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Resonance Raman Imagery of Semi-Fossilized Soft Tissues

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ABSTRACT

The discovery of soft structures in dinosaur bone with the morphological and molecular characteristics of blood vessels in extant vertebrates was both surprising and controversial. Mounting evidence suggests that these soft tissues are blood vessels, their preservation driven in part by reactive oxygen species derived from hemoglobin degradation. More data are needed to support this hypothesis. Raman spectroscopy, and resonance Raman in particular, can provide detailed information as to the chemical makeup of these samples. We used two different excitation wavelengths in microscale Raman measurements to look for lines characteristic of degraded heme molecules, both in ancient vessels and modern analogues taken from semi-fossilized, hemoglobin-soaked ostrich bones. In both samples, we observed two regimes: dark colored, stiff regions and more transparent, elastic regions. We discovered that the two apparent regimes in the samples had different strengths of Raman returns, and that resonance effects greatly affected the Raman intensity. In all cases, there was some evidence of degraded heme spectra, though the increased returns indicated that the dark regimes had reacted more strongly with the heme specie. The modern vessels displayed a resonance Raman intensity consistent with hemoglobin molecular structures, which indicated resonance spectra would provide understanding of the ancient heme molecule. To investigate the two regimes more thoroughly, we acquired Raman spectra over areas where the sample transitioned from one regime to another. Variable wavelength resonance Raman measurements over the whole sample were used to give more information about the heme species present, in both ancient and modern samples.

Keywords: Resonance Raman, hemoglobin, fossils, soft tissue

1. INTRODUCTION

Tissues are complicated from the chemical point of view, so the measurement of the materials present and how they are associated with each other is difficult. Raman spectroscopy can help to simplify analysis since each type of molecule has a 'fingerprint' Raman spectrum, since the vibration energies are distinct [1–8]. While the vibrations typically measured with Raman in the few hundreds to few thousands of wavenumbers generally correspond to functional groups, the structure of the overall molecule will often inject or extract charge form the functional group, resulting in an increase or decrease in the vibrational energy, respectively. These changes help make the fingerprints more unique. The whole-molecule vibrations tend to be slow, due to the size and hence lower restoring forces, so have lower energies. Although the Raman spectra can help with identification, its resolution is limited and the plethora of constituents can merge peaks and thus still make identification difficult. We therefore employ resonance Raman as well. When the wavelength of the light used to excite the Raman spectrum is tuned near an absorption feature of the material being studied, then the Raman signal becomes stronger [9–17]. Normally, this occurs over a wide wavelength range. On absorptions that are phonon-allowed (symmetry forbidden), we have found that the wavelength range for increasing Raman signal is very narrow, and the signal level increase over this narrow band is very large; this has been observed in benzene, toluene, and cytosine [14,18–20]. An extreme example is the observation, with a narrow line (1 wavenumber) DUV tunable laser, of a 3500-fold increase of the Raman signal as the laser was tuned by 0.01 nm (1 cm^{-1}) [21]. Since only the vibrations associated with that particular molecule are Raman-enhanced, it provides double-selectivity in identifying the molecule and the vibrations associated with it. Not all vibrations in a molecule show an increase in Raman signal at resonance. Typically, only those related to the bond being broken by the absorption will be impacted [22]. Thus careful choice of

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molecular absorption and vibrations monitored is required.

In this work, we used fixed wavelength lasers, so the broad enhancement of a symmetry-allowed absorption is appropriate. Further, we would like to investigate how much of the molecules remain in the ancient samples, and what sort of other chemical changes may have occurred on those molecules. Thus, we need to choose 'on' and 'off' resonance wavelengths for hemoglobin molecular absorption, and we would like to be able to discriminate other possible molecules that might be present. Data from reference samples and from modern analogues will provide a basis for comparison. The Raman spectra will be compared to see if there are substantive changes to the molecule even if it still resonates at the same laser frequency.

2. CHOICE OF EXCITATION WAVELENGTHS FOR RESONANCE

Figure 1 compares the absorption spectra of hemoglobin and heme, the two moieties that we would like to be able to discriminate. We find that the choice of a 473 nm laser and a 532 nm laser, both readily available, is sufficient. The hemoglobin molecule should show a Raman resonance in the green (532 nm) but not in the blue (473 nm), whereas the case for heme should be reversed.

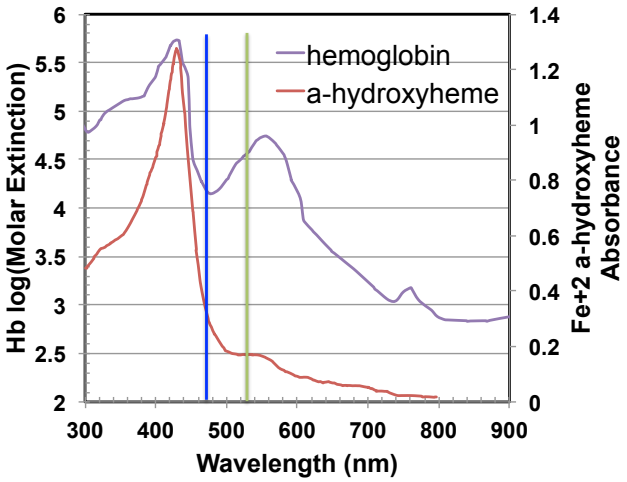

Figure 1. The absorption spectra of hemoglobin from Ref. [23] and heme from Ref. [24] are compared to the two laser wavelengths used so that resonance conditions can be evaluated.

Before we can compare the enhanced Raman spectra, there are two other factors that influence the data that we need to discuss. The first is the natural excitation-frequency-to-the-fourth-power dependence of the Raman signal. This arises from a dipole absorption and emission (square of frequency each), but needs to be normalized for before comparing Raman signal levels at different frequencies. The second factor of importance is fluorescence. Generally, higher frequencies (energies) cause more fluorescence than lower ones; so shorter wavelengths are more likely to have a larger fluorescence background under the Raman spectra than longer wavelengths. This stops being true as the deep-UV is reached [16]. In our case, it means that a background rising to the right, larger delta-wavenumbers, or lower detected Raman photon energy will be expected for the 473 nm excitation, but not as significant for the 532 nm excitation.

3. VESSEL PREPARATION

When working with potentially ancient samples, it is desirable to obtain a modern simulant to base conclusions from. For this purpose, cortical bone fragments from ostrich femur were demineralized in 500 mM EDTA until all mineral was removed, After demineralization, ~1 mm thick slices were cut and then washed 10-20 times with e-pure water to remove EDTA before collagenase digestion. Slices were then re-suspended in 2 ml of 1mg/ml collagenase A (Roche) in Dulbucco's phosphate buffered saline and allowed to digest overnight at 37 °C. The remaining vessels were washed with E-pure water 4 times to remove residual collagenase. To fully simulate ancient preservation, these modern vessels were then immersed in different environments: deoxygenated Hb in 2ml micro centrifuge tube under deoxygenated environment, and oxygenated water under same condition as control.

Our ancient samples were prepared in a laboratory dedicated to analyses of fossil tissues, exclusive of any extant material. Cortical bone fragments from the B. canadensis femur were demineralized in 500 mM ethylenediaminetetraacetic acid (EDTA) pH 8.0 for two weeks. We then isolated and collected the blood vessels from the demineralized bone fragments. We washed blood vessels with E-pure water 10 times to completely remove EDTA and stored them in water to complete preparation for further Raman analysis.

4. REFERENCE SPECTRA

Since we would like to be able to identify Raman from blood and particularly hemoglobin as the material that is resonating, we need to identify which Raman modes of hemoglobin have larger signals when they resonate on the absorption that 532 nm excitation is close to. Raman spectra of blood samples have been previously reported [25]. They identify the largest Raman peaks as belonging to hemoglobin when the near-resonant 532 nm is used as excitation. The fact that the hemoglobin dominates the spectrum is not surprising since resonance Raman can give increases in Raman signal levels by hundreds to thousands, as noted above. The Raman spectrum is shown in Fig. 2. Resonance gain is found over a wide range of delta-wavenumbers, for a large number of vibration modes. This is not unexpected, since the absorption region lies in the porphorin-like polymer structure that holds the iron atom, which that is central to the molecule, thus changes there effect vibrations throughout the molecule. We see the regions near ~1350 and 1600 cm^{-1} are dominant peaks. The much broader response of the non-resonant Raman indicates the larger number of vibration energies present on the range of molecules in the blood, when none are particularly enhanced over the others. Raman cross sections do differ between molecules and vibrations on molecules, of course, but still this illustrates the complications of using just a single-selectivity (vibration fingerprint) in complex samples. These spectra have not been corrected for the fourth power of the excitation frequency; doing so would increase the Raman from the 632 nm excitation by a factor of two, but it would still be weaker and not show the hemoglobin features.

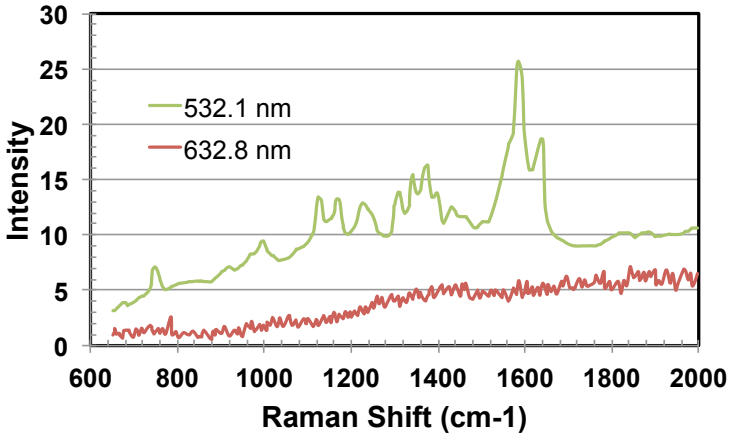

Figure 2. Raman spectra of blood on and off resonance after [25].

As another reference, we also acquired Raman spectra of blood samples on the wavelengths that we use in this experiment, Fig. 3. The results are similar: the on-resonance Raman spectra exhibits several more narrow vibration peaks and it is in general stronger. The increasing background due to fluorescence from the higher energy, non-resonant, 473 nm excitation is clearly evident in that data. If we compare to the non-resonant data in Fig. 2, at 632 nm, we find much less structure in the spectrum with the lower energy excitation (632 nm). This is consistent with lower frequencies being closer to some resonance. In particular, we see the absorption of hemoglobin ate 473 nm is much higher than at 632 nm, so there is probably some resonance in the 473 nm excitation Raman spectra. Since it is further from an absorption peak than the 532 nm excitation, the resonance is much weaker, so the peaks, although defined above those of other materials, are still quite small.

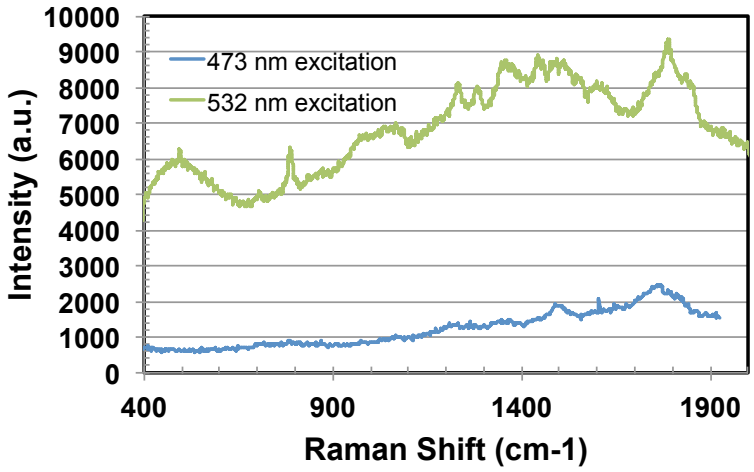

Figure 3. Micro-Raman spectra of blood using excitation energies of 473 nm (off-resonance) and 532 nm (on resonance for hemoglobin). The spectra have been corrected for power, but the background has not been subtracted.

5. OBSERVATION OF RESONANCE

In this section we will show that resonance is observed in the Raman signal from ancient vessel, fragments, which strongly suggests that a significant portion of the hemoglobin molecule is still intact. The Raman spectral features have changed, however, showing that the molecule has undergone some modification. Figure 4 contains Raman spectra of a particular region of what appears to be blood vessel remains of a brachylophosaurus. The on-resonance spectrum is somewhat familiar to that of Fig. 3, although clearly modified. It also has much higher signal levels than the off-resonance spectrum with 473 nm excitation, indicating that the parts of the molecule that are absorbing near 532 nm are still intact.

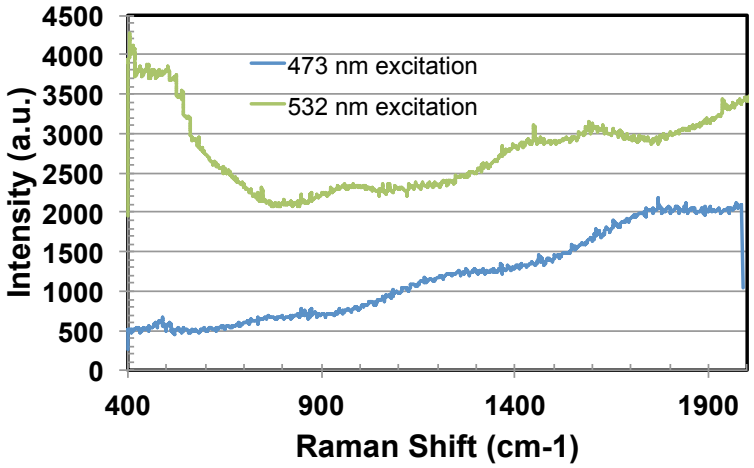

Figure 4. Micro-Raman spectra of a vessel-like region of a demineralized brachylophosaurus bone using excitation energies of 473 nm (off-resonance) and 532 nm (on resonance for hemoglobin). The spectra have been corrected for power, but the background has not been subtracted.

6. CONCLUSIONS

We have shown that the use of resonance Raman in complex biological materials provides a double-selectivity for the molecules of interest, which can simplify the analysis so that conclusions can be made about the presence of certain absorbing structures in the system. Comparison between on and off resonance spectra is useful. In our case, we demonstrate that enough of the hemoglobin molecule remains in soft tissue recovered from demineralized bone of ancient fossils that absorption still takes place and the enhanced Raman spectra indicates some similar features, and some modifications to the molecule.

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