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Atomic force microscope infrared spectroscopy of griseofulvin nanocrystals

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Abstract

The goal of this work was to evaluate the ability of photothermal induced resonance (PTIR) to measure the local infrared absorption spectra of crystalline organic drug nanoparticles embedded within solid matrices. Herein, the first reports of the chemical characterization of sub-100 nm organic crystals are described; infrared spectra of 90 nm griseofulvin particles were obtained, confirming the chemical resolution of PTIR beyond the diffraction limit. Additionally, particle size distributions via dynamic light scattering and PTIR image analysis were found to be similar, suggesting that the PTIR measurements are not significantly affected by inhomogeneous infrared absorptivity of this system. Thus as medical applications increasingly emphasize localized drug delivery via micro/nano-engineered structures, PTIR can be used to unambiguously chemically characterize drug formulations at these length scales.

Keywords

atomic force microscopy; AFM; AFM-IR; drug delivery; griseofulvin; photothermal induced resonance; PTIR

INTRODUCTION

Drug-polymer composites, such as the coatings of drug-eluting stents¹ and nanoparticulate formulations,^{2,3} are of widespread interest because the drug's release kinetics and bioavailability are controlled through proper polymeric selection and composite microstructure. To effectively engineer drug delivery systems, a fundamental understanding

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CONFLICT OF INTEREST

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of drug structure and distribution in the dosage form is required.⁴⁻⁷ This understanding necessitates methods to unambiguously chemically characterize drug formulations on the micro- and nanoscales.

Fourier transform infrared (FTIR) spectroscopy provides a wealth of chemical and structural information and is frequently used to characterize drug-polymer composites.^{4,8} However, nanoscale phenomena cannot be discerned with conventional FTIR spectroscopy because the spatial resolution is restricted to 3–30 μm by the optical diffraction limit.⁹⁻¹² In contrast, assumptions concerning the nanoscopic makeup of a system can be deduced from topographical and phase contrast analysis of solids obtained via atomic force microscopy (AFM), but this technique cannot describe compositional effects directly. Recent innovations have overcome these limitations by combining the two techniques.

Infrared scattering Scanning Near-field Optical Microscopy (sSNOM),¹³ a purely optical technique, measures the interaction between a sharp AFM tip and a substrate based on the local scattering of monochromatic light. This technique has effectively imaged polymer/polymer blends,¹⁴ polymer/inorganic systems,^{15,16} and nanoparticles embedded in biological materials¹⁷ with approximately 20 nm spatial resolution.^{13,15,16} However, the optical signal for the infrared sSNOM technique is approximately six times weaker than that of infrared spectroscopy based on the absorptivity of the sample¹⁸ and requires a substantial amount of post-processing to relate the scattered light to comparable FTIR spectra,¹⁹ especially for unknown samples.

In contrast, photothermal induced resonance (PTIR) exploits the increased signal caused by infrared absorption and is directly comparable to FTIR data. It allows for spatial resolution of chemical information on the nanoscale,^{12, 20-23} and has successfully assessed compositional variations in polymer blends^{8, 24-31} and living cells.^{20-22, 32, 33} As a result, PTIR has been used to analyze the drug-polymer blends in this study.

In PTIR spectroscopy, a sample is deposited onto a mid-infrared transparent ZnSe prism which is then irradiated by electromagnetic energy of defined wavelengths. When the wavelength of the incident radiation corresponds to one of the sample's absorption bands, the sample absorbs energy and converts it into heat. The resulting thermal stress induces a mechanical expansion in the sample. An AFM cantilever in contact with the sample will deflect as a result of this expansion. An optical parametric oscillator (OPO) nanosecond laser creates fast thermal expansions, or pulses, in the material. These can cause an AFM cantilever in contact mode to oscillate. By recording the cantilever deflection as a function of time and taking the Fourier-transform of this signal, the cantilever's harmonic modes are determined. A local, composition-specific infrared spectrum may be obtained by examining the maximum amplitude of the cantilever's vibration modes as a function of the wavelength of the incident energy.²³ These spectra can be highly sensitive and quantitative when evaluated at the harmonic modes of the cantilever. At the same time, the cantilever acts like a spring-damper system where the tip resonant frequency changes with variations in surface stiffness. By tracking the contact resonant frequency as a function of position on a sample, one may simultaneously obtain high-resolution stiffness maps of heterogeneous samples.¹²

The goal of this work is to evaluate the use of PTIR spectroscopy to allow chemical and mechanical evaluation of organic drug particles with diameters ~ 100 nm embedded within solid matrices, thereby extending prior efforts with this technique into the characterization of drug formulations. To this end, griseofulvin (GF) particles embedded in hydroxypropyl methyl cellulose (HPMC) have been characterized with a lateral resolution up to the smallest particle measured (90 nm), which is below the diffraction limit at infrared wavelengths. Additionally, particle size distributions via dynamic light scattering (DLS) and PTIR image

analysis are shown to be similar, suggesting that the PTIR measurements are not significantly affected by inhomogeneous infrared absorptivity of the system. Finally, PTIR spectra well above the noise threshold from 3600 to 1000 cm^{-1} for 100, 350, and 800 nm GF particles have been obtained.

METHODS

Materials

All chemicals were used as obtained without further purification. Griseofulvin (GF), a poorly water-soluble drug (EP/BP grade, solubility in water: 8.9 mg/l) was purchased from Letco Medical (Decatur, AL). Hypromellose, a.k.a., hydroxypropyl methyl cellulose (HPMC), was used as neutral polymeric stabilizer in suspension preparation and film/matrix former during spin-casting. Two grades of HPMC with identical chemistry but with slightly different viscosity, Methocel E15LV and E3, were purchased from Dow Chemical (Midland, MI). Sodium dodecyl sulfate (SDS) purchased from Sigma-Aldrich Co. (St. Louis, MO) was used as a wetting agent and for its known synergistic stabilizing action with cellulosic polymers in drug suspensions.^{2,3,34} All solutions were prepared in Milli-Q® ultrapure water (Millipore Corporation, Billerica, MA).

Suspension Preparation

Two aqueous suspensions of GF nanoparticles in HPMC/SDS were prepared via wet stirred media milling (WSMM) process to determine the potential of the PTIR technique for extracting chemical information on crystalline nanoparticles embedded in a polymer matrix. Sample 1 suspension with 10% GF, 2.5% HPMC E15LV, and 0.5% (w/w) (SDS) was prepared using a standard WSMM process, as described in [2]. To the best knowledge of the authors, drug particles with a median size below 100 nm have not been prepared using the WSMM process before. Sample 2 suspension was prepared using a novel, intensified WSMM process that utilizes 100 μm wear-resistant, yttrium-stabilized zirconia milling beads (Saint Gobain, Mountainside, NJ), synergistic stabilizing action of HPMC/SDS combination, and the low-viscosity HPMC grade. To this end, sample 2 suspension with 10% GF, 2.5% HPMC E3, and 0.2% (w/w) SDS was milled for 6 h.

Sample Preparation

The suspensions were stirred at 300 rpm for 30 minutes. The d_{50} were characterized by DLS (Zetasizer Nano-zs, Malvern) after diluting the suspensions with purified water (1:19 (v/v) for sample 1 and 1:30 (v/v) for sample 2). The d_{50} of sample 1 was 335.7 nm and for sample 2 was 85 nm. Next, 0.5 mL of sample 1 was added to an aqueous solution (4 mL) containing 2.5 % HPMC E15LV and 0.5% SDS by weight resulting in a 1:8 dilution (v/v). This solution was then spin-cast using a custom-made holder onto a ZnSe prism at 33.3 Hz (2000 rpm) for 20 s leaving a thin film of GF nanoparticles in HPMC. Similarly, 0.05 mL of sample 2 was added to 2 mL of the same aqueous solution as sample 1, resulting in a 1:40 dilution (v/v) and spin cast onto the prism at 33.3 Hz for 20 s. Film thickness was estimated by comparing the vertical displacement of the tip when moving from the coated to the uncoated regions on the substrate. As a result, sample 1 was ~500 nm thick and sample 2 was ~100 nm thick on average. The minimum heights in Figures 1 and 2 referred to these average film thicknesses and the maximum heights were due to the topography of the embedded GF clusters.

Image Analysis

The image analysis comparing the PTIR absorption data from Figure 2e to the DLS data of sample 2 was performed with MATLAB® (R2012a, The MathWorks, Inc.). Initially, each

pixel in Figure 2e was normalized between 0 and 1 by subtracting the image's minimum PTIR value and then dividing by the maximum PTIR value. The projected areas of the GF particles were determined by separating areas of signal intensity: large, medium large, medium small and small particles corresponded to areas with a normalized signal between 0.45–1, 0.38–0.45, 0.31–0.38, and 0.2–0.31, respectively. The equivalent diameters of each particle in the image were then calculated by estimating the diameter of a circular disc with the same area as the projected area of the particle.

PTIR Imaging

All measurements were performed with an Anasys Instruments nanoIRTM AFM (Santa Barbara, CA) using C-450 silicon cantilever probes in contact mode. Ten blank/background spectra, which normalized the signal intensity as a function of laser power, were obtained and averaged before each set of experiments. The sample was irradiated by an OPO nanosecond laser at a wavelength corresponding to the sample's absorption bands (e.g., 1710 cm⁻¹ or 1625 cm⁻¹ for griseofulvin), which rapidly expanded and contracted the sample causing the in-contact cantilever probe to oscillate. PTIR spectra and images were obtained by recording the peak-to-peak amplitude of the cantilever oscillations at varying locations on the sample. The first (61.0 kHz) and third (394 kHz) modes of the cantilever were used to distinguish between nanoparticles in Figures 1 and 2, respectively.²⁵

The images in Figure 1 were 2 μm × 2 μm and irradiated at 1710 cm⁻¹ with a resolution of 512 × 256 pixels. The pixel spacing on the AFM and infrared images was approximately 4 nm in the x-direction and 8 nm in the y-direction. Figures 2a–c were 10 μm × 10 μm images irradiated at 1625 cm⁻¹ with a resolution of 512 × 256 pixels yielding a pixel spacing of 20 nm in the x-direction and 40 nm in the y-direction. Figures 2d–f were 2.5 μm × 2.5 μm images scanned at 1625 cm⁻¹ with a resolution of 256 × 256 pixels yielding a pixel spacing of approximately 10 nm in both the x- and the y-directions. The AFM topographical map in Figure 4 was a 2.5 μm × 2.5 μm scan with a resolution of 512 × 256 pixels. A total of 16 spectra were collected and co-averaged at each pixel for the PTIR figures, which were scanned at 0.1–0.12 Hz. Individual spectra from 1800 to 1200 cm⁻¹ were co-averages of 128 laser pulses per wavelength in increments of 4 cm⁻¹. Individual spectra from 3600 to 1000 cm⁻¹ were co-averages of 256 laser pulses per wavelength in increments of 6.5 cm⁻¹.

RESULTS AND DISCUSSION

Figure 1 shows the results of topographical and PTIR analysis to obtain simultaneous topographical and chemical assessment of HPMC films containing GF nanoparticles. The AFM topographical image (Figure 1a) reveals several micron-sized agglomerates in the polymer. To establish the chemical composition of these surface features, the sample was illuminated at 1710 cm⁻¹, a characteristic absorption band unique to GF ketone groups.³⁵ The light areas of the PTIR image (Figure 1b) indicate regions of high material infrared absorptivity while the dark areas are regions of low absorptivity. The images also demonstrate the dependence of the PTIR signal on sample thickness²² because infrared intensities generally varied directly with changes in local topography (*i.e.*, thicker samples/taller structures showed stronger intensities). The agreement between the topographical and chemical images confirms that the particles are GF. Furthermore, Figure 1c shows clear GF features, including (C=O) absorption bands at 1710 and 1664 cm⁻¹ and the (C=C) absorption band at 1625 cm⁻¹ for spectra obtained in the particle regions.³⁵ It also shows a complete absence of these features for spectra obtained outside the particle regions (in the HPMC matrix).

The high correlation between AFM topography and the PTIR signal of sample 1 suggests that the spatial resolution for PTIR on GF-HPMC films may be limited by the size of the

cantilever tip. Generally, variations in the thickness and thermo-mechanical properties of the matrix surrounding the particle can reduce this resolution^{26,29,32,36} by introducing thermal artifacts into the system response.^{8,20,22,24,26,28,32,37} For example, there is an unexpected increase in apparent spatial resolution and contrast from the topographical to the PTIR images in Figure 1 because of the greater sensitivity of the PTIR technique to the sample thickness than the topographical contact mode of the AFM. Yet this increase is only apparent in the higher regions. The lower regions have lower contrast in the PTIR image suggesting that as the topographical variation between the HPMC matrix and GF nanoparticles decreases, the spatial resolution and contrast of the PTIR images decreases as well. Therefore, smaller GF particles have been analyzed to determine the resolution limit of PTIR on sub-micron particles.

Topographical, PTIR, and stiffness images of a second GF nanoparticulate sample dispersed in HPMC were obtained by irradiating the material at 1625 cm^{-1} as shown in Figure 2. Figure 2a shows the topography of the sample, with many GF particles in the HPMC matrix. The blue areas in the PTIR images (Figures 2b and 2e) indicate areas of low infrared absorptivity, whereas the pink, yellow, and red areas (see color scale to right hand side of image) indicate regions of high infrared absorptivity, reflecting the presence of GF at these locations. Figures 2a–c show that a broad particle/cluster size distribution (PSD), ranging from tens of nanometers (likely primary particles) to approximately 1 micron (likely agglomerates), is present in the $10 \times 10\text{ }\mu\text{m}^2$ area studied. Figures 2d and e show a magnified area of sample 2 in which a 90 nm wide GF particle was distinguishable (shown by the solid white circle) in both the topographical and chemical maps. This demonstrates that sub-100 nm GF particles can be chemically characterized with the PTIR technique.

The stiffness images also confirm the presence of crystalline GF embedded in the HPMC matrix (Figures 2c and 2f) with increased spatial resolution because the change in the contact resonant frequency is independent of material absorptivity. In these images, the crystalline nanoparticles were noticeably stiffer than the surrounding HPMC as indicated by the bright areas on the maps. The nature of the soft regions surrounding each particle is not clear, but it is hypothesized that these are surfactant- and water-rich regions that form during the drying of the films. Independent of the nature of these soft regions, the results in Figure 2 demonstrate that PTIR measurements approaching spatial resolution limits can be greatly enhanced by analyzing shifts in the contact resonant frequency (stiffness maps) in addition to the PTIR signals themselves.

To verify that the particle sizes from the PTIR analyses are representative of the true particle sizes, the distribution of the particles' equivalent diameters via image analysis (of the PTIR results) and DLS has been compared (Figure 3). Appropriate particle diameters from the PTIR image (Figure 2e) have been calculated as equivalent diameters based on the particles' projected areas. The distribution of equivalent diameters from the DLS analysis has been calculated assuming spherical particles. In the curve in Figure 3a, the DLS results for sample 2 are presented. There is a broad particle size distribution with $d_{50}=85\text{ nm}$. After converting the projected areas of the particles in Figure 3b to equivalent spheres, the diameter distribution has been plotted as the histogram in Figure 3a. As can be seen, the size distribution has $d_{50}=96\text{ nm}$, and the general shape shows reasonable agreement with the DLS curve suggesting that the PTIR measurements on the sub-100 nm particles provide a reasonable representation of the particle distribution, in spite of the heterogeneous nature of the sample.

As shown in Figure 4, spectra were collected from HPMC and from GF particles approximately 100, 350, and 800 nm in diameter in sample 2 over the wavelengths 3600 to 1000 cm^{-1} . The color-coded crosshairs in the AFM topographical map in Figure 4 show the

spectra acquisition locations for each particle and HPMC. The griseofulvin absorption peaks are readily distinguishable from the baseline noise for all three particles, as well as from the HPMC spectrum for the spectral region 1700–1600 cm^{-1} . The self-consistent signal-to-noise ratios for the 100, 350, and 800 nm particles at 1625 cm^{-1} were 5:1, 9:1, and 15:1 demonstrating that the GF is quantifiably different from the background signal. In addition, the GF signals for the 100, 350, and 800 nm particles at 1625 cm^{-1} were 5, 8, and 26 times greater than the HPMC signal at 1625 cm^{-1} , respectively. These results indicate that the PTIR technique is sensitive enough to chemically characterize local areas of heterogeneity to at least the 100 nm scale in drug-polymer systems.

This study demonstrates that chemical information for sub-100 nm particles can be obtained via PTIR for drug particles dispersed in a solid polymer matrix. Because the resolution of this technique depends on the infrared absorptivity and the thermomechanical properties of the components, the true resolution will vary for other samples. For materials with sufficient variation in electronic and mechanical properties, this method offers great promise for both nanoparticle and nano/micro- structural characterization of drug delivery systems.

CONCLUSION

The resolution and applicability of the PTIR technique for drug-polymer systems was tested using crystalline GF nanoparticles embedded in a HPMC matrix. Sub-100 nm drug particles in a polymer matrix were chemically characterized for the first time. PTIR spectra distinguishable from a background HPMC spectrum were also obtained for GF nanoparticles 100, 350, and 800 nm in diameter. This means that as modern pharmaceutical applications continue to emphasize localized drug delivery via micro/nano-engineered structures, PTIR can be used to unambiguously chemically characterize drug formulations at these length scales.

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ABBREVIATIONS

AFM	atomic force microscopy
DLS	dynamic light scattering
FTIR	Fourier transform infrared
GF	griseofulvin
HPMC	hydroxypropyl methyl cellulose or hypromellose
OPO	optical parametric oscillator
PSD	particle size distribution
PTIR	photothermal induced resonance
SDS	sodium dodecyl sulfate
WSMM	wet stirred media milling

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Figure 1.

(a) AFM height image showing micron-sized GF agglomerates embedded in HPMC. (b) Simultaneous PTIR chemical image when sample was illuminated at 1710 cm⁻¹ (a characteristic frequency of the C=O bonds in GF). (c) The molecular structure of GF, the GF (bold green) and HPMC (light green) spectra from 1800 to 1200 cm⁻¹ and their locations (bold and regular crosshairs) in (a) and (b). Spectrum acquisition time: 3 minutes. Sample studied: sample 1 was a 1:8 (v/v) dilution of an aqueous solution originally containing 10 % GF, 2.5 % HPMC and 0.5% SDS (w/w) with d₅₀ = 335.7 nm.

Figure 2.

PTIR analysis of GF particles dispersed in HPMC. (a) $10 \times 10 \mu\text{m}^2$ AFM height image showing the broad GF particle size distribution. (b) Simultaneous PTIR chemical image illuminated at 1625 cm^{-1} , a characteristic frequency of the C=C bonds in GF; blue indicates low infrared absorptivity and pink, yellow, and red indicate areas of GF. (c) Simultaneous stiffness image, with nominal contact resonant frequency of 394 kHz; darker areas are softer than lighter areas. (d) A magnified AFM height image of sample 2 and its corresponding (e) PTIR and (f) stiffness images showing a 90 nm wide nanoparticle (solid white circle) and a 100 nm wide nanoparticle (dashed white circle). Sample studied: sample 2 was a 1:40 (v/v) dilution of an aqueous solution originally containing 10 % GF, 2.5 % HPMC and 0.2% SDS (w/w) with $d_{50} = 85 \text{ nm}$.

Figure 3.

(a) Equivalent diameter particle size distribution based on the percentage of the total volume as measured by DLS (curve) and projected areas from image analysis (histogram). (b) Projected particle areas from PTIR image (2e), the different colors represent different particle areas detected by the image analysis (*i.e.*, red for the largest particles and light blue for the smallest). (c) Figure 2e, replicated for ease of comparison with (b).

Figure 4.

PTIR spectra of 800 nm (green), 350 nm (red), 100 nm (blue) GF particles and HPMC (purple) from 3600 to 1000 cm^{-1} and their corresponding locations indicated by the color-coded crosshairs on the AFM height image (inset). Spectrum acquisition time: 6.5 minutes.