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Parts per Million Powder X-ray Diffraction

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Abstract

Here we demonstrate the use of second harmonic generation (SHG) microscopy-guided synchrotron powder X-ray diffraction (PXRD) for the detection of trace crystalline active pharmaceutical ingredients in a common polymer blend. The combined instrument is capable of detecting 100 ppm crystalline ritonavir in an amorphous hydroxypropyl methylcellulose matrix with a high signal-to-noise ratio (>5000). The high spatial resolution afforded by SHG microscopy allows for the use of a minibeam collimator to reduce the total volume of material probed by synchrotron PXRD. The reduction in probed volume results in reduced background from amorphous material. The ability to detect low crystalline loading has the potential to improve measurements in the formulation pipeline for pharmaceutical solid dispersions, for which even trace quantities of crystalline active ingredients can negatively impact the stability and bioavailability of the final drug product.

Graphical Abstract

The chemical complexity of emerging drug molecules is rapidly increasing, driven by the desire for more potent drugs with higher specificity and fewer side effects. However, the growing chemical complexity presents challenges in developing formulations that retain sufficient bioavailability. As a result of increased chemical complexity, approximately 80% of potential active pharmaceutical ingredient (API) candidates are in danger of being

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abandoned due to poor systemic exposure.^{1–3} APIs are typically required to be sufficiently hydrophobic to pass through cell membranes and enter the bloodstream to reach their targets. However, the hydrophobicity must be balanced by a sufficiently high aqueous solubility. As the size and complexity of the API increases, these mutually exclusive properties become increasingly difficult to simultaneously satisfy.

Amorphous APIs are an attractive option for increasing the bioavailability of poorly soluble APIs through the development of formulations containing higher free energy solid state forms, with correspondingly faster dissolution rates.^{2–9} However, amorphous APIs are typically metastable, with the potential to crystallize over time scales that vary considerably from compound to compound, with external conditions such as temperature and relative humidity, and with the matrix or formulation in which the API resides.^{10–13} Several strategies have been utilized to stabilize amorphous formulations, including the use of polymeric crystallization inhibitors to form amorphous solid dispersions (ASD), as opposed to amorphous API powders.^{5,6,14,15} Nevertheless, major challenges emerge in assessing the long-term stability of amorphous formulations, particularly for low API loadings and early stages of crystal formation. Underpinning all of these formulation efforts is the need for sensitive, precise, and accurate analysis tools for characterizing API crystallinity, particle size, and spatial distribution within formulations, since each of these parameters can affect the release profile.

The most widespread current approaches used for assessing crystallinity in APIs and API formulations include powder X-ray diffraction (PXRD),^{15,16} differential scanning calorimetry (DSC),¹⁵ scanning electron microscopy (SEM),^{16–19} and spectrochemical techniques such as Raman.^{16–25} However, each of these techniques faces significant practical limitations in characterizing the spatial distribution of APIs, the characteristic domain sizes, and even the presence or absence of crystallinity. The detection limits for PXRD are typically on the order of a few percent crystallinity by mass. Similar detection limits are generally observed for DSC, Raman, and electron microscopy. Currently, the detection limits for percent crystallinity range from 0.2 to 5% for existing methods. These limits can be problematic in studies of amorphous formulations for which the total drug loading within an amorphous matrix may only be a few percent. Recently, brighter X-ray sources from synchrotron facilities have been utilized as one route for improving the detection limits of PXRD. Efforts using synchrotron X-ray sources for powder diffraction have lowered the limits of detection for trace crystallinity of APIs to approximately 0.2% crystallinity.²⁶

Second harmonic generation (SHG), or the frequency doubling of light, is particularly well-suited for quantitative analysis of API formulations for several reasons. First, the symmetry requirements for even ordered nonlinear processes requires SHG to be highly selective for noncentrosymmetric crystals, with negligible background contribution from amorphous materials.²⁷ The inherent chirality in most emerging APIs guarantees that the resulting crystals will satisfy the symmetry requirements for SHG. Second, SHG shares the same advantages as two-photon excited fluorescence by allowing imaging in turbid media, as only the “ballistic” unscattered light surviving to the focal volume contributes to the detected signal. Lastly, SHG exhibits a high intrinsic dynamic range of detection, allowing

quantification spanning ppm levels through 100% crystallinity. Previous studies of model systems have suggested detection limits on the order of ppm for crystallinity within amorphous solid dispersions.^{28–32}

Despite the demonstrated capabilities of SHG for quantitative analysis in amorphous pharmaceutical formulations, the nature of the signal provides little qualitative information on unknowns. Many pharmaceutical formulations contain a multitude of ingredients that can potentially produce SHG-active crystalline materials. However, the SHG intensity alone is a relatively information-poor indicator for composition within the crystalline components. Furthermore, reliable quantitative analysis by the SHG intensity has previously required prior calibration on a material-specific basis. As a result, methods that can provide qualitative determination of crystalline composition at trace (ppm) levels would significantly improve the broader utility of SHG for analysis of amorphous pharmaceutical formulations. Orthogonal analyses at these low levels of crystallinity present their own analytical challenges. The following article describes a method capitalizing on the complementary capabilities of SHG and X-ray diffraction (XRD) to yield crystallinity detection limits in the ppm range by XRD. By using an SHG microscope to guide targeted XRD analysis, the detection of crystalline materials can be achieved for samples that are deemed amorphous by conventional benchtop PXRD analysis.

METHODS

A combined instrument for SHG and XRD in a single, integrated platform has been described in detail previously.³³ Briefly, the SHG microscope was integrated into GM/CA@ APS beamline 23-ID-B at the Advanced Photon Source, Argonne National Laboratory. The unattenuated photon flux for a 12 keV, 5 μm diameter beam using the minibeam collimator³⁴ was 1.5×10^9 photons/sec/ μm^2 . The SHG microscope utilized a FemtoPower 1060 laser (Fianium) with a repetition rate of 50 MHz, pulse width of <150 fs, and power at the sample of ~90 mW. The laser was focused to a spot size of 2 μm with a 20 μm depth of field by a 10 $\times$ objective with a numerical aperture of 0.35 (Optem, 28–21–10). The SHG signal was separated from the fundamental by a series of dichroic mirrors (Semrock FF555-Di03–25 $\times$ 36, Chroma z1064rdc-sp) and filters (Chroma HQ530/30m, and CVI, 03FCG567/KG3), and detected using photon counting modules (Hamamatsu, H10722–10). Beam scanning was achieved by use of an 8 kHz resonant scanning mirror (Cambridge Technology, 1–003–3002509) moving along the vertical (y) axis and a galvanometer stepping mirror (Cambridge Technology, 6210H) moving along the horizontal (x) axis to generate images with a 300 $\times$ 300 μm field of view and a 2 μm pixel size. All SHG images were integrated for 1000 sweeps per line (~20 s/image). All software for the scan mirror control and data acquisition was written in-house. The general workflow for the SHG-guided XRD analysis is shown in Figure 1.

The diffraction patterns were analyzed and indexed by the software packages *Fable* and *GrainSpotter*.³⁵ The positions of the diffracted peaks are retrieved frame by frame based on a single intensity threshold and combined into a g-vector file. The g-vector file stores the peak positions in 3D coordinates, where individual frames are sitting in the x – y planes with the tilt angles in the third dimension. *GrainSpotter* then generated numbers of theoretical

diffraction patterns based on the reported Ritonavir (RTV) crystal structure among the different crystal orientations and built a lookup table for indexing. A built-in algorithm automatically matched the peaks in the g -vector space with the lookup table and reported the crystal orientation when it found an overlay in good agreement with the observed experimental diffraction pattern. Once the crystal orientation was obtained, the theoretical diffraction patterns at this particular orientation were generated by the *PolyXSim* and compared with the experimental data.

1D powder-type patterns are recovered via azimuthal autocorrelation (i.e., along a constant radius, or 2θ value) of the 2D image. As only discrete spots are present in these images, integrating or averaging along this dimension artificially diminishes the observed signal-to-noise ratio (SNR) for these reflections. Rather, autocorrelation preserves the 2θ locations and intensities of individual reflections while removing the amorphous scatter arising from the glass substrates surrounding the sample.

The physical mixture of 100 ppm RTV (Astrix Laboratories, India) in hydroxypropyl methylcellulose (HPMC; Shin Etsu, Japan) was prepared via serial dilutions with geometric mixing. Portions of each sample were then sandwiched between two number 1 coverslips to achieve a uniform sample thickness of $\sim 150\ \mu\text{m}$, with two coverslips serving as spacers on either end. The coverslips were then mounted on the goniometer for combined SHG and XRD analysis. RTV was chosen as a representative model system, in part because it exhibits midrange SHG activity compared to a suite of small molecule APIs³⁶ and the polymorphic purity of the commercial product is well established.³⁷ The RTV was used as received from the manufacturer.

SHG imaging for assessment of overall crystallinity was performed on a system described in detail previously.³⁸ Each of 110 fields of view were comprised of multiple images corresponding to adjacent planes in the z direction (i.e., parallel to beam propagation), with each plane integrated for approximately 2 s at a laser power of 50 mW. Post-acquisition, the in-focus plane from each field of view was isolated for analysis. NIH ImageJ was used to threshold each representative plane and assess particle number and size.³⁹

RESULTS AND DISCUSSION

Particle sizing and counting was performed on a large (>100) set of SHG images to independently quantify the level of crystallinity within a physical mixture (PM) of RTV in HPMC. Figure 2A,B shows two representative SHG images of 100 ppm (w/w) RTV in HPMC. Figure 2C shows a histogram of particle volumes for the pooled data set.

Comparisons of the SHG active volumes to the total probed sample volumes can be used to provide estimates of the overall crystallinity present within the physical mixture. An overall crystallinity of 230 ppm (v/v) was recovered for the imaged fraction of the 100 ppm (w/w) PM.

While the recovered crystallinity by SHG differs from the expectations based on gravimetric analysis by approximately a factor of 2, several potential sources of error could account for this difference. First, the PM was prepared as 100 ppm by weight. However, using the SHG

images to estimate the crystallinity produces an estimate by volume. Second, the relative densities and packing densities of both RTV and HPMC could affect the calculated crystalline content. While the combined effect of these variables is difficult to estimate, observed variations in the packing density of HPMC and other pharmaceutical excipients suggest that variations in these quantities, in addition to the absolute density difference between HPMC and RTV, could introduce sufficient error to account for this difference.^{40,41} Third, although the number density of crystallites observed between the >100 raw images suggests a sufficiently wide sampling of the powder aliquot via SHG, it is possible this ~mg aliquot is not representative of the ~kg bulk PM. Finally, the total volume of material probed by SHG was approximately 0.5 μL . At a loading (by volume) of 230 ppm, this corresponds to ~100 pL of total crystalline material probed. Considering these combined factors and the exceedingly small volume of crystalline sample probed, the recovery of a percent crystallinity within 130 ppm of the anticipated value based on gravimetric analysis is encouraging for future quantitative studies.

Prior to combining SHG with synchrotron XRD, diffraction data were acquired for a single crystal of RTV across 120 different crystal orientations within the diffractometer. Figure 3 illustrates the effect of preferred crystallographic orientations in XRD. Total time required to acquire the full tilt series was 2 min per location, with another ~2 min of total dead time for sample repositioning between exposures. While only a fraction of all possible reflections are observed for the single frames (1° integrations) in Figure 3A,B, summation of the diffraction patterns through the 120 crystal orientations begins to recover the anticipated PXRD pattern retrieved from the Cambridge Structural Database (CSD; Figure 3C, red trace). It is noted that the relative peak heights differ from the theoretical pattern from the CSD. This observation is expected, as only a subset (varying only one rotation axis through 120°) of all possible orientations is contributing to the summed pattern in Figure 3C (blue trace).

From both the raw diffraction data and the pooled results shown in Figure 3, preferred orientation effects from a single orientation of the sample can complicate analysis by synchrotron XRD, as only a subset of all possible Bragg reflections will appear simultaneously if only one or a few orientations from a finite number of crystallites are probed in the course of a measurement. Such effects can complicate both qualitative chemical identification of a crystal and quantitative determination of volume fraction, as both peak location and relative amplitude for a given reflection can vary significantly based on the orientation of the crystal. For trace crystal detection, in which only a relatively few total number of crystals may be used in the complete analysis, these crystal orientation effects are particularly pronounced.

In single crystal diffraction analysis, indexing provides a route for effectively allowing crystal orientation to be used as an advantage rather than a limitation in diffraction analysis. Figure 4A shows zoom-in of the diffraction image in Figure 3A. Figure 4B shows the indexing of the diffraction image shown in Figure 4A using the *Fable* software package.³⁵ Experimental reflections are shown in black, while theoretical reflections for the determined crystal orientation are shown in red (Figure 4B). The indexing process successfully recovers the known crystal orientation across each of the 120 diffraction images, assuming explicitly the lattice constants obtained from the CSD (entry ID YIGPIO03). Such an approach

suggests the validity of indexing to subsequently confirm the identity of a crystal by XRD, after being pre-identified for analysis by SHG.

The combination of SHG microscopy with synchrotron XRD has the potential to significantly lower the limits of detection for trace crystallinity within an amorphous medium, as demonstrated in Figure 5. Analysis of the same 100 ppm (w/w) mixture of RTV in HPMC, shown in Figure 5, by conventional benchtop PXRD results in no significant experimental evidence for the presence of crystallinity (Figure 5C). However, through SHG guided synchrotron XRD, the trace crystallinity is readily detected as seen in Figure 5B. The diffraction pattern for the region with the greatest signal in the SHG image (Figure 5A) is shown in Figure 5B. The anticipated reflection positions based on the indexing results and recovered single crystal orientation are also included in Figure 5B (solid red spots). The red circles in Figure 5B indicate the areas where the indexing and experimental diffraction match. The observed reflections are consistent with the assumed lattice parameters for RTV, supporting the ability to characterize crystallinity within a trace (100 ppm) crystalline sample by SHG-guided synchrotron XRD. Diffraction images of multiple SHG-inactive regions, taken with the identical beam size and exposure time, do not show any observable reflections, further suggesting that the observed diffraction is unlikely to be arising from the excipient polymer matrix.

SHG-guided diffraction analysis also offers two key quantitative advantages regarding the observed SNR in the measurements. The first advantage is through background reduction. The rapid identification of crystal location by SHG allows X-ray diffraction analysis with a small ($10\text{ }\mu\text{m}$) beam. Significant background from amorphous scatter is arguably one of the most common factors contributing to the relatively high detection limits of traditional PXRD measurements. The volume of amorphous material probed is reduced by using the minibeam collimator to pair the size of the X-ray source to that of the crystalline sample, resulting in a lowering of the background scatter from amorphous contributions.⁴² The second SNR advantage arises from the reduction in spectral congestion. The diffraction features in the single-crystal measurements are well separated in angle from other diffraction features, while multiple peaks can be highly overlapping in conventional powder XRD. The observed SNR in the diffraction measurements in Figure 5B is ~ 5000 from a 1 s exposure with a $5\text{ }\mu\text{m}$ diameter beam. Given the sixth power dependence of diffraction on crystal diameter, this SNR corresponds to a minimum detection limit for a single crystal on the order of $2\text{ }\mu\text{m}$, assuming a $5\text{ }\mu\text{m}$ diameter beam.

In addition to the SNR advantage in the X-ray analysis, the integration of SHG imaging also offers a significant time savings in the identification of crystalline domains. Although in principle, background suppression could be broadly achieved by localization of the X-ray source, the relatively long integration time per pixel (1 s in the present study) represents a considerable cost. Some time savings can be expected through iterations first using a spatially broad source to identify regions of interest based on observed diffraction, then progressing to smaller diameter beams for SNR optimization. However, this strategy will be limited to relatively large crystals to overcome the background with a wide-area source and can still require several hours of synchrotron beam time. By first targeting the diffraction to

regions of interest (ROIs) within $\sim 2\ \mu\text{m}$ resolution rapidly identified by SHG, high SNR diffraction can be collected in a matter of seconds.

Although all the measurements reported in this study were performed with an integrated instrument combining SHG with synchrotron XRD in a single platform, this general strategy for SHG-guided diffraction is not limited to this one architecture. If the SHG-active ROI can be reliably and accurately located through the use of any set of inherent or artificial fiducial markers, combined measurements could in principle be obtained through prescreening on a benchtop nonlinear optical microscope followed by diffraction on an independent beamline. For small crystals, the integrated system has the advantage of absolute accuracy in the positioning between SHG and synchrotron XRD to within $1\ \mu\text{m}$. However, for larger crystals off-line analysis may still provide the accuracy required for structural characterization.

Off-line measurements would also have the advantage of allowing for integration with complementary methods for structural analysis, such as Raman spectroscopy. SHG-guided Raman spectroscopy has recently been used to study a commercial formulation of nanoparticle albumin-bound paclitaxel.⁴³ Increasing the number of orthogonal measurements of the same sample significantly improves the overall confidence of the conclusions drawn, with Raman potentially serving as a relatively simple first-pass analysis approach prior to synchrotron XRD. XRD is arguably the gold standard for crystal characterization, in many cases showing a more sensitive response to subtle variations in crystal structure and form than common orthogonal techniques such as Raman, DSC, and SEM. The high (>5000) SNR observed in the diffraction images of 100 ppm RTV in HPMC illustrate these advantages. Additionally, the detector integration time required for thorough characterization of crystal form by synchrotron XRD ($\ll 1\ \text{s}$) is significantly lower than that required for the Raman measurements performed in the previous study (on the order of several minutes). As construction of new synchrotron facilities continues worldwide, the overall availability and utility of SHG-guided XRD has the potential to increase concurrently.

The generalizability of this technique to other samples can be assessed from previous measurements done using benchtop instrumentation. The integrated diffraction for any small molecule organic API crystal is likely to be similar, with much greater anticipated variability in the SHG activity. Fortuitously, previous experiments have shown a 7 orders of magnitude detectable dynamic range for the observed SHG intensity of 42 different APIs under comparable imaging conditions.³⁶ The SHG activity of RTV falls in the midrange of this scale, suggesting viable detection for the majority of homochiral crystalline APIs. From previous studies of common excipients, it is reasonable to suggest that the approach described herein will have few constraints arising from the selection of polymer matrices in amorphous solid dispersions. From these combined results, it is reasonable to suggest that SHG-guided microdiffraction is likely to be a broadly applicable approach for both detection and analysis of trace homochiral crystallinity within amorphous solid dispersions.

CONCLUSIONS

SHG microscopy and synchrotron XRD were coupled to detect trace crystallinity of an API in a common polymeric excipient. The combined instrument was able to detect 100 ppm (w/w) ritonavir in HPMC, decreasing the limit of detection by more than 1 order of magnitude over previously reported values.²⁶ Using SHG to guide synchrotron XRD analysis provides two main advantages over PXRD. First, SHG allows for rapid detection and localization of areas containing crystalline APIs. The SHG images can be acquired in a matter of seconds, whereas the same field of view would be prohibitively time-consuming to image via XRD raster scanning. Second, SHG-guided diffraction allows background suppression in the XRD through the use of a 5 μm diameter source better matched to the dimensions of the crystalline domains. Such low detection limits have the potential to enable substantially faster turnaround times in optimization of amorphous solid dispersions, in which even trace crystallinity can substantially impact the bioavailability in final dosage forms.

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Figure 1.

Experimental workflow for SHG-guided XRD analysis of trace crystallites. First the amorphous solid dispersion is imaged using an IR source. The resulting SHG image is then used to guide the synchrotron to the regions of interest for diffraction analysis.

Figure 2. SHG images (A, B) and generated histogram (C) of particle volumes. The SHG-active fraction of material can be used to assess overall crystallinity of the powdered blend.

Figure 3.

Single crystal diffraction patterns of RTV were obtained for 120 different angles with representative diffraction patterns from two different angles shown in A and B. A simulated powder diffraction is obtained by summing all of the single diffraction images together (blue trace in C) and compared to the predicted powder pattern obtained from the CSD (red trace in C). Even though the experimental pattern was obtained from only a small subset of crystal orientations it is beginning to approach that of the predicted pattern.

Figure 4.

(A) Single crystal diffraction pattern for RTV. Single crystal diffraction pattern generated by the *Fable* software suite base only on lattice parameters from the CSD entry for RTV is shown in B. Excellent agreement is observed between experimental and theoretical diffraction patterns indicating that the indexing method can correctly recover single crystal diffraction patterns without knowing the orientation *a priori*.

Figure 5.

Analysis of a 100 ppm sample of ritonavir in HPMC (0.01% w/w) with SHG-assisted synchrotron XRD, where (A) is epi-SHG signal from ritonavir, with the red crosshairs denoting the center of the synchrotron X-ray beam. An XRD pattern of the SHG active region (circled in yellow) is shown in (B) along with the results from the auto indexing (red dots). Areas where the auto indexing results and experimental diffraction pattern agree are highlighted by the red circles. The powder XRD spectrum using a conventional PXRD benchtop is shown in (C) and indicates that the sample is amorphous by conventional PXRD.