1 Introduction

Constant progress in pharmaceutical sciences regarding the improvement and new development of drug delivery systems as well as *in vitro* test systems necessitates a simultaneous advancement in analytics as well. Here, analytical techniques with high flexibility and versatility without sacrificing high-precision detection especially for visual sample examination are beneficial to be established in the pharmaceutical context. Confocal Raman microscopy represents such a highly qualified analytical technique. It combines a label-free, non-destructive working principle with chemically selective, high-resolution visualization for contactless analysis.

1.1 Basic Principles of Raman Spectroscopy

Raman spectroscopy is based on the detection of scattered light occurring upon the irradiation of a sample with monochromatic light. The majority of scattered light is generally scattered elastically. This is referred to as Rayleigh scattering and the scattered light has the same frequency as the incident light (Figure 1). However, a small portion of the light is scattered inelastically at a different frequency than the incident light. This event is called spontaneous Raman scattering or Raman effect, named after the Indian Physicist Sir C. V. Raman, who was awarded the Nobel Prize in 1930 for this discovery. [1]

If a photon strikes a molecule in its ground state, a part of its energy can be transferred to the molecule allowing it to change its excited state. Thus, the photon loses a portion of its energy and is scattered at a longer wavelength compared to the incident photon (red shift). This is called Stokes scattering (Figure 1). In contrast, light interacting with a molecule which is in an already excited vibrational state will be scattered at shorter wavelength (blue shift). This opposite event is referred to as anti-Stokes scattering.

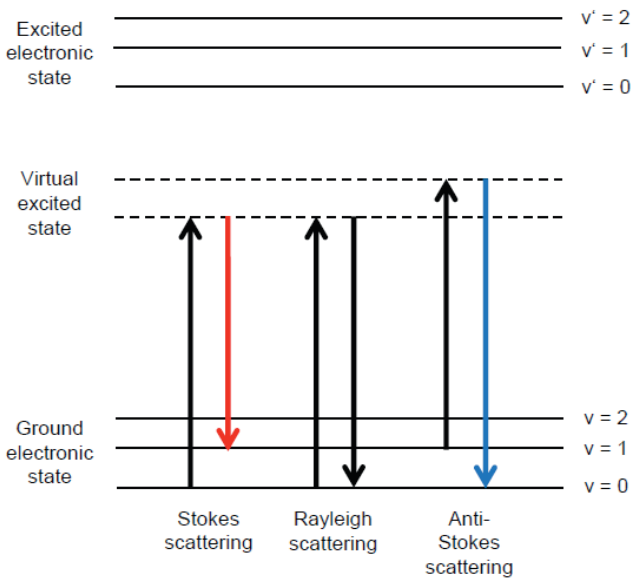

Figure 1. Energy level diagram of Rayleigh, Stokes and anti-Stokes scattering. The Raman effect comprises Stokes and anti-Stokes scattering.

The frequency shift between incident and scattered photon is equal to the frequency of the vibrational mode of the chemical moiety in the irradiated molecule. The resulting frequency difference is displayed in the Raman spectrum by plotting wavenumbers (reciprocal wavelengths) against the intensity of the scattered radiation (Figure 2). A Raman spectrum can be split into three parts. [2] Peaks at frequencies below 1800 cm^{-1} are highly specific for functional groups. Therefore, this region is referred to as the fingerprint region of the Raman spectrum. As barely any molecular bonds oscillate between 1800-2600 cm^{-1}, this spectral region is termed silent region. The high frequency region located above 2600 cm^{-1} is dominated by vibrations arising from carbon-hydrogen groups. Based on substance specific scattering, Raman spectroscopy is suited for chemically selective detection, enabling label-free compound identification.

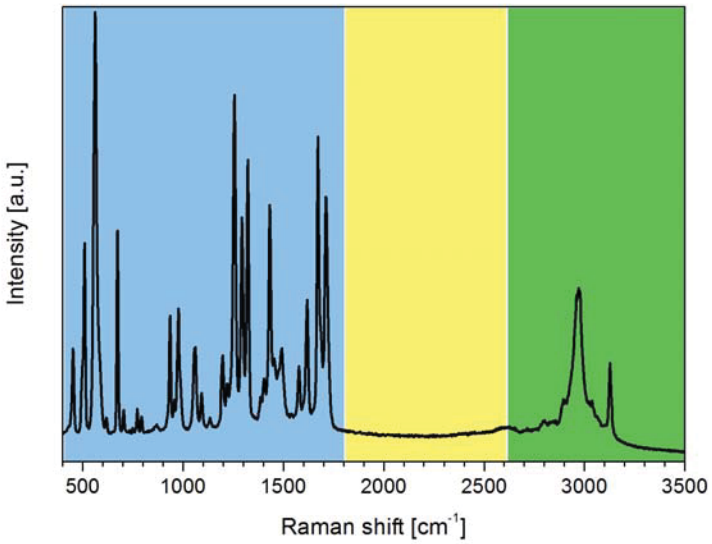

Figure 2. Exemplary Raman spectrum of a chemical compound. The fingerprint region is highlighted in blue, the silent region in yellow and the high frequency region in green.

The Raman activity of a compound is determined by its change in polarizability upon interaction with the alternating field of laser light (electromagnetic field). The more elastic the electron cloud of the molecule, the higher the Raman activity.

In spontaneous Raman spectroscopy, Stokes scattering is predominantly detected, as anti-Stokes scattering occurs rarely because the chance of striking a molecule in an excited vibrational state is low. Nevertheless, the overall probability of observing Raman scattering is by far lower than for example the observation of fluorescence. However, significant advancements in lasers and detection technology have made Raman spectroscopy an effective analytical tool.

1.2 Confocal Raman Microscopy for Chemical Imaging

The combination of Raman spectroscopy with optical microscopy is entitled Raman microscopy. Thereby, chemically selective detection is united with spatially resolved analysis.

The center piece of a Raman microscope is a conventional optical microscope as sampling device. It is equipped with a laser light source and a detection unit composed of a spectrometer and a detector. The laser light is focused onto the sample through an objective and the

scattered light is detected to record the Raman spectra. By implementing a confocal microscope, out-of-focus light is rejected by the pinhole. Thereby, lateral and more importantly depth resolution of the Raman microscope increase and background signals are effectively reduced. The spot size of the irradiated volume defines the spatial resolution of the confocal Raman microscope. As shown in the Abbe equation (Equation 1) this diffraction limited spot size (d) is determined by the excitation wavelength (λ) and the numerical aperture (focal length, NA) of the objective. [3]

$$d = \frac{\lambda}{2\,NA} \qquad (Equation\ 1)$$

Confocal Raman microscopy delivers spatially resolved chemical information of a sample by probing intrinsic properties of the molecules while irradiating the sample with monochromatic laser light. Subsequently, a color-coded distribution map of the sampled area can be generated from the recorded spectral data set to elucidate the composition of the sample. The color-code assigned to a spectral band of a component is depicted as image pixel which represents the spatial element in the false color map equal to the location from which the Raman spectrum was recorded. This is referred to as chemical imaging.

The principal procedure of creating a chemical image is schematically illustrated in Figure 3. Exemplarily, an extrudate composed of an active pharmaceutical ingredient (API) embedded in a lipid matrix is shown. The Raman spectra are recorded by stepwise rasterizing the sample with laser light. Next, a different color is assigned to the spectrum of each component. In this case, the lipid matrix spectrum is depicted in blue, whereas the API's Raman spectrum is portrayed in red. All spectral data points recorded from the sample represent one or the other compound. Thus, each spectrum is translated into an image pixel with the aforementioned color classification. Thereby, two separate false color images are constructed, each representing one compound within the extrudate cross section. By merging the two images, an overview of the component distribution within the drug delivery system is obtained.

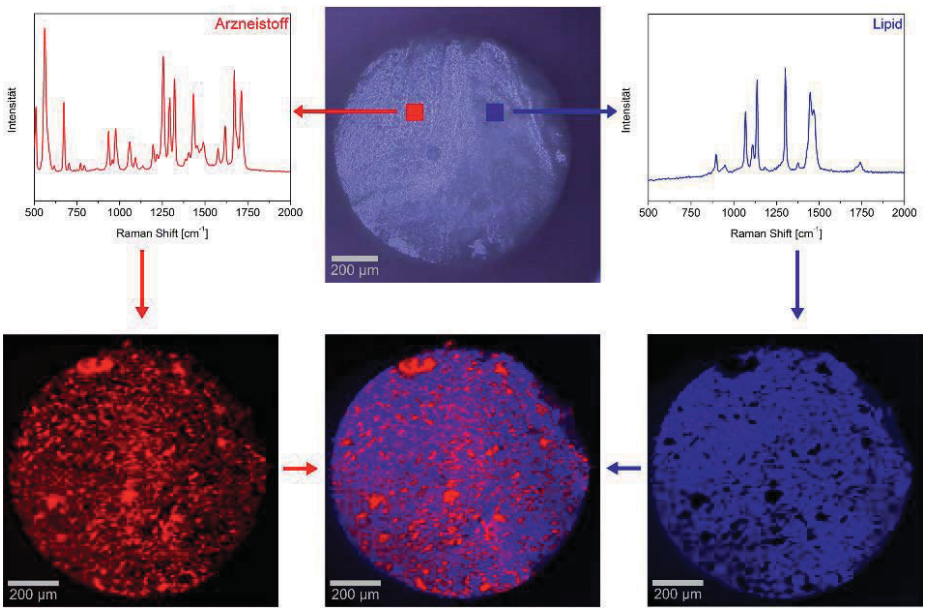

Figure 3. Schematic illustration of the sequentially performed steps to create a false color map from a Raman spectral data set. Spectra assigned to API are depicted in red, whereas Raman spectra assigned to the lipid matrix are shown in blue. This figure was first published in TechnoPharm 3, No. 3 (2013) 146-149 and is reprinted with permission. [4]

Chemical images can be acquired by point mapping, line scanning or global illumination. While the latter requires no sample transition, this is needed for point mapping and line scanning. [5] Here, the sample is moved through the focal point of the probing laser beam in a predefined raster in order to collect the spectra from the individual sample positions. Consequently, the terms ‘imaging’ (stationary sample) and ‘mapping’ (sample transition) are discussed controversially although they are generally used synonymously. [6-8]

The recorded hyperspectral data set contains manifold information about pixel coordinates where the spectra were collected, the wavelength as well as the intensity of the scattered radiation. Thus, the transformation from a spectral data set to a color-coded image necessitates the processing of the acquired raw data. The first step is often referred to as pre-processing and aims at removing spectral artefacts originating from experimental conditions. [5, 9, 10] For example, Raman peaks generated by cosmic rays are eliminated from the data set. Further, the baseline of the spectra is corrected by reducing the background noise. Additionally, the sample surface texture may lead to the detection of varying spectral intensities across the sample. To account for this, the scattering intensity may be normalized.

The pre-processed spectral data set is then further processed by chemometric tools to generate the color-coded chemical image. Univariate methods only consider one component and require therefore a component specific spectral band which is preferably rather prominent in the spectrum. More importantly, it cannot be masked or interfered by any other compound in the sample. Either the position, the intensity (height) or the area (integral) of the identified band in combination with the spatial coordinates are the basis for constructing the map. In contrast to univariate data analysis, the multivariate approach employs all spectral information from the data cube. [9, 11] The scores or parameters which are yielded for each Raman spectrum by these multivariate methods are subsequently plotted as a function of the spatial coordinates of each spectrum in the sample. [9] The most prominent algorithms comprise two complementary approaches. Cluster algorithms sort all spectra of the data set based on similarity of the spectra, whereas unmixing algorithms are sorting according to greatest dissimilarities finding the most extreme spectra. In multivariate data analysis the algorithms can work supervised or unsupervised. The latter is impossible for univariate methods, as comparison with reference data is necessary to identify the component specific spectral band. [5, 9] Especially for the analysis of low content samples, multivariate processing is needed as shown in an imaging study by Šašić et al. [12] Here, univariate methods failed to construct reliable chemical images of low alprazolam loaded tablets as the drug’s Raman signal was too weak.

Further spectroscopic techniques which are also suited for chemical imaging are infrared microscopy and fluorescence microscopy. Infrared microscopy is another vibrational spectroscopic technique. Unlike confocal Raman microscopy it is based on light absorption and not on scattering (Figure 4). When referring to IR spectroscopy, it has to be differentiated between near and mid infrared spectroscopy. While near infrared light (λ = 800 nm - 2,5 µm) excites vibrational overtones and combinations of vibrations which lead to broad and overlapping spectral bands, mid infrared light (λ = 2,5 – 50 µm) generates sharp individual bands. The most prominent example to demonstrate the difference between IR and Raman spectroscopy is the water molecule. The water molecule is a strong dipole. It shows an intense IR activity. The water absorption band covers nearly all other IR bands in the spectrum and impedes IR analysis of other molecules in aqueous surroundings. As Raman scattering depends on a change in polarizability of the molecule and not on a change in dipole, Raman microscopic investigations of wet samples or in aqueous surroundings are feasible.

Consequently, strong IR activity is often equal to weak Raman activity and vice versa. Furthermore, the resolution in confocal Raman microscopy is superior to IR microscopy due to the long excitation wavelength necessary for IR investigations. [13]

The chemical selectivity of fluorescence microscopy is based on the detection of emitted light (Figure 4) which originates in the majority of cases from a marker molecule that has been linked to the compound of interest and not from the constituent itself. However, the labeling process represents both, the up- and downside of the technique. On the one hand, it provides the great opportunity for differentiated sample visualization, which made fluorescence microscopy the state-of-art analytical technique for biological applications. On the other hand, labeling of molecules bears the risk of altering their physicochemical properties leading to data misinterpretation. Further, the fluorophore might not sufficiently bind to the target molecule and migrate within the sample. In general, the number of analytes is limited to the microscopic setup, as each analyte requires a different excitation wavelength in order to detect multiple compounds simultaneously. Moreover, many fluorescent dyes are susceptible to photo-bleaching hampering long term analysis. Nevertheless, some components fluoresce intrinsically and can be probed directly. When a sample with an autofluorescent component is investigated by spontaneous Raman microscopy, Raman bands are generally obscured, because the autofluorescence of a molecule is much stronger than its Raman scattering intensity. By increasing the excitation laser wavelength, the chance of exciting fluorescence decreases, because the energy of the incident photon is reduced. Thus, the excitation to the higher electronic energy state causing fluorescence becomes less likely. Further, photo-bleaching or quenching are alternatives to circumvent intruding fluorescence during Raman analysis.

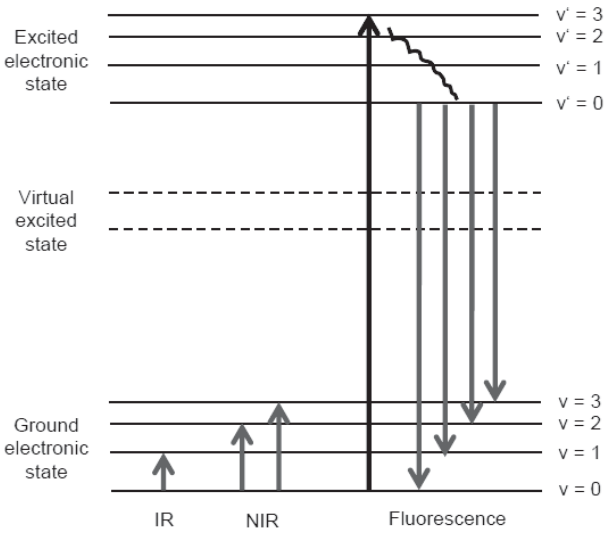

Figure 4. Energy level diagrams of IR and NIR absorption and fluorescence.

Other techniques, which are often employed to depict pharmaceutical samples, are atomic force microscopy (AFM) and scanning electron microscopy (SEM). Both of them do not offer chemically selective visualization of the complex composition of samples, but provide morphological information with extreme high resolution in the low nanometer range. However, they are restricted to the analysis of outer surfaces. Whereas AFM enables direct sample analysis on a very small scale, samples for SEM analysis generally have to be sputter-coated first. Consequently, the sample in its native state cannot be analyzed repeatedly as the applied

metal layer impairs subsequent processing steps. Both AFM and SEM have been successfully combined with Raman microscopy. The analytical AFM - Raman combination is known as tip-enhanced Raman spectroscopy (TERS), whereas only recently a correlative microscope for SEM and Raman analysis was introduced to the market.

Overall, spontaneous confocal Raman microscopy represents an advantageous and highly beneficial alternative to established analytical techniques or complements them. In contrast to staining or sputter coating processes, confocal Raman microscopy requires little if any sample preparation. It is based on a non-destructive working principle and allows repeated examination. Here, analysis in not limited to external areas, as virtual planes can be spanned through the sample depending on its opacity. Consequently, consecutive steps such as multiple processing steps can be monitored in air as well as in aqueous surroundings.

Nevertheless, the acquisition of spectral data sets using spontaneous confocal Raman spectroscopy can be time consuming due to the rare occurrence of Raman scattering. Here, non-linear Raman technologies such as stimulated Raman spectroscopy (SRS) and coherent anti-Stokes scattering (CARS) microscopy are alternatives. The linear correlation of incident light and scattering intensity in spontaneous Raman scattering is abolished, as multiple photons are involved in generating these non-linear processes. As a consequence, the probability of light scattering increases and the Raman signal is enhanced reducing the time for analysis.

In SRS, two laser beams at different frequencies (ω_{pump} and ω_{Stokes}) are irradiating the sample. The excitation of a vibrational transition is stimulated when the frequency difference corresponds to a molecular vibration of the sample. The SRS signal is detected as a loss or gain in energy of one of the incident laser beams.

In CARS microscopy the probed anti-Stokes scattering is detected. It is generated in a four photon process (Figure 5). Two laser beams irradiate the sample with photons at different frequencies (ω_{pump} and ω_{Stokes}) simultaneously. A third photon, which is usually of the same frequency as the pump photon and therefore originating from the same source, interacts with the molecule as well and the anti-Stokes photon as the fourth photon is generated. When the frequency difference between the pump photon and Stokes photon coincides with a molecular vibration of the sample, the CARS signal is resonantly enhanced. This process is coherent as all transitions are driven.

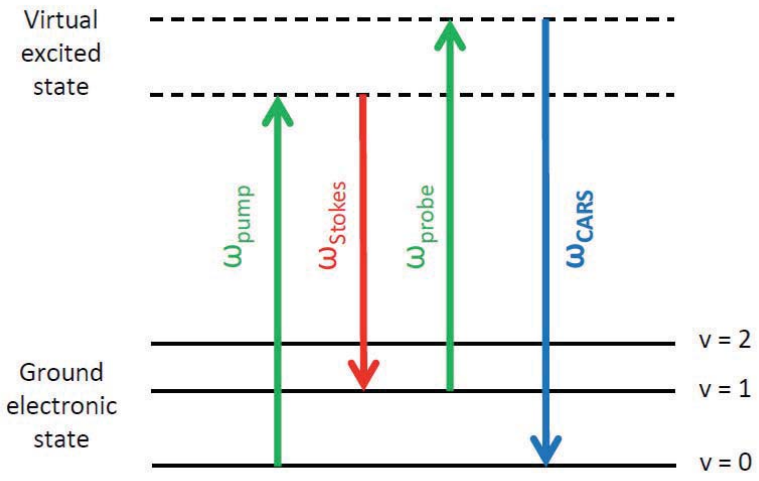

Figure 5. Energy level diagram of the CARS signal generating process.

As the frequency difference is tuned to coincide with a specifically chosen vibrational resonance, different molecules of interest can be visualized instantaneously. This is exemplarily demonstrated by CARS imaging of a transparent polymeric bead mixture composed of polystyrene and polymethylmethacrylate (PMMA) beads at different Raman shifts shown in Figure 6. The surrounding structure is revealed by a non-resonant background which accompanies the CARS signal. The spectral information is reduced in CARS imaging because only one vibrational resonance is probed at a time. In contrast to long integration times in spontaneous Raman microscopy where the full spectral information is collected, this spectral expense facilitates fast image acquisition up to video rate. Consequently, *in situ* processes in a sample can be visualized. The signal-generating process makes non-linear Raman techniques inherently confocal, and fluorescence is not interfering.

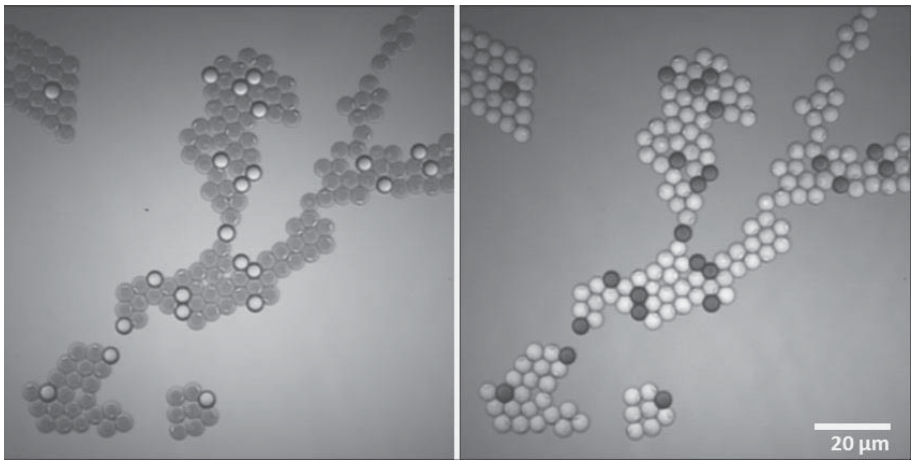

Figure 6. Chemically selective CARS images of a transparent polymeric bead mixture. Polystyrene beads (left panel) and PMMA beads (right panel) are depicted with high precision.

Depending on the sample and the focus of investigation, different variations of Raman microscopy are available for chemical imaging. Regular confocal Raman microscopes have become commercially available due to immense technical progress ever since a first self-built regular confocal Raman microspectrometer was reported in 1990. [14] Although the Raman effect was described as early as 1928 [1], non-linear Raman techniques such as CARS and SRS were first reported as early as 1982 and 2007, respectively. [13, 15] These microscopes are still predominantly reserved to specialized laboratories, which limits their availability to the general research community. However, the techniques are on the verge of being marketed commercially besides their linear counterpart.

1.3 Pharmaceutical Applications of Confocal Raman Microscopy

The variety of pharmaceutical samples is large. It reaches from classic oral solid dosage forms like tablets to modern drug delivery systems on the nanometer-sized scale. Cell culture based and artificial *in vitro* test systems are comprised as well as drug coated medical products. As a versatile analytical technique, confocal Raman microscopy is generally suited to cover this manifold sample spectrum. However, its application only started to emerge in the pharmaceutical community over the last decades. This is mainly due to the immense progress in laser technology and the development of sensitive detection units, which led to the commercial availability of confocal Raman microscopes and therefore to a greater access to

Raman analysis. Despite the longtime utilization of Raman spectroscopy, confocal Raman microscopy has not yet become a standard analytical technique.

One crucial parameter hampering a broad use is the challenging interpretation of the spectra. For confocal Raman microscopy, the recorded hyperspectral data set requires explicit knowledge about spectral processing methods to generate reliable false color images.

As the majority of employed raw materials (both excipients and active pharmaceutical ingredients) for manufacturing drug delivery systems is simply white, visual component discrimination in the final delivery system becomes impossible due to the uniform appearance. Similarly, biological systems show the same difficulty. While drug delivery systems, especially the classic solid ones, are opaque, biological samples rather show a gradual transparency. Consequently, color-coded chemical images of pharmaceutical samples are highly desirable to gain an in-depth understanding of the often complex structures and to elucidate fabrication and interaction processes. Such visualization approaches can add valuable information to an ongoing study because of clarifying the context of puzzling analytical results.

The drug distribution in a delivery system impacts the drugs dissolution rate and consequently its bioavailability. In this context, chemical imaging is of great value to evaluate changes in drug distribution before and after dissolution experiments to understand the release mechanism in order to design delivery systems with tailor-made release profiles. Matrix systems are often utilized when sustained release is requested. The effect of adding a pore former in an extruded solid lipid matrix system on drug dissolution was observed by Raman mapping. [16, 17] Pores started to form immediately in the water insoluble lipid matrix upon contact with the dissolution medium as visualized in comparative false color maps of the drug delivery system before and after dissolution testing. Consequently, the contact area for the dissolution medium with the API increased and the drug was released significantly faster than from extrudates which were lacking the pore former. Here, Raman images barely showed any difference in component localization after dissolution.

Solution-mediated solid-state transformations have been observed spectroscopically *in situ* during dissolution. As these processes occur at the interface of delivery system and dissolution medium, they can be visualized by Raman microscopy. However, spontaneous Raman microscopy is too slow to image these rapid transformations. Here, coherent anti-Stokes Raman scattering microscopy has been successfully employed to monitor the transformation of the model drug into its monohydrate. [18-20] Due to the generation of a coherent signal, events can be followed with video rate.

Active coatings are not only applied to oral solid dosage forms but also to biomedical implants to deliver drugs to specific body regions. One popular example are stents, which are covered with drug-eluting coatings to locally prevent restenosis. [21] Here, the analytical strength of confocal Raman microscopy becomes evident as it facilitates the non-destructive visualization of the polymeric coat and the embedded drug throughout the entire thickness of the film coating in virtual cross section images. [22] Thus, API diffusion during stent elution was characterized without laborious sample preparation, and segregation of drug enriched regions inside the coating was detected. Another study by Dong et al. [23] depicted the distribution of rapamycin in another therapeutic coating by exploiting all axes of the Raman microscope for chemical imaging. Lateral images showed a homogenous distribution of rapamycin domains in the polymer, whereas cross section images portrayed the formation of drug columns spanning vertically through the coating. The acquisition of lateral scans at different distances from the water coating interface depicted a clear drug migration towards this interface during elution.

The recording of spectral data sets was performed with a water immersion objective to capture drug diffusion during the actual elution process. Due to the aqueous surroundings, IR investigations of the coating are impossible. To gain deeper insight in the effects of drug elution on surface properties of drug-eluting stents, confocal Raman microscopy analysis was correlated with atomic force microscopy studies. [24] Whereas Raman imaging facilitates the surface and subsurface characterization of chemical distribution, AFM complements surface analysis by high resolution profiling. Thereby, the drug elution dependent change of voids in the coating is visualized. Three dimensional drug redistribution during its release from polymers used for stent coatings was visualized *in situ* by depth resolved CARS imaging. [25]

These studies on stents nicely demonstrate the potential and versatility of Raman microscopy for non-destructive analysis along all three axes of the microscope, which facilitates access to internal sample structures. As the polymer used for coating is gradually transparent, virtual planes can be spanned within the coating. Thus, the laser light penetrates through the material and Raman signals are recorded from within the sample without destroying it. Thereby, internal structures of the sample as well as e. g. changes upon drug elution were successfully characterized.

Although especially the field of classic drug delivery system is predestined for the analysis with linear as well as non-linear Raman techniques as the molecules are generally strong Raman scatterers, the number of studies employing Raman imaging is low. This is mainly due to the fact that IR microscopy is a long established technique in this area of pharmaceutics and samples are rather large, which often does not necessitate the use of analytical techniques with higher resolution. However, the few studies employing both vibrational techniques demonstrate the potential benefit of adapting confocal Raman microscopy in this field on a larger scale.

A comparative chemical imaging study of a tablet with five components showed the superiority of Raman microscopy over NIR microscopy. All five components were retrieved by multivariate data analysis of the Raman spectral data set, whereas a maximum of three compounds was identified in the NIR spectral data set. [26] Furthermore, the distribution of an API with a content of less than 1% was shown by Raman mapping. [12] Model tablets containing polymeric beads of defined sizes demonstrated the accuracy of Raman chemical imaging to evaluate API particle sizes within tablets. [27] The domain size of API in a chemically selective map of a tablet with an API concentration of 0,5 % w/w was shown to be dependent on the size distribution of raw API material. [28] Even polymorphic impurities were still detectable from the Raman spectral data set. Due to broad overtone bands, NIR spectroscopy is not accurate enough for polymorph detection. This is however possible in MIR spectra, but the low dose API causes detectability problems as signals from excipients are competing with API bands if not even shadowing them. Consequently, confocal Raman microscopy is a suited alternative technique showing high precision. Besides, the detectability of amorphous compounds in multi-component solid mixtures by Raman as well as NIR spectroscopy was shown in several studies. [29-31]

NIR and Raman spectroscopy are also competing as process analytical technology (PAT) tools. Its goal is to gain fundamental understanding of fabrication processes, which are often a black box up to today. Here, vibrational molecular spectroscopy can contribute significant insight into fabrication processes as fiber-optic probe instruments for NIR and Raman spectroscopy can be implemented into process streams of various unit operations for continuous real-time monitoring in a non-destructive manner.

Especially probing in aqueous surroundings pointed at the different analytical focus of the two techniques. While NIR spectroscopy is sensitive to different water states, Raman spectroscopy solely probes the molecular vibrations of the analyte. Thus, the stepwise dehydration of theophylline monohydrate during fluid-bed drying was monitored on line by following the free water content as well as lattice-bound water. [32] On the contrary, Raman spectroscopy was shown to be superior to NIR spectroscopy during wet granulation. [33] The broad water band in the NIR spectra covered the peak which indicated the hydrate formation of theophylline (1686 cm^{-1}). The sensitivity of Raman spectroscopy for structural changes of drug molecules in water rich environments was also demonstrated in a solution-mediated crystallization study of carbamazepine. [34]

Besides the investigation of solid states, Raman spectroscopy as a PAT tool was applied to observe other parameters of pharmaceutical production processes. Blend homogeneity was determined in powder mixtures as well as in aqueous suspension. [33, 35-37] Furthermore, coating processes of tablets were spectroscopically analyzed by correlating Raman spectra to coating time representing the weight gain of the tablet. [38-40] Active coating processes were also successfully evaluated. [41, 42] The endpoint of coating was determined by detecting the API quantity applied with the coating. [41] Raman spectroscopy was also feasible to monitor the active coating of a two layer tablet which contributes different spectral bands to the signal depending on the side facing the laser beam. [42] By implementing Raman probes in the extruder barrel during hot melt extrusion, profound process understanding regarding the influence of different settings and formulations on the solid state of polymer and drug was gained and in-line drug quantification was possible. [43-46] The development of protein drug delivery systems, which are often manufactured by lyophilization, is steadily increasing. The therapeutic performance of such protein formulations is highly dependent on the protein's physical stability. Here, Raman spectroscopy was used *in situ* to investigate the degree of protein denaturation and thus inactivation, based on process parameters during freeze drying and stabilization strategies using trehalose. [47, 48] Further, the suitability of Raman spectroscopy as sensitive PAT technology was shown by determining the secondary structure of fourteen model proteins during freezing, thawing as well as during frozen storage. [49]

Although the variety of investigated samples indicates the potential of confocal Raman microscopy, the field is still wide open. Besides its application for the characterization of classic solid drug delivery systems and for quality assessment, another rapidly evolving field are biological systems.

In this regard, rapidly evolving therapeutic concepts for curing diseases on the subcellular level have resulted in developing novel, small so-called modern carrier systems for adequate intracellular drug delivery. As IR microscopy suffers from poor spatial resolution of several micrometers and as water shows an interfering strong IR absorption, its application is limited for biological investigations. The analysis of cell culture models and their interaction with the micro- to nanometer sized drug carriers as well as potential internalization is predominantly performed by fluorescence microscopy as state-of-the-art technique in the biomedical field. However, recently investigations including single cell imaging as well as drug carrier uptake visualization with Raman microscopy emerged. In fact, Puppels et al. [14] introduced confocal Raman spectroscopy for the analysis of single living cells and chromosomes already in 1990. Cell cycle phases were spectroscopically determined *in situ* as well as apoptotic changes. [50, 51] Further, various cell types and their subcellular structures were imaged solely based on molecular vibrations of chemical moieties inside the cell. [52-57] The investigations were not limited to any specific cell type and included human embryonic stem cells as well as diverse