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Using 2D Correlation Analysis to Enhance Spectral Information Available from Highly Spatially Resolved AFM-IR Spectra

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Abstract

The recent combination of atomic force microscopy and infrared spectroscopy (AFM-IR) has led to the ability to obtain IR spectra with nanoscale spatial resolution, nearly two orders-of-magnitude better than conventional Fourier transform infrared (FT-IR) microspectroscopy. This advanced methodology can lead to significantly sharper spectral features than are typically seen in conventional IR spectra of inhomogeneous materials, where a wider range of molecular environments are coaveraged by the larger sample cross section being probed. In this work, two-dimensional (2D) correlation analysis is used to examine position sensitive spectral variations in datasets of closely spaced AFM-IR spectra. This analysis can reveal new key insights, providing a better understanding of the new spectral information that was previously hidden under broader overlapped spectral features. Two examples of the utility of this new approach are presented. Two-dimensional correlation analysis of a set of AFM-IR spectra were collected at 200-nm increments along a line through a nucleation site generated by remelting a small spot on a thin film of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate). There are two different crystalline carbonyl band components near 1720 cm⁻¹ that sequentially disappear before a band at 1740 cm⁻¹ due to more disordered material appears. In the second example, 2D correlation analysis of a series of AFM-IR spectra spaced every 1 micrometer of a thin cross section of a bone sample measured outward from an osteon center of bone growth. There are many changes in the amide I and phosphate band contours, suggesting changes in the bone structure are occurring as the bone matures.

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Keywords

atomic force microscopy; infrared spectroscopy; two-dimensional correlation analysis; poly(hydroxyalkanoate); osteonal bone; bone nanostructure

1. Introduction

Two-dimensional correlation spectroscopy (2DCOS) has been applied to many different types of data sets in an effort to help accentuate small spectral variation occurring as a result of some perturbation, such as temperature, pressure, pH, spatial sequence, etc. [1]. In the case where infrared (IR) spectroscopy is the measurement technique used, the correlation analysis performed at each pair of wavenumbers in the dataset serves to emphasize the variation between IR bands and within individual band contours. Condensed phase IR spectral line broadening typically occurs, because there is a wide distribution of molecular environments within the sample, each of which can result in a slight peak wavenumber shift. When IR spectra are collected from sample volumes over 100-times smaller traditional IR measurements, it stands to reason that fewer molecular states may be sampled, and the spectra could sharpen as a result. In this work, narrowly spaced (1- μm or less apart) AFM-IR spectra have been collected along a line through a specific area of interest in the sample. In the case of the microdomain-forming semicrystalline biodegradable polymer poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) or P(HB-*co*-HHx), the spectra were collected at 200-nm increments moving away from a small location where the sample was remelted.

In a second application, 2D correlation analysis of closely spaced AFM-IR data of bone tissue cross sections are examined. Osteons, representing the newly remodeled cortical bone, are formed by resorption of existing bone coupled with circumferential apposition of mineralized collagen fiber lamellae surrounding a Haversian canal. These tree ringlike structures with the newest mineralized layers deposited nearest to the blood vessel canal (osteonal center) present a natural gradient in tissue age, from the center (youngest tissue) to the periphery (oldest tissue). In this study, AFM-IR spectra were collected at 1-micrometer increments moving away from an osteon center of bone growth. Variation in the line of sequential spectra was then displayed as 2D correlation maps.

2. Experimental

The instrumentation used to collect the spatially resolved AFM-IR spectra has been described in detail elsewhere [2–9]. A film of poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) or P(HB-*co*-HHx) was solution cast from chloroform onto a zinc selenide (ZnSe) prism. The sample studied had a weight averaged molecular weight of 624,000 and contained 7.6 mol% of the 3-hydroxyhexanoate co-monomer. The sample was then melted (heated to 160 °C for one hour) and annealed at 90 °C overnight to generate crystalline lamellae. The film thickness was determined to be 450 nm by AFM, resulting in an estimated spatial resolution for the IR spectra measured of about 100 nm. A nucleation site for polymer recrystallization was generated by bringing a self-heated AFM tip held at a temperature of 350 °C to a distance of 3 μm from a specific location on the sample for 90 s and then withdrawing it. The sample film was allowed to equilibrate thermally with the

sampling system prior to commencement of the measurements, which were performed using a nanoIR™ AFM-IR instrument (Anasys Instruments, Santa Barbara, CA). More detail regarding AFM-IR data collection parameters used to analyze this sample has been reported previously [5,7].

The bone sample studied was taken from a 13-year-old baboon femur. The animal was from the colony at the Southwest National Primate Research Center/Southwest Foundation for Biomedical Research (SNPRC/SFBR, San Antonio, TX), and all procedures during its life at SNPRC/SFBR were approved by the Institutional Animal Care and Use Committee in accordance with established guidelines. The details of bone preparation have been reviewed elsewhere [10]. Briefly, femurs were collected during routine necropsies, wrapped in saline-soaked gauze and maintained in frozen storage at -20°C . The bones were initially fixed with 80% ethanol, slowly dehydrated through a series of increasing concentrations of ethanol, cleared with xylene, and finally infiltrated and embedded in polymethylmethacrylate (PMMA) using the Erben method [11]. A $0.5\text{-}\mu\text{m}$ -thick section was cut horizontally from the embedded undecalcified bone block on a Reichert_Jung Ultracut E ultramicrotome (Vienna, Austria) equipped with a diamond knife (Diatome Ltd., Bienne, Switzerland). The section was then transferred onto a ZnSe prism and mounted onto the sample stage of the same AFM-IR instrument. All data were obtained in contact mode with an Arrow cantilever (Arrow-FM, NanoWorld, Switzerland). The tunable IR laser produced laser pulses of 10 ns at a repetition rate of 1 KHz, with a spectral resolution of better than 8 cm^{-1} over the whole tuning range. The IR power levels incident on the ZnSe was about 0.5 mW, and the focused laser spot size was about $50\text{ }\mu\text{m}$. The AFM-IR spectra were collected from 900 to 1800 cm^{-1} , with a data point spacing of 4 cm^{-1} . A total of 128 cantilever ringdowns [7] were coaveraged for each data point.

3. Results and Discussion

3.1 Poly(3-hydroxybutyrate-co-3-hydroxyhexanoate)

Figure 1 shows an AFM topography image and a series of 13 AFM-IR spectra of P(HB-co-HHx) collected every 200 nm as a function of distance from a nucleation site produced by a heated AFM tip, as described above [5,7]. The arrows on the spectra indicate wavenumbers where the intensity is either increasing or decreasing as a function of distance from position 24. This spot is the most crystalline location in the sample, as determined from the relative intensity of the 1720 cm^{-1} carbonyl stretching band. Figure 2 shows the corresponding synchronous and asynchronous 2D correlation maps produced from the spectra of P(HB-co-HHx) shown in Fig. 1 from positions 24–36. There is clearly a lot of spectral variation apparent as a function of spatial location, much of which would not be observable in a diffraction-limited dataset generated using conventional FT-IR microspectroscopy.

Figure 3 shows a blow up of the synchronous and asynchronous 2D maps of Fig. 2 highlighting the carbonyl stretching vibration region. From these 2D maps, it is clear that there are three main carbonyl stretching bands in this region of the spectrum: two relatively sharp bands that are split centered at 1720 cm^{-1} due to crystalline-like structures; and a broader band due to more disordered structures at 1740 cm^{-1} . The intense autpeak in the synchronous spectrum at 1720 cm^{-1} indicates that most of the spectral changes in the dataset

arise from the decrease in intensity of crystalline contribution (i.e., crystallinity reduction and thinning). Some increase in amorphous component was also detected as negative synchronous cross peaks. There are two distinct crystalline contributions (i.e., highly ordered primary crystals and less ordered secondary ones formed between lamellae) resulting in the appearance of asynchronous cross peaks. Signs of cross peaks indicate the highly ordered crystals disappear first followed by the decrease of less ordered ones, as the AFM probe tip moves away from the most crystalline point of the AFM IR map (position 24). These results suggest the following mechanism of morphological evolution. Initially, dense highly ordered crystal lamellae were formed from the spot which is now located at the probe position 24. As the lamellae grew outward toward position 36, less ordered secondary crystal were formed in the interstitial spacing between sparse lamellae. The population of both primary and secondary crystals decreases, which is accompanied by the increase in amorphous component.

Figure 4 shows a blow up of the synchronous and asynchronous 2D maps of Fig. 2 highlighting the C-O-C backbone stretching vibration region of the P(HB-co-HHx) IR spectrum. The spectral changes observed in this region are much more dramatic and more difficult to unambiguously interpret at the molecular level. It is clear, however, that the band centered at 1276 cm^{-1} increases in intensity as a function of distance from the point of highest crystallinity.

3.2 Bone osteon

Figure 5 shows an AFM image of a cortical bone cross section in the region of an osteon. The center of the osteon where the haversian canal is located is at the left edge of the image. The locations where 55 AFM-IR spectra were collected with $1\text{-}\mu\text{m}$ spacing are shown in the image. Also shown is a graph generated from ratioing the peak heights of the 1044 cm^{-1} phosphate band and the 1660 cm^{-1} amide I band in the full AFM-IR spectra. The plot shows that the mineral-to-matrix ratio increases as a function of distance from the osteon center where new bone is deposited.

Figure 6 shows an overlay of all the individual $1\text{-}\mu\text{m}$ -spaced AFM-IR spectra collected from the locations indicated in Fig. 5. Note that the spectra are scaled to all be the same intensity at 1014 cm^{-1} . The reason for doing this rescaling procedure is that the overall intensity of an AFM-IR spectral intensity depends not only on the amount of IR radiation absorbed at each wavenumber, but also on the thermal conductivity and mechanical stiffness of the region of the sample being interrogated by the AFM tip. As part of the bone sample preparation procedure, the center of the osteon (which appears at the left hand edge of the AFM image shown in Fig. 5) consists entirely of the PMMA embedding material. Since PMMA is much stiffer than the surrounding bone material, it gives a larger IR spectral response. Thus, any 2D IR map generated from and an unscaled set of these spectral data would be dominated by the PMMA signal. By forcing all the spectra to have the same intensity at one wavenumber in the phosphate stretching band region, we are better able to focus the analysis on spectral changes within the bone regions of interest which extend along a radius originating from the osteon center. By doing this data pretreatment, however, we will not observe any synchronous or asynchronous 2D IR peaks at 1014 cm^{-1} .

Figure 7 shows the overall synchronous and asynchronous 2D correlation maps produced from the bone cross-section spectra shown as scaled in Fig. 6. These maps clearly show that there is a lot of spectral variation occurring within this dataset. In particular, the strong negative crosspeaks showing up in the asynchronous 2D correlation map between the phosphate bands around 1040 cm^{-1} and the amide I and II protein bands at 1660 and 1585 cm^{-1} indicate that the mineral content is increasing as you move further from the osteon center of bone growth.

Figure 8 shows a blow up of the synchronous and asynchronous 2D maps of Fig. 7 in the amide I stretching vibration region. There appear to be at least five different contributions to the amide I band intensity contour likely due to different protein secondary structures in the matrix component of the bone. Figure 9 shows a blow up of the synchronous and asynchronous 2D maps of Fig. 7 in the phosphate stretching vibration region due to the bone mineral component. Once again, there are clearly a lot of differences in the bone mineral spectrum as a function of spatial position. Closer examination of these spectra does not suggest any sequential changes beyond the gradual increase in the mineral-to-matrix ratio already noted. It appears there may be important spectral changes occurring on a much finer spatial scale than we are able to pick up with such a broad (1 micrometer) spacing of spectral data points. Studies with a much finer spacing of AFM-IR spectra have already been initiated to test this hypothesis.

4. Conclusion

Two-dimensional correlation analysis of highly spatially resolved AFM-IR spectra looks to be a promising methodology for sorting out and understanding spectral variations arising from different molecular environments occurring at sub-micrometer spatial scales. In the case of the microdomain-forming semicrystalline biodegradable polymer poly(3-hydroxybutyrate-co-3-hydroxyhexanoate), two different crystalline carbonyl band components near 1720 cm^{-1} sequentially disappear before a band at 1740 cm^{-1} , due to more disordered material, appears. In the second example, 2D correlation analysis of a series of AFM-IR spectra of a thin cross section of bone measured outward from an osteon center of bone growth shows that there are many changes in the amide I and phosphate band contours occurring as the bone matures.

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Highlights

- 2D correlation analysis is applied to two sets of closely spaced AFM-IR data
- A PHB copolymer has three separate carbonyl band components that change sequentially
- A bone sample exhibits many changes in the amide I and phosphate band contours

Figure 1.

AFM image of an annealed film of P(HB-co-HHx) (top) showing the locations where AFM-IR spectra (the last 13 of which are shown) were collected (bottom). The dark area near the middle of the image is an indentation created when the sample film was remelted by a heated AFM tip at that location. The arrows on the spectra indicate whether the intensity of the band at that wavenumber is increasing or decreasing as a function of distance from position 24, the most crystalline location.

Figure 2. Synchronous and asynchronous 2D correlation maps produced from the spectra of P(HB-co-HHx) shown in Fig. 1 from positions 24–36.

Figure 3.
Blow up of the synchronous and asynchronous 2D maps of Fig. 2 showing the carbonyl stretching vibration region.

Figure 4.
Blow up of the synchronous and asynchronous 3D maps of Fig. 2 showing the C-O-C backbone stretching vibration region.

Figure 5.

AFM image (top) of a cortical bone cross section in the region of an osteon. The center of the osteon where the haversian canal is located is at the left edge of the image. The locations where 55 AFM-IR spectra were collected with 1-μm spacing are shown in the image. A graph generated from ratioing the peak heights of the 1044 cm⁻¹ the phosphate band and the 1660 cm⁻¹ amide I band in AFM-IR spectra (bottom).

Figure 6.
Overlay of the 55 1- μm -spaced AFM-IR spectra taken from the locations shown in Fig. 5.
The spectra are all scaled to have the same intensity at 1014 cm^{-1} .

Figure 7.
Synchronous and asynchronous 2D correlation maps produced from the bone cross-section spectra shown in Fig. 6.

Figure 8.

Blow up of the synchronous and asynchronous 2D maps of Fig. 7 showing the amide I stretching vibration region due to the protein matrix of the bone.

Figure 9.

Blow up of the synchronous and asynchronous 2D maps of Fig. 7 showing the phosphate stretching vibration region due to the bone mineral.