Video Article

Coherent anti-Stokes Raman Scattering (CARS) Microscopy Visualizes Pharmaceutical Tablets During Dissolution

Andrew L. Fussell[1], Peter Kleinebudde[2], Jennifer Herek[1], Clare J. Strachan[3], Herman L. Offerhaus[1]

[1]Optical Sciences Group, MESA+ Institute, University of Twente

[2]Institute of Pharmaceutics and Biopharmaceutics, Heinrich-Heine University

[3]Division of Pharmaceutical Technology, Faculty of Pharmacy, University of Helsinki

Correspondence to: Andrew L. Fussell at A.L.Fussell@utwente.nl

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Abstract

Traditional pharmaceutical dissolution tests determine the amount of drug dissolved over time by measuring drug content in the dissolution medium. This method provides little direct information about what is happening on the surface of the dissolving tablet. As the tablet surface composition and structure can change during dissolution, it is essential to monitor it during dissolution testing. In this work coherent anti-Stokes Raman scattering microscopy is used to image the surface of tablets during dissolution while UV absorption spectroscopy is simultaneously providing inline analysis of dissolved drug concentration for tablets containing a 50% mixture of theophylline anhydrate and ethyl cellulose. The measurements showed that *in situ* CARS microscopy is capable of imaging selectively theophylline in the presence of ethyl cellulose. Additionally, the theophylline anhydrate converted to theophylline monohydrate during dissolution, with needle-shaped crystals growing on the tablet surface during dissolution. The conversion of theophylline anhydrate to monohydrate, combined with reduced exposure of the drug to the flowing dissolution medium resulted in decreased dissolution rates. Our results show that *in situ* CARS microscopy combined with inline UV absorption spectroscopy is capable of monitoring pharmaceutical tablet dissolution and correlating surface changes with changes in dissolution rate.

Video Link

The video component of this article can be found at https://www.jove.com/video/51847/

Introduction

During the development of oral pharmaceutical dosage forms such as tablets and capsules there is a strong emphasis on dissolution testing. Oral dosage forms are required to dissolve before they can be absorbed for therapeutic efficacy. Poorly soluble drugs generally have issues reaching an adequate concentration which makes dissolution testing particularly important[1]. Pharmacopoeial dissolution methods are most commonly used for dissolution analysis. In most cases this requires preparing the drug as a tablet or capsule which is then placed into a beaker of flowing dissolution medium. The dissolved drug concentration is then determined by analyzing samples of the dissolution medium using a standard spectroscopic technique such as UV absorption spectroscopy[2]. These traditional pharmaceutical dissolution methods do not provide any direct analysis of the sample or any changes that might be occurring on the dissolving surface of the dosage form. Direct analysis of the sample during dissolution can provide more information about the dissolving dosage form and potentially identify problems causing dissolution test failure.

Direct analysis of dissolving dosage forms requires the use of *in situ* analytical techniques which are capable of monitoring the dissolution process. To record *in situ* during dissolution the analytical technique must not be influenced by the presence of the dissolution medium and the technique needs a high temporal resolution to reliably measure changes to the dissolving dosage form in the order of seconds. Attenuated total reflectance UV spectroscopy has been shown to be suitable for measuring changes during dissolution but lacks spatial resolution provided by imaging techniques[3]. Traditional pharmaceutical imaging techniques such as scanning electron microscopy (SEM), and spontaneous Raman mapping both have limiting factors preventing their use *in situ* for dissolution.

SEM imaging is a high-resolution rapid imaging technique capable of imaging the surface of pharmaceutical dosage forms. However, SEM imaging is generally performed under vacuum conditions and requires sample coating making it unsuitable for *in situ* dissolution imaging. Fiber-coupled spontaneous Raman spectroscopy combined with a flow through cell and UV flow-through absorption spectroscopy, has been performed to monitor various drug systems *in situ* during dissolution, including theophylline[4], carbamazepine, and indomethacin[5]. Raman spectroscopy was capable of identifying surface changes occurring during dissolution but it gave no spatial information about where the surface changes were occurring. Spontaneous Raman mapping uses Raman spectra and provides spatial information about the surface of the sample but imaging takes on the order of minutes to hours depending on image area, making it unsuitable for *in situ* dissolution imaging.

Coherent anti-Stokes Raman scattering (CARS) microscopy is a rapid imaging technique and combined with inline UV absorption spectroscopy, it has allowed us to develop a technique capable of *in situ* dissolution analysis. CARS microscopy provides rapid chemically selective imaging which is not influenced by the presence of dissolution medium making it a suitable technique for *in situ* dissolution analysis. CARS techniques are divided roughly into two groups based on the pulse duration of the lasers; one being narrowband CARS (picosecond pulsed lasers), and the other being broadband CARS (femtosecond pulsed lasers). A typical CARS microscope system consists of two pulsed laser sources and an inverted microscope. To produce a CARS signal, one of the pulsed lasers needs to be tunable so there is a frequency difference between the two lasers which matches a Raman vibration. Additionally, the two lasers are required to overlap in space (spatial) and time (temporal), with pulses from both lasers arriving at the same area of the sample at the same time. As Raman vibrations are chemically specific and CARS signal is only generated within the focal volume of the microscope, CARS microscopy is capable of chemically selective imaging with a resolution down to the diffraction limit.

Narrowband CARS microscopy using a single Raman vibrational mode allows about 100x faster imaging compared to spontaneous Raman mapping techniques[6]. Broadband CARS microscopy images over a wider spectral range (600-3,200 cm^{-1} vs. ~4 cm^{-1}) but has a lower spectral resolution (around 10 cm^{-1} vs. ~4 cm^{-1}) and slower imaging speed (50 msec/pixel vs. ~5 µsec/pixel) compared to narrowband CARS microscopy[7].

Narrowband CARS microscopy has been used to image drug release from some pharmaceutical systems. In the area of pharmaceutical formulations, Kang *et al.*[8-10] imaged drug loaded polymer films. Initially they imaged the distribution of the loaded drug, which was followed by imaging of the drug release from a static dissolution medium. Jurna *et al.*[11] and Windbergs *et al.*[12] went a step further and imaged firstly the theophylline distribution in lipid dosage forms followed by imaging the drug dissolution using a dynamic dissolution medium.

We have developed a new analytical method to simultaneously monitor surface changes on the tablet undergoing dissolution with narrowband CARS microscopy while recording the dissolved drug concentration with UV absorption spectroscopy. We illustrate the use of this method imaging tablets containing the model drug theophylline combined with ethyl cellulose undergoing dissolution with water as dissolution medium.

Protocol

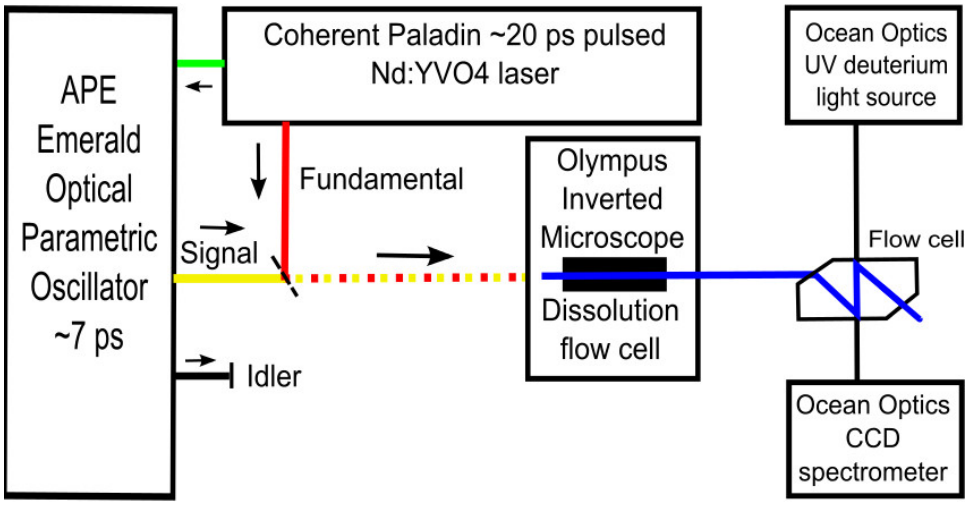

Figure 1. Schematic illustrating the CARS microscope setup with the intrinsic flow through dissolution setup. This figure has been modified from Fussell *et al*[13].

1. System Startup

1. Turn on the 20 psec pulsed 1,064 nm CARS laser and allow the laser to warm-up (approx. 1.5 hr).
2. Turn on the deuterium lamp UV light source and allow it to warm-up (approx. 10 min).
3. Open the shutter on the deuterium lamp UV light source by setting the shutter switch to "open".
4. Turn on the microscope control PC and open the microscope control software.
5. Turn on the UV spectrometer PC and open the spectrometer control software.

2. Microscope Setup

1. Select the desired microscope objective. Use a 20X/0.5 NA objective to achieve the results presented in this work.
2. Set the filters in the filter set turret to transmit excitation lasers and reflect the CARS signal. Select a 775 nm long-pass dichroic mirror and a 650 nm band-pass 40 nm filter to replicate the results shown in this work.
3. Place appropriate filters in front of the photomultiplier tube (PMT) detector that transmit CARS signal and filter unwanted light. Filter the light with a 750 nm short-pass filter and a 650 nm band-pass 40 nm filter to reproduce the experiments conducted in this work.

3. System Testing

1. Turn on the peristaltic pump and pump dissolution medium for a few minutes through the Z-shaped UV flow cell to clear previous liquid from the piping.
2. Determine the flow rate of the pump by weighing the amount of dissolution medium pumped in 2 min. Adjust pump speed until desired flow rate is reached. Pump the dissolution medium at a flow rate of 5 ml/min to achieve the results reported in this work.

4. UV Dissolution Measurement

1. In the UV spectrometer control software, click the “File” menu then click “New absorbance measurement” to open a window which lists all available spectrometers.
2. Click on the correct UV spectrometer, and then click “Next” to open a window which displays the data acquisition parameters.
3. Define both the integration time and the spectral averaging. Choose an integration time of 150 msec with 200 averages to replicate the results shown in this work.
4. Click the button labelled “Next” to bring up the screen used to record the reference spectrum.
5. Click on the button that appears as a yellow light bulb to record a reference spectrum. Pump dissolution medium continuously during this measurement.
6. Close the shutter on the deuterium lamp UV light source by setting the switch to “closed”.
7. Click the button labelled “Next” to bring up the screen used to record the dark spectrum.
8. Click on the button that appears as a grey light bulb to record a dark spectrum. Pump dissolution medium continuously during this measurement.
9. Click on the button that says “Finish” to begin the UV absorbance measurements.

5. CARS Dissolution Video

1. In the CARS microscope control software click on the button that selects an “XYT” measurement.
2. Click the drop-down box and select the image size in pixels. Select an image size of 512 x 512 pixels to reproduce the images reported in this work.
3. Drag the imaging speed slider to either the “fast”, “medium”, or “slow” position. Use the fast scanning speed (1.12 sec per image) to achieve the results shown in this work.
4. Click the arrows labelled “zoom” to adjust the zoom level. Select “2x” zoom to replicate the level of zoom and field of view (350 x 350 µm) used for these results.
5. Click the drop-down box and select the objective used.
6. Click the input box and type the amount of frames required for the CARS dissolution video (depending on length of experiment). Conduct dissolution for about 15 min by recording 900 frames to reproduce the results shown in this work.

6. CARS Wavelength Tuning

1. Using the optical parametric oscillator (OPO) controller adjust the settings of the OPO such as temperature, piezo position, and Lyot filter position until maximal laser output at the desired Raman frequency is reached. Tune the OPO to 2,960 cm^{-1} to record the same results as those presented in this article.

7. The Dissolution Experiment

1. Place a tablet into sample holder of the custom built CARS flow cell, screw the sample holder closed tightly to prevent leakage.
2. Attach the piping to the CARS flow cell connecting the CARS flow cell to the beaker containing the dissolution medium and the peristaltic pump.
3. Place the CARS flow cell containing a tablet on the microscope stage.
4. Check that the CARS flow cell is connected to the dissolution medium beaker, the peristaltic pump, the Z-shaped UV flow cell and the waste collection beaker.
5. Click the “XY repeat” button to start the microscope system scanning in a continuous scan mode.
6. Adjust the focus of the microscope by moving the objective until the surface of the tablet is in the field of view on the microscope control computer screen.
7. Click on the slider in the microscope control software labelled “PMT”. Adjust the detector sensitivity by increasing/decreasing PMT voltage until a satisfactory image (neither too dark nor saturated) is visible on the screen. NOTE: Take care not to overload the PMT by using high voltage. For this work, we used a PMT voltage around 600 V but this can vary depending on the PMT used.
8. Click “Stop” in the microscope control software to stop the continuous scan.
9. Simultaneously (or as close together as possible) start pumping dissolution medium, start recording a single XYT scan, and start collecting UV absorbance spectra.
10. During the dissolution experiment, monitor the video recording and manually adjust the microscope focus to ensure the tablet is continually in focus.

8. Post Dissolution

1. Stop the peristaltic pump by turning it off.
2. Click the “File” menu and then click “Save as video” on the microscope control software to save the XYT scan as a video.

3. Click the "File" menu, then click "Save" and then click "Stop Export" on the spectrometer control software to stop the collection of UV absorption spectra.
4. Remove the CARS flow cell from the microscope stage and remove the tablet from the CARS flow cell.
5. Wash the CARS flow cell using water and ethanol, and then dry using tissue paper.

Representative Results

In situ dissolution analysis using CARS microscopy was conducted on tablets (12 mm diameter, flat-faced) containing a 50:50 mixture of the model drug theophylline anhydrate and ethyl cellulose with distilled water pumped at 5 ml/min as the dissolution medium. CARS images (512 x 512 pixels) were collected every 1.12 sec at the Raman vibrational frequency 2,960 cm^{-1} which is selective for the theophylline content in the tablet for the duration of the dissolution experiment. **Figure 2** shows selected frames from the dissolution video. At the beginning of dissolution (**Figure 2**, time 0 sec) there are areas of green showing the theophylline content of the tablet and there are also dark areas where there is only ethyl cellulose present on the surface of the tablet. In the dark areas on the surface of the tablet it is possible to faintly see the ethyl cellulose content. This is because it is reported that ethyl cellulose has Raman vibrational frequencies with maxima around 2,930 and 2,975 cm^{-1} [14]. After about 60 sec there appears to be the beginning of theophylline monohydrate crystal growth on the surface which can be seen as narrow needle-shaped crystals growing outwards from at least one crystal nucleus at the center of the frame (**Figure 2**, time 60 sec). The monohydrate crystal growth can be much more clearly seen after 130 sec (**Figure 2**, time 130 sec). Additionally, at time point 130 sec it can be seen that the monohydrate crystal has not spread entirely across the surface of the tablet. It appears as though the presence of the ethyl cellulose regions has physically blocked the lengthening of the monohydrate needles. After 250 sec, it can be seen that the monohydrate coverage of the surface is not as prominent which suggests that the monohydrate crystals are themselves beginning to dissolve.

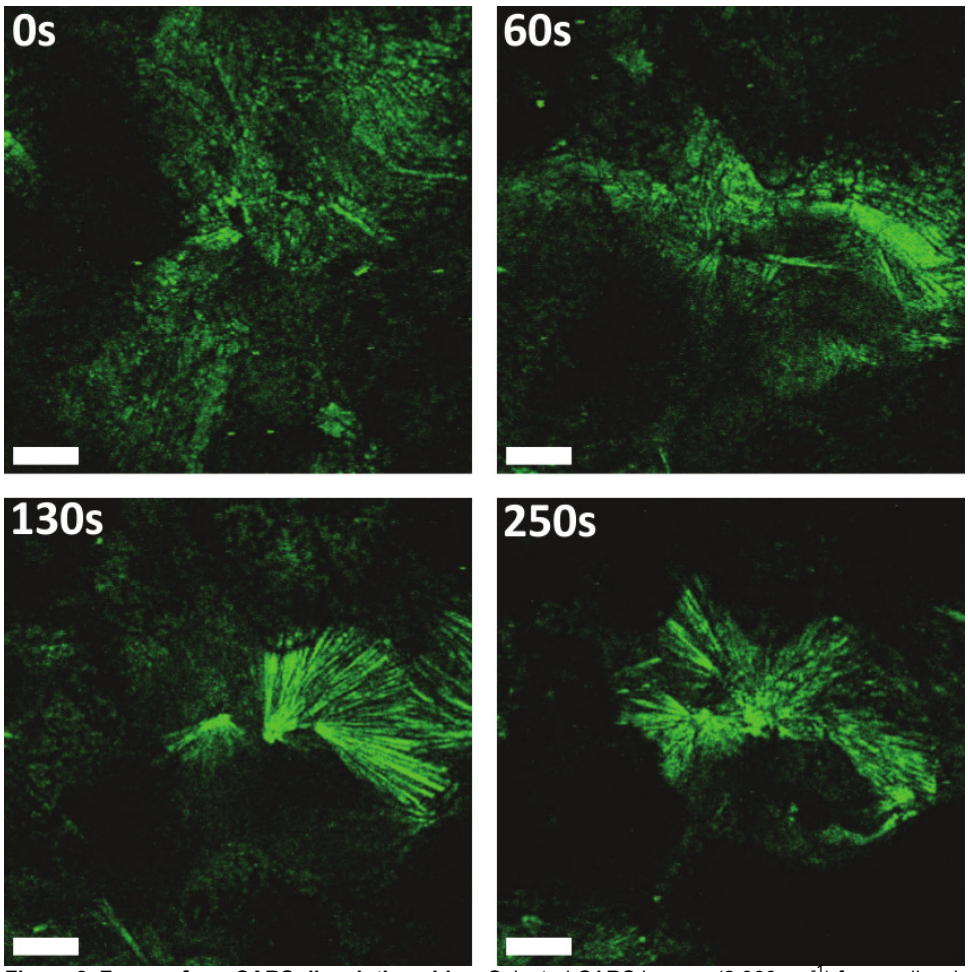

Figure 2. Frames from CARS dissolution video. Selected CARS images (2,960 cm^{-1}) from a dissolution video for a theophylline anhydrate with ethyl cellulose tablet. The 0 sec image is recorded at one area of the sample while the 60, 130, and 250 sec images are recorded at another area of the sample. The CARS video is available as supplementary information. Scale bar is 50 µm.

Ultra-violet (UV) spectroscopy is a form of absorption spectroscopy using UV light as the excitation source. UV spectroscopy measures electron transitions from the ground state to an excited state[15]. Theophylline has a broad peak centered around 270 nm while ethyl cellulose is practically insoluble in the dissolution medium so is not expected to contribute to the UV spectrum recorded. Analysis of the dissolution medium using the inline z-shaped UV flow cell allows us to quantitatively determine the amount of drug dissolved during dissolution. **Figure 3** shows the UV-dissolution profile for the dissolution of the theophylline anhydrate with ethyl cellulose tablet. The UV dissolution profile (**Figure 3**) shows that dissolution of theophylline anhydrate begins quickly reaching a maximum concentration of around 90 µg/ml within 120 sec; after this time point

the dissolution rate begins to decrease. The decrease in the dissolution rate could be due to the presence of the theophylline monohydrate (solubility 6 mg/ml at 25 °C [16]) crystals on the surface which are less soluble than theophylline anhydrate (solubility 12 mg/ml at 25 °C [16]) and clearly seen in the CARS dissolution video (**Figure 2**) at this time point. The gradually reducing dissolution rate could also partially be explained by a reduction in theophylline exposure to the flowing medium. This reduction occurs because ethyl cellulose is practically insoluble in water, so as the theophylline dissolves the remaining ethyl cellulose hinders theophylline exposure to the dissolution medium.

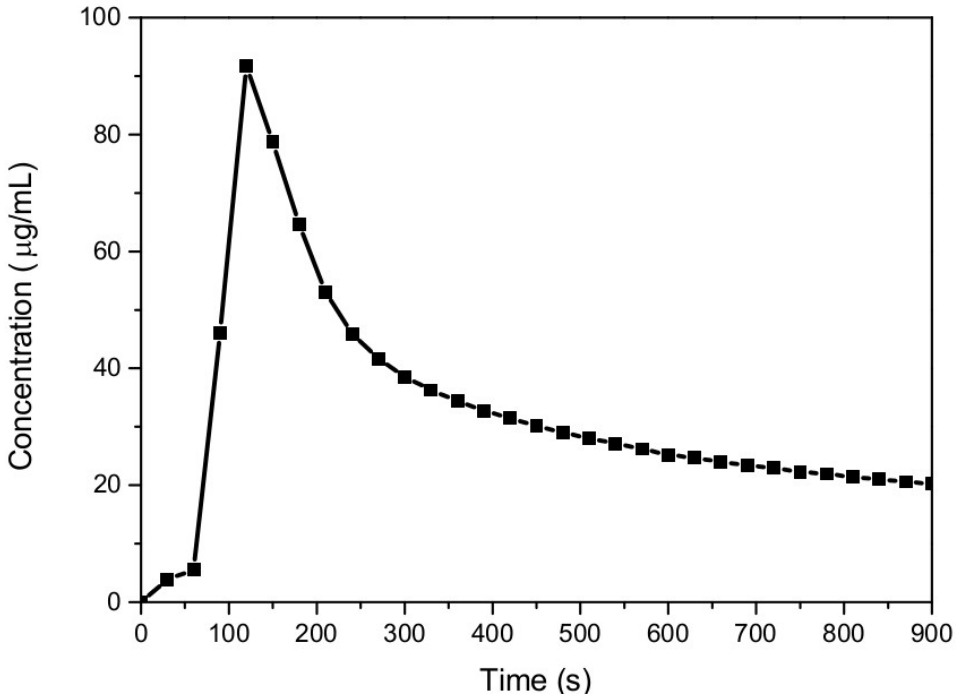

Figure 3. UV dissolution profile. Concentration vs. time plot for a theophylline anhydrate combined with ethyl cellulose tablet showing the concentration of theophylline in the dissolution medium during the dissolution experiment.

Discussion

When performing CARS microscopic dissolution experiments there are a few critical aspects that need to be monitored during the experiment. Firstly, introducing the dissolution medium to the CARS flow cell causes the focus to move. This means that the image is immediately lost and it takes a few microns of objective adjustment to find the surface again. Secondly, there is risk of liquid leakage from the CARS flow cell if the glass cover breaks during the experiment. This can potentially cause liquid damage to the optics, so it is important to listen for any cracking sound that could mean the glass has broken. Finally, there is also a small chance that the piping can become blocked due to particulate matter in the system during the experiment, this can be seen as a sudden unusual change in the UV spectra and also through periodically checking the flow during the experiment.

Particulate blockage of the piping is mainly an issue with tablets that have been designed to disintegrate during dissolution. This is one of the limitations for this technique as this system requires the surface of the tablet to remain intact throughout the dissolution to allow imaging. In addition to disintegrating tablets, it is currently not possible to image tablets that are designed to swell during dissolution as this can lead to breakage of the CARS flow cell.

Imaging tablets during dissolution provides a greater understanding of what is occurring on the surface of a dissolving tablet. Conventional pharmaceutical dissolution methods focus only on the drug content dissolved in the dissolution medium which can identify whether the tablet passes or fails the required standard. However, in the case of a failed test it is difficult to determine what caused the failure. The case of a failed dissolution test is potentially where *in situ* dissolution analysis using CARS microscopy can provide answers.

Future applications for *in situ* dissolution analysis using CARS microscopy could include investigations using more complicated tablets containing more than one drug or excipient, in particular non-swelling sustained or controlled release dosage forms during formulation development. Additionally, it could be possible to investigate samples using biorelevant dissolution media creating conditions more closely related to *in vivo*.

In conclusion, this work shows that CARS microscopy is capable of rapid chemically specific imaging based on Raman vibrational frequencies allowing selective imaging of the drug in a tablet containing both drug and excipient. Additionally, CARS microscopy combined with inline UV absorption spectroscopy is a powerful tool capable of monitoring the surface of tablets undergoing dissolution and correlating surface changes seen using CARS with changes in dissolution rate.

Disclosures

The authors declare that they have no competing financial interests.

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