text{ mJ} / \text{cm}^2$ and pulse duration $\tau_{pump} = 5 \text{ ps}$, which is within the ANSI safety standards). The result is $\beta_{SRS} \cong 850 \text{ cm}^{-1}$, which is about two orders of magnitude greater than a typical tissue absorption in the near IR spectral region (800–1200 nm) [20].

One of the important features of stimulated Raman photoacoustics is that the amplitude of the generated acoustic waves will depend linearly upon the local concentration of molecular species of interest, and that the spatial location of those molecules can be recovered using a standard photoacoustic method. However, to make this approach practical, several things have to be satisfied. The amplitude of the acoustic signal should be strong enough to ensure high signal-to-noise detection. Signal detection in optically transparent medium is not problematic, but as light scattering increases there should be a substantial degradation of

amplitude of the signal, especially considering the nonlinear dependence of the resultant signal on the incident pulse intensity. In addition to linear optical scattering, signal attenuation is also a consequence of linear absorption in the tissues, which depends upon wavelength-specific chromophore abundance.

Clearly, the stimulated Raman photoacoustic signal, which is chemically specific to a molecular vibration of a given target molecule, must be detectable above the non-specific background due to linear absorption and scatter. In this report, we address all the above issues in order to make a compelling case for applying stimulated Raman photoacoustic imaging to the diagnosis and progress of osteoporosis in humans.

Traditional Raman imaging

We performed a feasibility study using traditional Raman spectroscopy to evaluate the signal strength and potential markers for stimulated Raman photoacoustic imaging as applied to imaging bone tissue. Our analysis is important because, unlike spontaneous Raman scattering spectroscopy which provides the entire vibrational spectrum for a single wavelength excitation, stimulated Raman scattering excitation is limited to a single vibrational line. The full spectrum acquisition can be a time consuming procedure diminishing the advantages of stimulated Raman photoacoustic imaging.

We used bones from beef ribs, purchased in a local supermarket, as model system for our measurements. They were placed in a vinegar solution, and a series of Raman hyperspectral images were taken at a given location with a time interval of several hours to watch the variation of local chemical distribution. As expected, the acidic environment changed the mineral content of the bone, as evidenced by the reduced phosphate vibration (960 cm^{-1}). This is clearly seen in Fig. 2, where Raman spectra at a single location were taken with a time difference of 24 hours. Both spectra are normalized to the strength of the amide I band, which is associated with a protein content of bone and which is assumed to be unchanged (the later was verified by monitoring the strength of this vibration over an extended period of time over which the strength of the Raman signal for this band did not vary significantly).

The major and the most pronounced difference was in the ratio of peaks at 960 and 1650 cm^{-1} . While this ratio exhibits a rather strong variation along the surface of a bone (see Fig. 3, on the left) and has a different rate of decrease as a result of exposure to vinegar (see Fig. 3, on the right), it provides a good measure of chemical and structural integrity of the bone. We also note that the Raman signal is rather strong, and for a typical incident power of 5 mW ($\lambda=532\text{ nm}$) focused into $1\text{-}\mu\text{m}$ spot on the bone surface, we get high signal-to-noise signal with 1-s exposure on a CCD detector (Jobin Yvon, Inc.) attached to a spectrograph (Jobin Yvon, Inc.). Strong Raman signal is essential for stimulated Raman photoacoustics, and the ability to get vital information from a ratio of just two measurements at distinct Raman peaks greatly simplifies the task.

Nonlinear Raman imaging with optical detection

Coherent anti-Stokes Raman scattering (CARS) spectroscopy is often employed to substantially increase the strength of the Raman signal, when there is a large number of highly Raman scattering molecules in the focal volume (see, for example, [22–24]).

We used the experimental set-up, which is described in detail earlier [23–24], and put a bone sample on a translational stage that could be moved in all three dimensions. A layer of chicken skin (approximate thickness of 2-mm) was placed on top of the bone to mimic *in vivo* imaging conditions. While taking the full CARS spectrum, we only display here the ratio of Raman peaks at 960 and 1650 cm^{-1} (see Figure 4). The amplitude of the CARS signal was found to be decaying quickly with respect to imaging depth, and at the depth of

about 10 μm into the bone, we observed essentially no signal above the background noise (this area is shown in dashed line in Figure 4). One of the problems of CARS microspectroscopy is the third-power signal dependence on the excitation power, and that it requires phase-matching. Strong light scattering in bone tissue results in substantial degradation of the signal, similar to the one observed earlier in a model system [25]. This provided us with an additional motivation to explore stimulated Raman scattering with ultrasound detection, because it does not require phase-matching conditions and has only quadratic power dependence.

Results and Discussion

Laser system for stimulated Raman photoexcitation

The complexity of stimulated Raman scattering imaging comes from the necessity of having two synchronized light sources exciting a vibrational transition of a given molecule. Traditionally it is achieved by having an optical parametric or dye amplifier, which allows tunability of one beam with respect to the other beam. However, such an approach is often associated with difficulty in controlling the bandwidth of the amplified pulse and in adjusting the wavelength differences between the two beams in order to match the vibrational frequency of the desired molecular transition. Therefore, we propose to simplify our approach by utilizing strong Raman transitions known to be present in our target tissues, such CH_2 vibrations in lipids (at around 2850 cm^{-1}) and phosphate (PO_4) vibration in bones and microcalcifications (at around 960 cm^{-1}). To attain those frequencies, one can make use of stimulated Raman process in a medium having a vibrational frequency that is the same or close to the frequency of the desired molecular transition. For example, to interrogate selective excitation of lipids one can use mineral oil, a widely available substance whose high-frequency vibrational spectrum provides a good match for a typical lipid. Phosphate vibration can be matched fairly well with a sulfate solution, which can be prepared at very high concentrations.

A schematic of the experimental set-up is shown in Fig. 5. In brief, a picosecond Nd:YAG laser (GCR-3RA, Spectra Physics, Inc.) generated pulses as strong as several mJ with a pulse duration of 40 ps at 532 nm (the second harmonic of the fundamental radiation). As a first example, we used a mineral oil placed in a 40-mm long quartz cell (Helma Cells, Inc). The incident laser radiation was focused into the cell using a 20-cm focusing lens, and the generated Stokes radiation was separated from the pump radiation using a dichroic mirror and an absorbing filter. We observed a linear rise of the Stokes radiation above the threshold pump energy of 50 μJ with a slope efficiency of about 20%. At the pump energy of about 3 times the threshold energy, the output pulse energy fluctuations (10% peak-to-peak) are similar to those of the pump laser with no observable spectral shape variations.

Very similar results were observed when the mineral oil was replaced with a saturated solution ($\sim 250\text{ g/L}$, or about 2M) of magnesium sulfate (MgSO_4). The major difference was the color of the generated Stokes beam, which was shifted from about 630 nm (corresponding to 2950 cm^{-1} vibrational frequency shift with respect to the pump 532-nm radiation) in the case of mineral oil, to about 560 nm (corresponding to 980 cm^{-1} vibrational frequency shift) in the case of magnesium sulfate. The later Stokes shift is somewhat offset from the targeted 960 cm^{-1} Raman line of hydroxyapatite, the major inorganic component of bone, although it has roughly the same signal strength and can be used as a model system to study stimulated Raman photoacoustic signal formation in bone tissues.

Stimulated Raman photoexcitation

The pump (532 nm) and the Stokes (at around 630 nm for mineral oil and at around 560 nm for magnesium sulfate solution) were combined using a dichroic beamsplitter. The combined beam was focused using a 10-cm focal length lens into another cell, which contained the same liquid as in the Raman cell used for generation of the Stokes pulse. A translational stage was used to adjust the time delay between two pulses to ensure their simultaneous arrival on the sample.

A needle hydrophone (HNC1500 and AH-2010-20-025, Onda, Inc.) was used to detect the ultrasonic waves generated in liquid by placing its tip approximately 1 cm away from the line of the incident beam propagation. The position along this line was chosen by maximizing the photoacoustic signal amplitude while translating the detector along the beam propagation. The acoustic signal, after being converted to an electrical signal, was displayed on a fast-digitizing oscilloscope (TDS3054B, Tektronix, Inc.), and the peak amplitude of the generated wavefront was used for quantifying the amplitude of the acoustic wave. We realize the existence of a bandwidth mismatch of our detector (20 MHz) and the generated acoustic wave-front (25 GHz, which corresponds to the inverse pulse duration of the excitation pulse). By using a higher-frequency response detector, a much stronger signal can be recorded, thus improving the sensitivity of all the measurements described below. Alternatively, a multiple-pulse excitation sequence could be used to enhance the generated photoacoustic signal [21]. Nevertheless, even the use of this off-the-shelf ultrasonic transducer provided us with enough sensitivity to characterize the photoacoustic signals generated here.

We first established that the detected signal was indeed the result of the acoustic wave propagating in solution. By varying the position of the hydrophone with respect to the focal point, we confirmed that the arrival time of the signal was equal to the distance between the focal point divided by the speed of sound in the medium. Next, we confirmed that the detected photoacoustic signal originated from the presence of two beams in the sample. Either by misalignment of the combined beams or by blocking either of the beams, we observed the complete disappearance of the photoacoustic signal, manifesting the combined action of two pulses on the medium. When the neutral density filters were placed in front of the sample, the signal's amplitude was dramatically attenuated, as shown in Figure 6. The dashed line indicates a quadratic dependence, which is in an excellent agreement with Eqs. (1–2). By moving the position of a translational stage we were able to adjust the time delay of the pump beam with respect to the Stokes beam. It was expected that at large delay times, the signal should disappear since stimulated Raman excitation occurs (see Figure 1) only when the two pulses are simultaneously present. The results of these time-delayed experiment are shown in Figure 7, where the photoacoustic signal (red dots) is shown as a function of a time delay between two pulses together with the sum-frequency signal generated in a nonlinear crystal (BBO, Type I), when the later was placed in the place of a sample cell. Since both the stimulated Raman excitation and the sum-frequency generation are two-photon processes, the shapes of those two temporal cross-correlations are about the same. We also note that at the peak of the signal ($t=0$), the generated photoacoustic signal is more than two orders of magnitude greater than the signal at very large delays, which was thought to be attributed to the noise of the detection system. Finally, when the sample is replaced with some other solution, which does not have the same vibrational lines, as the liquid used in the Raman cell, the signal completely disappeared, indicating the chemical specificity of the excitation.

The effect of scattering

Our results demonstrate an ability to effectively generate photoacoustic signals in optically transparent media. However, biological tissues are characterized by significant light scattering, which will certainly affect the measurements because light scattering will substantially decrease the intensity of both the pump and Stokes beams, resulting in a magnified reduction of the photoacoustic signal, as indicated in Figure 4. To understand the limitations introduced by the light scattering, we performed a simple experiment in which stimulated Raman photoexcitation was used to generate a photoacoustic wave in a clear solution of magnesium sulfate. We positioned the hydrophone approximately 2 cm inside the cell and started to gradually increase the scattering by adding a concentrated solution of Intralipid to mimic the scattering properties of a real tissue [26]. As expected, the amplitude of the signal gradually decreased (see Figure 8), and at about 0.2% of the Intralipid concentration, the signal almost reached the noise level, indicating the detection limit using the available ultrasound transducer.

It is evident that the sensitivity of our ultrasound transducer is the limiting factor in those measurements, and we made an attempt to compare ultrasound detection with optical detection. In this experiment, we simultaneously measured both the Stokes output on the exit of the Raman cell filled with a mineral oil and the photoacoustic signal generated as a result of this interaction [27]. We found that the sensitivity of photoacoustic measurements was about the same as the sensitivity of the energy meter (EPM1000 with J4-09, Coherent, Inc.) used for those measurements. It corresponds to about the approximate minimum limit of about 1 nJ of the deposited energy, which could be measured using the currently available hydrophone. This is well above the single-photon sensitivity which is currently used routinely for optical detection. One way to improve the sensitivity of ultrasound detection is to combine the action of a series of picosecond laser pulses arriving at the target before the acoustic wave leaves the excitation volume [21]. Such an approach potentially allows as much as 3 orders of magnitude increase of the signal amplitude without sacrificing the safety limits for high-intensity laser exposure. To attain even higher sensitivity, one must significantly improve the current high-frequency ultrasound transducers, and there are several promising ongoing efforts which should lead to a dramatic improvement in the performance of the next generation of transducer [28–30].

Conclusion

In conclusion, we introduced the stimulated Raman photoacoustic imaging technique, which combines the chemical specificity of Raman spectroscopy with the deep tissue optical imaging attainable through photoacoustic detection. The proposed technique is ideally suitable for assessing the mineral content of bone tissues. The amplitude of the signal is sufficient to collect high resolution photoacoustic images through several millimeters of scattering tissue, and the proposed way of temporally discriminating against the non-specific background provides sufficient contrast for imaging both the mineral and protein content of a deep-laying bones.

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Figure 1.
Schematic energy diagram for stimulated Raman excitation.

Figure 2. Raman spectra of a selected bone area before (solid black line) and after (dashed red line) exposure to an acidic solution. The fluorescence background was digitally subtracted and spectra were normalized to the signal intensity of amide I band.

Figure 3.

The ratio of intensities of Raman lines at 960 and 1650 cm^{-1} for a selected region of a beef bone before (on the left) and after (on the right) exposure to acidic solution.

Figure 4.
The ratio of intensities of CARS lines at 960 and 1650 cm⁻¹ for a selected three-dimensional region of a beef bone.

Figure 5.

Experimental optical set-up for stimulated Raman photoacoustic measurements. Raman cell is indicated by a quartz cell filled with a liquid, which generates the Stokes pulse.

Figure 6. The stimulated Raman photoacoustic signal as a function of the incident input power (pump and Stokes combined). The dashed line is a guide to an eye and shows quadratic power dependence.

Figure 7. Stimulated Raman photoacoustic signal (red dots) and sum-frequency generation signal (blue squares) as a function of the time delay between the pump and Stokes pulses. Dashed red line and solid blue line show fits with the Gaussian function.

Figure 8. Stimulated Raman photoacoustic (SRPA) signal as a function of Intralipid concentration in the solution.