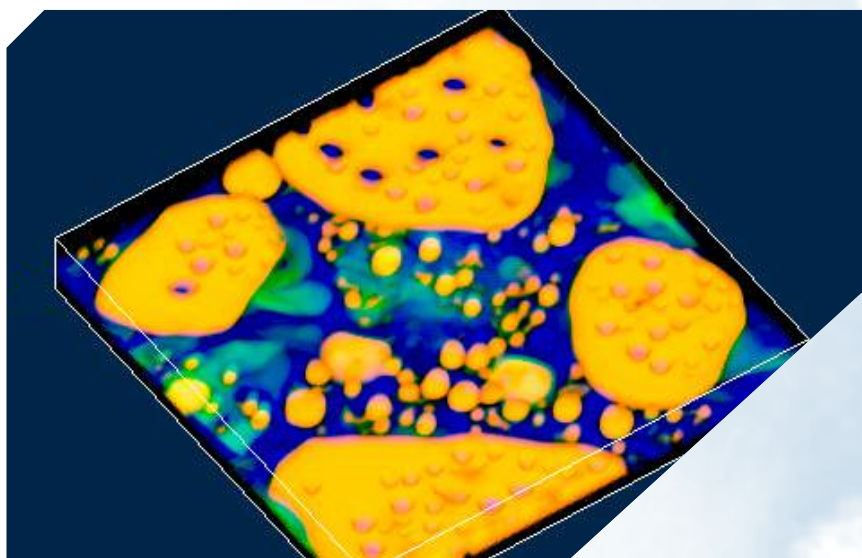
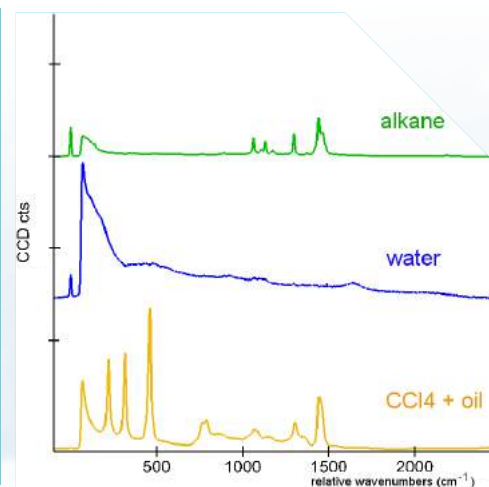
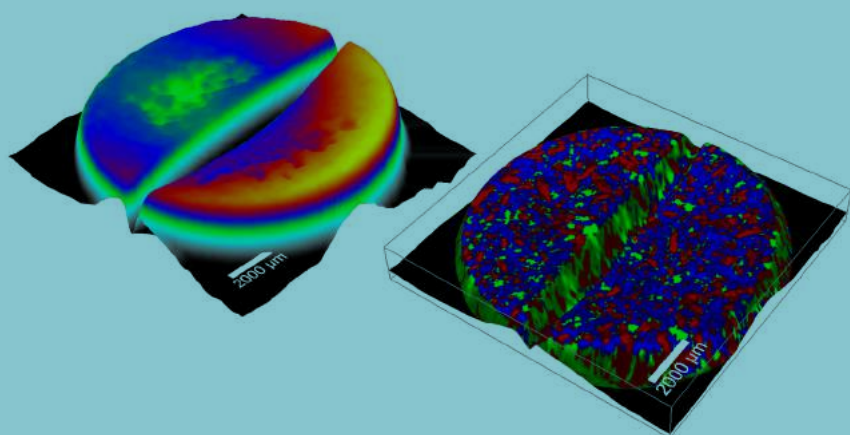


Chemical Characterization of Pharmaceutical Samples by Confocal Raman Microscopy and Correlative Techniques



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Introduction

Development, production and quality control in the pharmaceutical industry requires efficient and reliable control mechanisms to ensure the safety and the therapeutic effect of the final products. These products can vary widely in composition and application. Several of their properties can be difficult to study with conventional techniques due to the inability of these methods to chemically differentiate materials with sufficient spatial resolution and without damage or staining. Therefore analyzing methods that provide both comprehensive chemical characterization and the flexibility to adjust the method to the investigated specimen are preferred in pharmaceutical research.

Confocal Raman Microscopy (CRM) is a well-established and widely-used spectroscopic method for the investigation of the chemical composition of a sample. In pharmaceutical research, CRM can be used to probe the distribution of components within formulations, to characterize the homogeneity of pharmaceutical samples, to determine the state of drug substances and excipients and to characterize contaminants and foreign particulates [1]. The information obtained by CRM is also useful for drug substance design, for the development of solid and liquid formulations, as a tool for process analytics and for patent infringement and counterfeit analysis [2, 3, 4]. Being a non-destructive method it is possible to combine CRM with other imaging techniques such as Atomic Force Microscopy (AFM), Scanning Electron Microscopy (SEM) or fluorescence analysis.

The Aim of the Study

Based on various samples the article will demonstrate how CRM can be beneficially applied in the pharmaceutical field. With high-resolution, large-area Raman images,

topographic 3D Raman images, depth-profiles and correlative Raman-AFM images the analyzing capabilities of CRM and its correlative techniques will be shown. Furthermore an example of a statistical quantitative evaluation of a spectral Raman dataset will be given.

Materials & Methods

Confocal Optical Microscopy

The primary benefit of confocal microscopy is the suppression of light from outside the focal plane. This is realized firstly by a point illumination in the focal plane and secondly by a pinhole in the conjugate plane of the detection beam path.

Due to the point illumination and detection, only information from a single point is determined at a time. In order to generate an image the sample is scanned point-by-point and line-by-line.

The advantages of confocal microscopy over conventional wide-field microscopy are an improved resolution in the lateral plane, a high axial discriminatory power, a reduced background signal and the ability to collect optical sections in series from different focal planes to generate depth profiles or 3D images.

Confocal Raman Microscopy

By combining a research-grade confocal optical microscope with a high-sensitivity Raman spectrometer, the resolution of the optical microscope can complement the analytical power of Raman spectroscopy. Thus it is not only possible to obtain Raman spectra from extremely small sample volumes, but also to collect high-resolution Raman images that show the distribution of chemical species and crystallographic properties with sub- μm resolution. The sample is scanned point-by-point and line-by-line, and at every image pixel a complete Raman spectrum is acquired. This process is called hyperspectral imaging.

These multi-spectrum files are then analyzed to display and image the distribution of chemical sample properties.

Atomic Force Microscopy

Atomic Force Microscopy (AFM) provides topographic high-resolution information by recording the interaction forces between the surface and a sharp tip mounted on a cantilever. In addition, local material properties such as adhesion or stiffness can be investigated.

Combining CRM with AFM the high spatial and topographical resolution obtained with an AFM can be directly linked to the molecular information provided by confocal Raman spectroscopy.

Topographic Confocal Raman Imaging

Although confocal Raman imaging is considered to be a non-destructive method it is common to create a smooth and even sample surface in order to keep the surface in the focal plane while measuring in confocal Raman imaging mode. However, this intervention can lead to the misinterpretation of the analytical results. To avoid any sample preparation topographic confocal Raman imaging (TrueSurface Microscopy, WITec GmbH, Germany) can be applied. It is especially useful for samples with rough or inclined surfaces. The highly precise sensor scans the topography of the sample. The topographic coordinates are used to trace the sample's surface in confocal Raman imaging mode to ensure that the Raman laser is always kept in focus. The result is a confocal Raman image that can be overlaid on the topographic image and visualized three dimensionally.

Experimental Setup

A confocal Raman microscope (alpha300 R, WITec GmbH) was used to study the chemical composition of various pharmaceutical samples. The laser light is guided to the microscope through an optical

fiber. It can be focused to a diffraction-limited spot and therefore acts as a point light source. This light is focused onto the sample using a dichroic beam splitter that reflects the exciting laser beam, but is fully transparent for the frequency-shifted Raman light. The sample is scanned with a piezo-electric scanning table with capacitive feedback correction for high resolution. The Raman scattered light is collected with the same objective and is focused into the core of another optical fiber that is connected to a spectrometer. The light is dispersed inside the spectrometer and the spectra are acquired with an ultra-sensi-

ve, back-illuminated CCD camera.

Results & Discussion

High-Resolution, Large-Area Confocal Raman Imaging, Volume Scans and 3D Visualizations

In the first example a pharmaceutical emulsion was investigated. The active pharmaceutical ingredient (API) was dissolved in water. In figure 1a a large-area, high-resolution Raman image of the emulsion is shown. The image scan range is $180 \times 180 \mu\text{m}^2$ with 2048×2048 pixels. At each image pixel a complete Raman spectrum

was acquired, therefore the large image is the result of an evaluation of 4,194,304 Raman spectra. The integration time per spectrum was 2 ms and the raw data file size is 12.5 GB. The consecutive zoom-in images (figure 1b and 1c) of the same dataset illustrate the extremely high-resolution of the large-area scan shown in figure 1a. The Raman data were acquired, evaluated and processed with the Project FOUR Software (WITec GmbH, Germany). In the resulting color-coded images the water and API-containing phase is presented in blue, whereas with green the oil-matrix is displayed. In addition to the distribution of

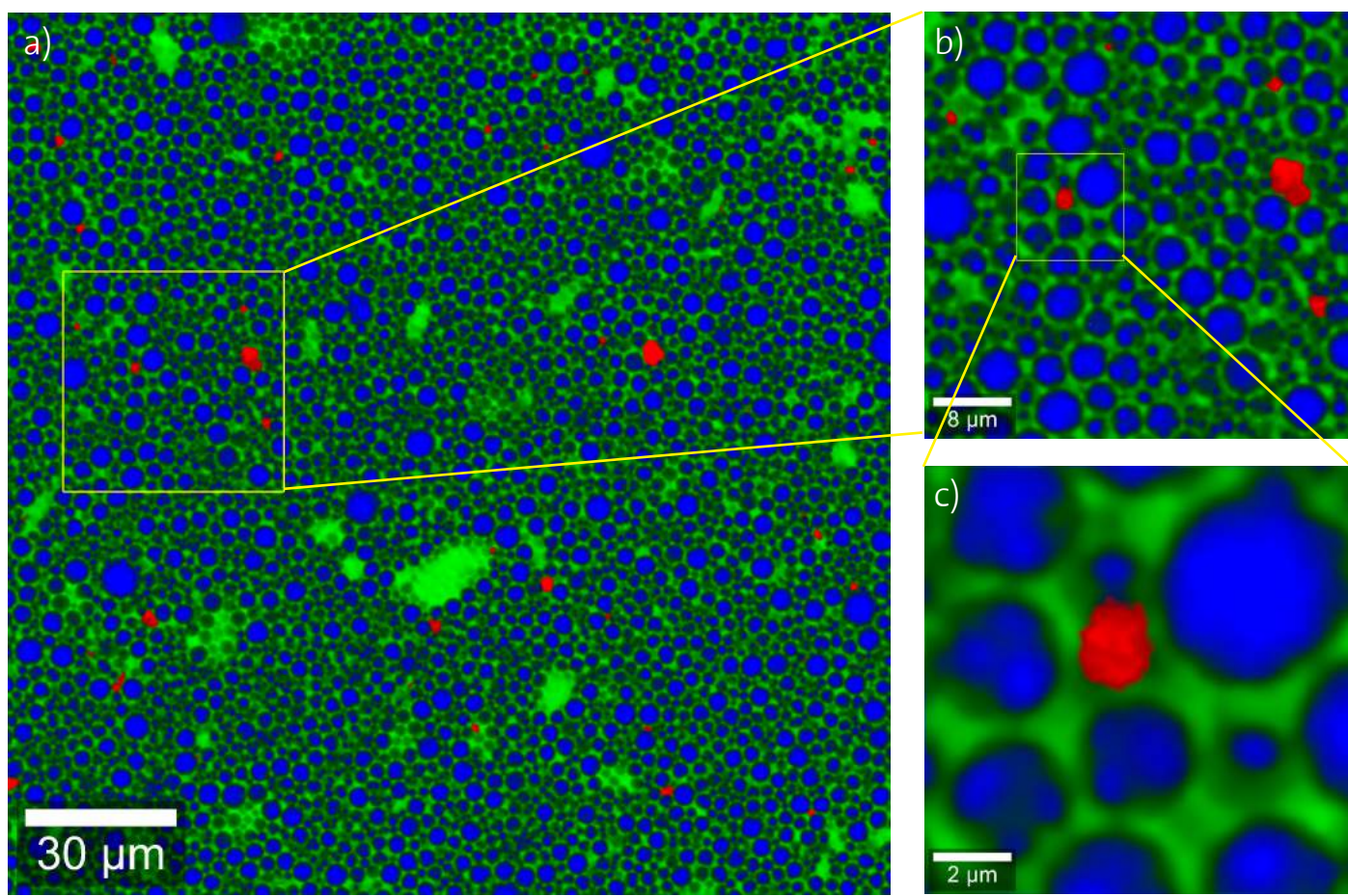


Figure 1: a) Large-area, high-resolution confocal Raman image of a pharmaceutical emulsion. Scan range: $180 \mu\text{m} \times 180 \mu\text{m}$; 2048×2048 pixels = 4,194,304 Raman spectra; raw data file size: 12.5 GB. Blue: active pharmaceutical ingredient; green: oil; red: silicon impurities. b) and c) consecutive zoom-in images of the same dataset.

the known materials, silicon-based impurities could be visualized (red in the images). Volume scans are a valuable tool for providing information about the dimensions of objects or the distribution of a certain compound throughout the sample. The generation of volume scans and 3D images always requires large data sizes. In order to generate 3D images, confocal 2D Raman images of parallel focal planes are acquired by scanning throughout the sample in the z-direction. The 2D images are then combined into a 3D image stack.

To investigate the volume of the impurities of the emulsion from figure 1a in more detail, a 3D scan was performed. For that a volume of $25 \times 25 \times 20 \mu\text{m}^3$ was analyzed with $200 \times 200 \times 50$ pixels. At each image pixel a complete Raman spectra with 10 ms integration time per spectrum was acquired. The image stack contains the information of a total of 2 million Raman spectra and is based on a raw data file size of 6 GB (figure 2).

In the following an example emulsion was studied. Therefore carbon tetrachloride (CCl_4) was mixed with an alkane, water and oil. A high-resolution 3D Raman image of the emulsion was acquired (figure 3). It shows the three-dimensional distribution of the components and reveals CCl_4 dissolved in the oil phase.

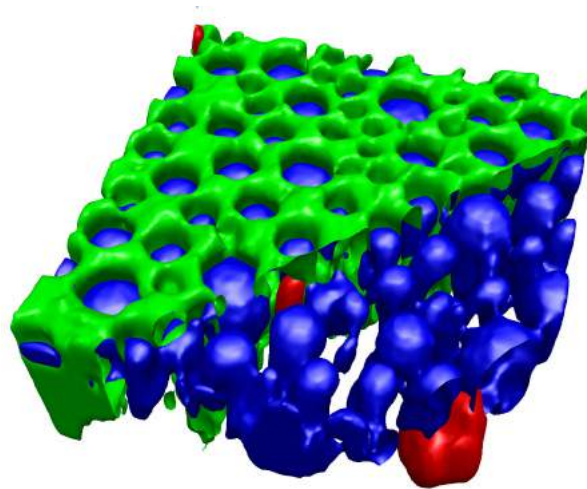


Figure 2: Confocal 3D Raman volume image. The green oil is partially removed in the image to facilitate a better identification of the red impurities. Scan range: $25 \times 25 \times 20 \mu\text{m}^3$, $200 \times 200 \times 50$ pixels = 2,000,000 Raman spectra, integration time per spectrum: 10 ms, data file size: 6 GB.

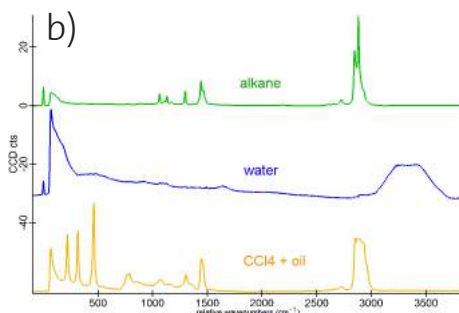
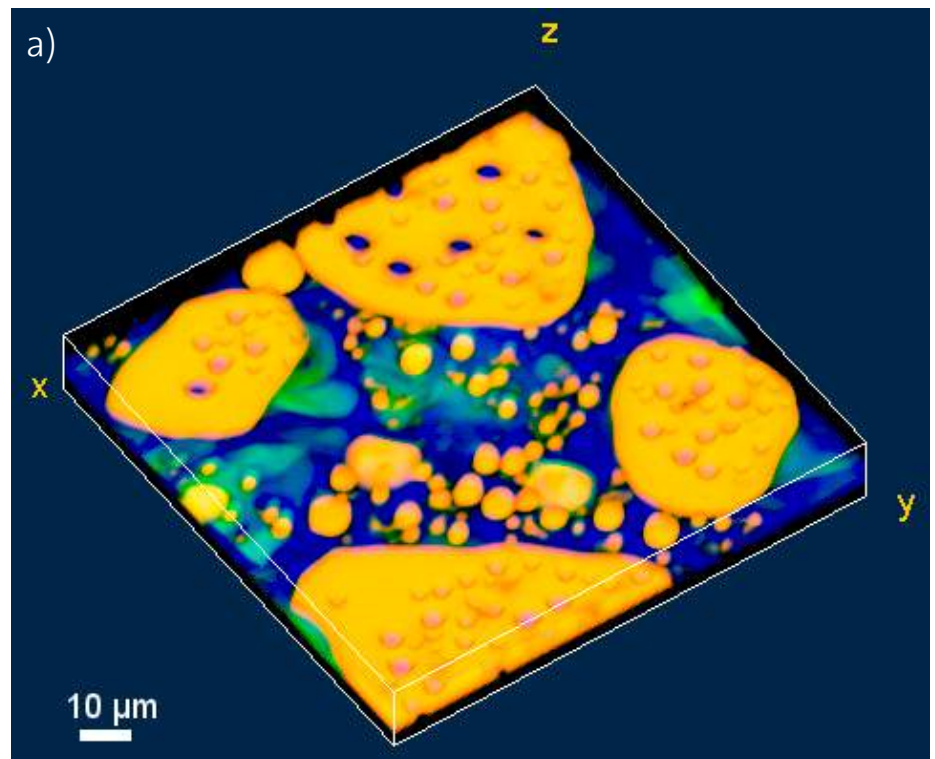


Figure 3: Confocal, high-resolution Raman microscopy of an emulsion of CCl_4 , alkane, water and oil. a) Three-dimensional confocal color-coded Raman image of the emulsion; alkane: green, water: blue, CCl_4 + oil: yellow. Image parameters: $200 \times 200 \times 20$ pixels; scan area: $100 \times 100 \times 10 \mu\text{m}^3$; integration time per spectrum: 0.06 s; excitation laser wavelength: 532 nm. b) corresponding Raman spectra.

Topographic Raman Imaging

A pharmaceutical tablet with height variations of 300 µm on the surface was investigated. Therefore topographic confocal Raman imaging was applied [5, 6]. Figure 4a shows the resulting height profile of the pharmaceutical tablet. The color code displays the varying heights. Following the sample's topography during confocal Raman image generation keeps

the sample's surface in focus over the entire scanned area without any Raman signal decrease. The resulting confocal Raman image reveals the distribution of the sample's components three dimensionally. Figure 4b shows the height profile with the chemical information from the confocal Raman measurement overlaid. The drugs are labeled red and blue respectively, while the excipient is shown in

green. In this way the generated 3D Raman image provides the full information about the chemical composition and additionally combines topographic and spatial sample characteristics. Figure 5 shows the results of the same method applied to study the distribution of the protein conformation of a highly structured lyophilisate section (for more information please refer to the image caption).

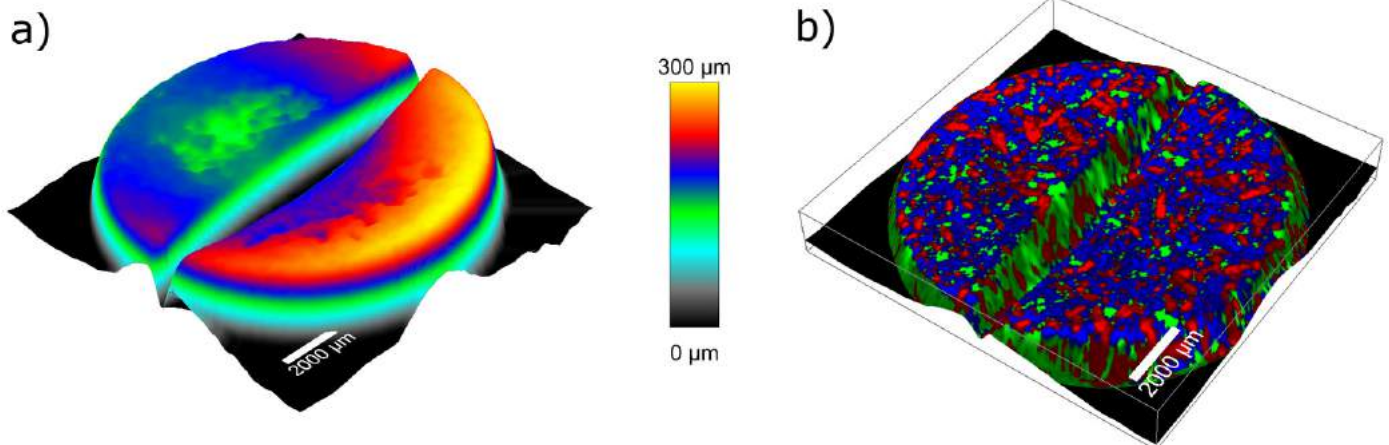


Figure 4: a) Height profile of a pharmaceutical tablet scanned with TrueSurface. The color code displays the varying heights. Topographic variation is more than 300 µm. b) Topographic profile from a) overlaid with the confocal Raman image. The drugs are labeled red and blue, while the excipient is shown in green.

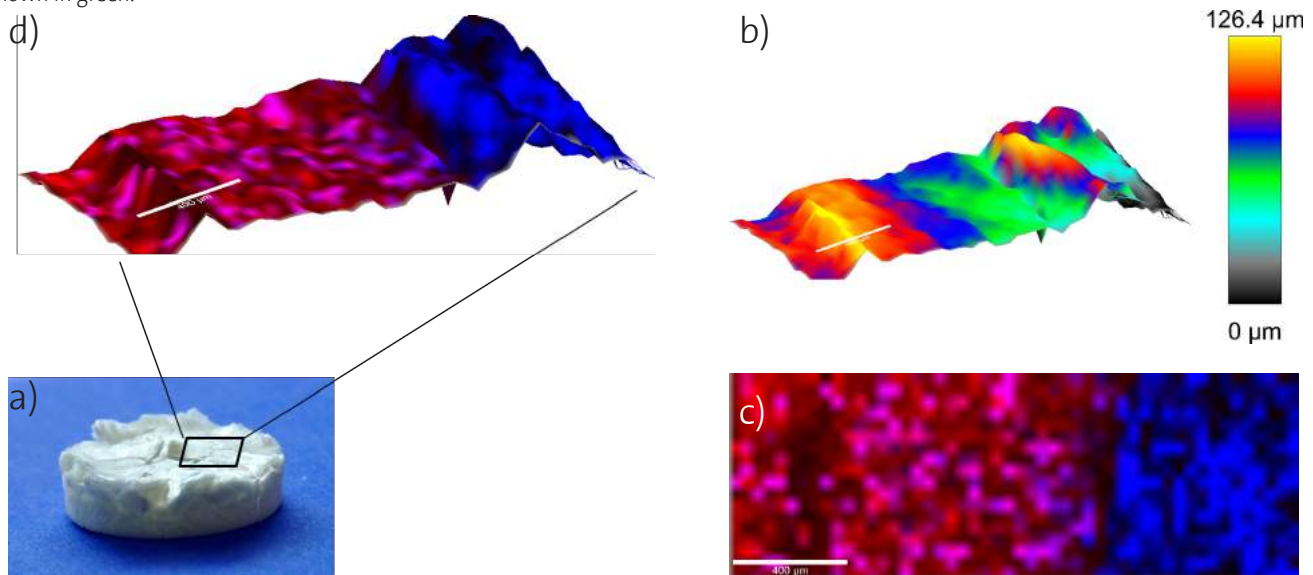


Figure 5: Investigation of the protein conformation of a highly structured lyophilisate section. (a) Lyophilisate image visualizing the highly structured surface. (b) Surface topography profile of a lyophilisate section acquired with TrueSurface® Microscopy. (c) Plain confocal Raman image of the investigated area. (d) Overlay of the topography profile with the corresponding confocal Raman image. The resulting three dimensional, color coded image indicates the native protein conformation in blue and the denatured protein conformation in red. Scale bars: 400 µm.

Raman Depth Profiling & AFM of a Drug Delivery Coating

For the characterization of drug delivery coatings, nondestructive confocal Raman imaging provides the ability to analyze the chemical composition and spatial distribution of the material within the coating. Due to the confocal principle even the thickness of these coatings can be measured. In figure 6a the spectral Raman image of a drug-eluting coating on top of a stent is shown. In this case the confocal Raman microscope was used to perform a depth scan along the x-z axis through the coating. As a result the chemical composition of the pharmaceutical sample as well as the distribution of each individual chemical compound can be spatially distinguished and displayed. In addition to Raman imaging the microscope allows AFM analysis. The obtained AFM image shows the drug distribution on top of the polymer substrate structure of the stent (figure 6b). The high-resolution AFM image provides a precise topographic description and provides additional information about the surface properties [7].

Ultrafast Confocal Raman Imaging

With the Ultrafast Confocal Raman Imaging option the acquisition time for a single Raman spectrum can be as low as 760 microseconds and 1300 Raman spectra can be acquired per minute. As a confocal Raman image typically consists of tens of thousands of spectra, the option reduces the total acquisition time for a complete image to only a few minutes. For example, a complete hyperspectral image consisting of 250 x 250 pixels = 62,500 Raman spectra can be recorded in less than a minute. The latest spectroscopic EMCCD detector technology combined with the high-throughput optics of a confocal Raman imaging system are the keys to this improvement, which can also be advantageous when performing measurements on delicate and precious samples requiring the lowest possible levels of excitation

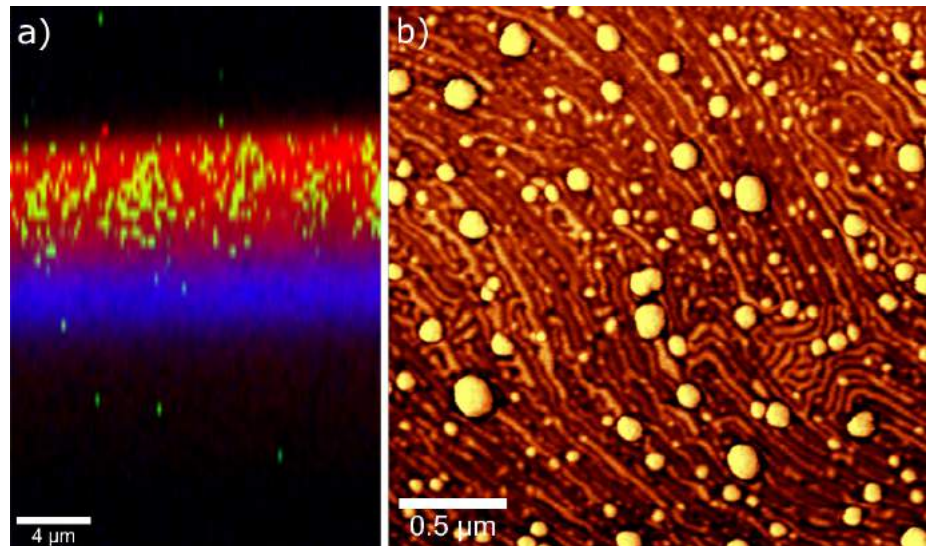


Figure 6: a) Confocal Raman image (depth scan x-z) of a drug-eluting stent. The drug-eluting coating (drug: green, polymer: red) on top can be distinguished from the substrate polymer layer (blue). b) High-resolution AFM surface topography of a drug-eluting stent surface showing the polymer substrate structure with the embedded drug particles.

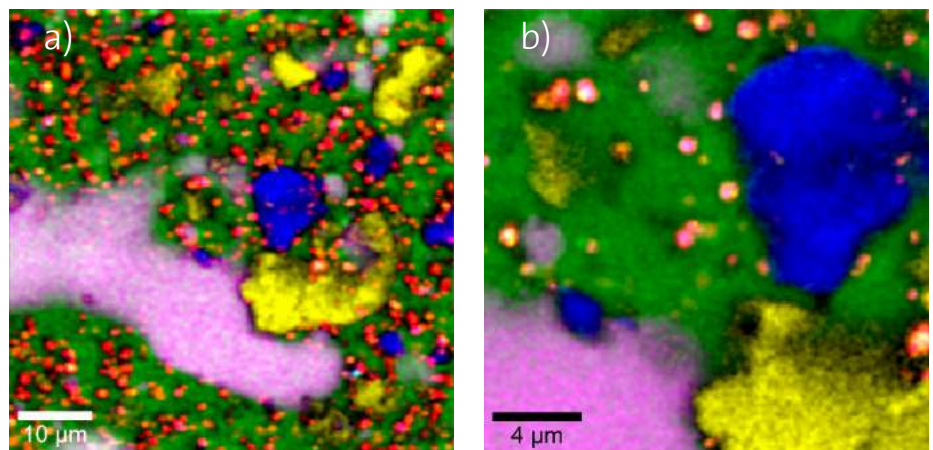
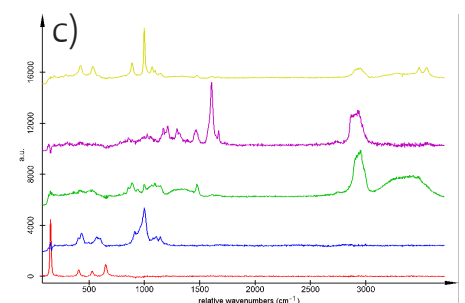


Figure 7: a) Raman image of toothpaste. Scan range: 60 x 60 μm^2 , 200 x 200 pixels, 40,000 spectra, 760 microseconds/spectrum, 42 seconds/image. b) Zoom-in of the marked area in Fig 5a, scan range 20 x 20 μm^2 , 200 x 200 spectra, 40,000 spectra, acquisition time: 0.76 ms/spectrum, 40 s/image. c) Corresponding Raman spectra.



power. Time-resolved investigations of dynamic processes can also benefit from the ultrafast spectral acquisition times. A standard, commercially available toothpaste was imaged using the Ultrafast Confocal Raman Imaging option. The scan range was $60 \times 60 \mu\text{m}^2$ and $20 \times 20 \mu\text{m}^2$ respectively at 200×200 pixels (40,000 Raman spectra). The acquisition time for a single spectrum was 760 microseconds, resulting in 42 seconds for the complete image. The Raman images in figure 7a and 7b show the distribution of the toothpaste's main compounds. Figure 7c shows the corresponding spectra.

Statistical Analysis - Dexpanthenol Ointment

For further detailed analysis of the distribution of a sample's chemical components it is useful to apply statistical methods. Cluster analysis is a typical example of a statistical evaluation method with which spectral characteristics can be extracted, classified and imaged. Furthermore histograms can serve as effective visualization tools to quantitatively evaluate spectral datasets.

In the following example two dexpanthenol ointments for different areas of topical

application (eye and skin) were analyzed. Alkane, oil and water were identified as the primary components of both ointments by confocal Raman microscopy (figure 8a). However, the confocal Raman images clearly show that the components occur in different concentrations in the ointments (figures 8b and 8c).

For further validation and a more detailed quantitative examination a histogram analysis was performed. The intensity of the detector counts was plotted against the number of pixels. Hence, the frequency of the appearance of a component (pixels) relative to its intensity (detector counts)

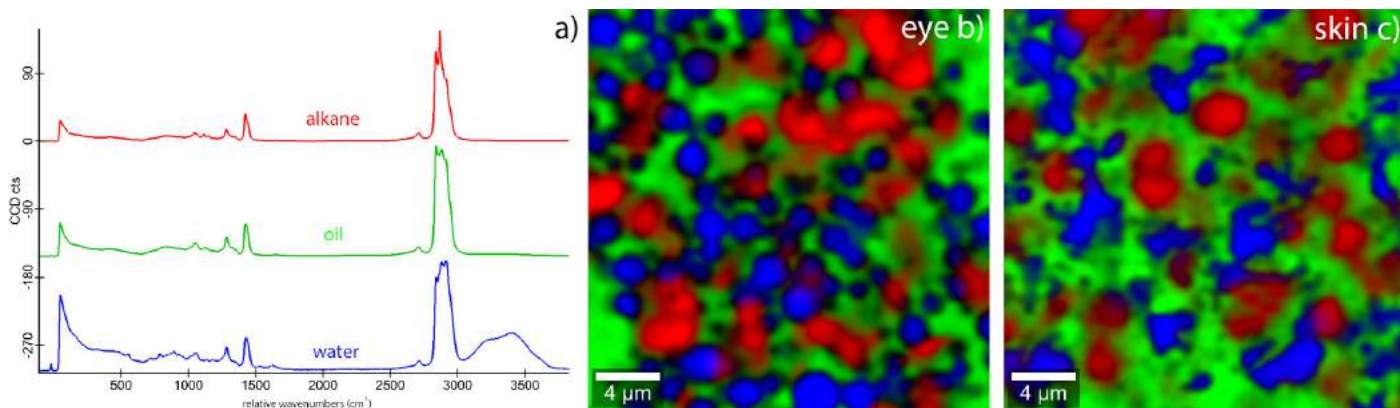


Figure 8: a) Example Raman spectra of the main components of two dexpanthenol-containing ointments for different areas of topical application. b) and c) corresponding confocal Raman images of the ointments (alkane red, oil green, water blue; $25 \mu\text{m} \times 25 \mu\text{m}$, 150×150 pixels = 22,500 spectra, 0.05 s integration time/spectrum, 532 nm excitation wavelength, 25 mW laser power).

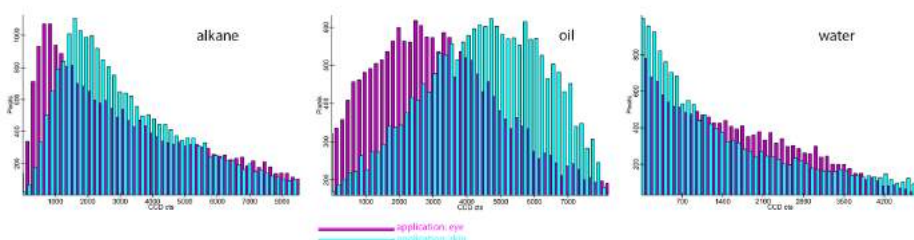


Figure 9: Histogram analysis of the intensity distributions of the main components (alkane, oil, water) relative to the area of topical application (eye in purple; skin in cyan) of two dexpanthenol ointments. The intensity of the detector counts (abscissa) is plotted against the number of pixels (ordinate).

is displayed. Figure 9 shows the resulting histograms of the two dexpanthenol ointments for the appearance of the main components: alkane, oil and water. The histogram analysis reveals that the components' mixture differs between the ointments depending on their areas of topical application. The eye ointment contains less oil, but on average more water. With statistical analysis of spectral datasets, pharmacists in research and development can gain insight into the chemical composition of a product and obtain significant information to improve the required pharmaceutical characteristics.

Conclusion

In pharmaceutical development it is of great importance to acquire information on drug composition and distribution of chemical components. With confocal Raman images the distribution of the chemical components in diverse drug systems can be clearly visualized. Combining CRM with other techniques such as topographic Raman imaging and AFM expands its analyzing capabilities. Thus, a variety of different pharmaceutical samples can be investigated and the chemical information from spectral Raman analyses can be linked to topographic data and further surface characteristics. CRM and its correlative analyzing methods contribute to a comprehensive understanding of a pharmaceutical product and facilitate its development.

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