CARS microscopy as a tool for studying the distribution of micronised drugs in adhesive mixtures for inhalation†

Andrew L. Fussell,a* Floris Grasmeijer,b† Henderik W. Frijlink,b Anne H. de Boerb and Herman L. Offerhausa*

Drug particles can be produced in the proper aerodynamic particle size distribution (PSD) for inhalation by techniques such as micronisation or spray drying (1–5 μm). However, mixing with coarse carrier particles may change the PSD by agglomeration. Furthermore, the spatial distribution of the drug particles on the carrier particles in adhesive mixtures is highly relevant to the dispersion performance of inhalation powders. Coherent anti-Stokes Raman scattering (CARS) microscopy is capable of chemically selective imaging, allowing the distribution of drug particles on the surface of carrier particles to be visualised. We used CARS microscopy to image the drug distribution for budesonide and salmeterol on the surface of lactose carrier particles. Image analysis was performed to determine the drug PSD that was then compared with the PSD obtained from laser diffraction. Additionally, comparative CARS and scanning electron microscopy (SEM) images were recorded to allow a direct comparison of the images obtained from CARS microscopy and from SEM. CARS microscopy revealed the drug to be in clusters on the surface of the carrier particles, while the image analysis identified 68% of the particles to have a median area of 0.4 μm². Image analysis resulted in measurement of larger particles than laser diffraction, which may be caused by agglomeration during mixing. The combined chemical and morphological information from comparative CARS and SEM analysis resulted in unambiguous identification of the spatial drug distribution over the carrier surface. Our results indicate that CARS microscopy is a useful tool to study adhesive mixtures for inhalation. Copyright © 2014 John Wiley & Sons, Ltd.

Additional supporting information may be found in the online version of this article at the publisher’s web site.

Keywords: powder for inhalation; coherent anti-Stokes Raman scattering microscopy; scanning electron microscopy; drug distribution; particle size distribution

Introduction

CARS and hyperspectral CARS microscopy

Coherent anti-Stokes Raman scattering (CARS) is a third-order nonlinear optical technique that probes the same molecular vibrational frequencies as spontaneous Raman techniques. Coherent Raman techniques such as CARS have about 100 times faster imaging speed when compared with spontaneous Raman mapping techniques.[1] Spontaneous Raman techniques collect information over a wide spectral range, while narrowband coherent Raman techniques collect information from only a single Raman shift. Broadband coherent Raman techniques are able to probe a wider spectral region but with a reduced spectral resolution.[2] To compromise, hyperspectral narrowband CARS imaging techniques have been developed allowing increased chemical specificity.[3] As CARS produces anti-Stokes shifted signal (blue shifted with respect to excitation pulses), it is free from single-photon fluorescence that hampers spontaneous Raman measurements.

Microscopes that employ the CARS technique allow imaging with chemical specificity and selectivity have been widely applied in biological and medical imaging.[4,5] So far, the application of CARS microscopy to pharmaceutical systems has been scarce. Kang et al.[6–8] imaged in situ release of paclitaxel from polymeric films in a static medium using CARS microscopy. In the first work focusing on orally administered drugs and dosage forms, Windbergs et al.[9] and Jurna et al.[10] used CARS microscopy to image the distribution of theophylline in lipid dosage forms. Additionally, they monitored the release of theophylline during dissolution in a flow through cell set-up. More recently, the capabilities of broadband CARS for imaging of indomethacin tablets were demonstrated by Hartshorn et al.[11]

Adhesive mixtures for inhalation

Adhesive mixtures for inhalation are pharmaceutical systems that are well suited for chemical-specific imaging by CARS microscopy. These powder mixtures contain drug particles in the aerodynamic size range of 1–5 μm. Only such small particles can effectively reach
the target deposition sites in the airways on inhalation. To improve the dose reproducibility of the fine and thus cohesive drugs, they are mixed with relatively coarse alpha-lactose monohydrate particles. The lactose excipient dilutes the drugs and improves the flowability of the powder, which enables reproducible metering of doses as low as only a few micrograms. During mixing, the small drug particles will adhere spontaneously to the large lactose ‘carrier’ particles, predominantly by means of Van der Waals forces. This adhesive behaviour is favourable with respect to content uniformity and physical stability of the mixtures, but the ‘adhesive units’ are too large for effective transport to the target deposition sites in the airways. Hence, the performance of adhesive mixtures for inhalation depends on how well the drug particles are detached from the carrier during inhalation and consequently dispersed into the inhaled air stream.

Despite many research efforts, the exact relationship between formulation variables and the dispersion performance of adhesive mixtures is to a large extent unknown. This is partly because of difficulties concerning the qualitative and quantitative measurement of certain critical mixture properties. Two such properties are thought to be the particle size distribution (PSD) of drug containing particles on the carrier surface (including agglomerates with or without fine lactose particles) and the spatial distribution of the drug containing particles over carrier surfaces with different binding activities. Drug particles may deagglomerate and reagglomerate during the mixing process (in conjunction with lactose fines present in the mixture), which changes the size distribution of the adhering particles. The heterogeneous character of the carrier surfaces results in sites with a wide range of binding activity and sites from which drug particles can be liberated with different efficacy. These effects influence the drug–carrier interaction forces as well as the separation forces during inhalation and, therefore, the dispersion performance of the mixture. Scanning electron microscopy (SEM) may be considered the standard imaging technique used for the characterisation of the appearance of adhesive mixtures. It has proven to be valuable in studying the morphology of particles in adhesive mixtures for inhalation because of its high resolution. However, with this nonchemical-specific imaging technique, drug particles are difficult to distinguish from fine lactose excipient particles (fines), which are always present in the formulation. To date, it has thus not been possible to accurately assess the composition of agglomerates or the spatial distribution of the drug over the carrier surface. Therefore, a combination of a high-resolution imaging technique like SEM with a chemical-specific imaging technique such as CARS may greatly aid in the study of adhesive mixtures.

Comparative CARS and scanning electron microscopy

Combining the imaging modalities of light microscopy and electron microscopy is well established in the area of biomedical sciences, with fluorescence microscopy commonly being combined with SEM. Correlative light and electron microscopy allows a cell to be imaged from the micron to nanometre scale while maintaining spatial orientation. Additionally, spontaneous Raman microscopy and spectroscopy have been combined with SEM. CARS microscopy has also been combined with electron microscopy but, to the best of our knowledge, has not yet been demonstrated in a correlative manner with SEM. Here, we present results that combine CARS microscopy with SEM in a correlative manner, allowing direct comparison of images recorded by both modalities.

Aims

In this work, we demonstrate CARS microscopy for chemically selective imaging of micronised drug that is distributed over the surface of lactose carrier particles. We performed image analysis using Image J to determine the drug particle distribution that is compared with the standard method of laser diffraction. Additionally, we have performed correlative CARS and SEM allowing us to compare the images obtained using CARS microscopy with images from SEM.

Materials and methods

Materials

Alpha-lactose monohydrate (Pharmatose 80M) was donated by DFE Pharma (Goch, Germany). The drugs used in this study are micronised salmeterol xinafoate (Novartis, Germany) and budesonide (Fagron, The Netherlands).

Mixture preparation

All drugs were passed through a 90-μm sieve to break up larger agglomerates prior to mixing with the lactose carrier. Carrier size fractions of 63–90 and 250–315 μm were sieved from the Pharmatose starting material (Retsch AS 200 control, Germany). The carrier fractions were subsequently subjected to an air jet sieving process to remove as many residual contaminating lactose particles (known as fines) as possible from the carrier particle surface (Alpine AS 200, Augsburg, Germany). Budesonide (0.4% w/w) was mixed for 10 min at 90 rpm with the 250- to 315-μm carrier material using a Turbula blender (WA Bachofen, Basel, Switzerland). Salmeterol (1.48% w/w) was mixed with the 63- to 90-μm carrier fraction for either 0.5 or 600 min using the same mixer and mixing intensity.

Hyperspectral and z-stack CARS methods

CARS microscope set-up

The CARS microscopy system is described in detail elsewhere. Briefly, a neodymium-doped yttrium orthovanadate picosecond pulsed laser (Coherent, USA) operating at a fundamental wavelength of 1064 nm was frequency doubled to pump an optical parametric oscillator (OPO; APE, Germany) that produced two dependently tunable laser beams. The fundamental laser beam was combined with one of the beams from the OPO and directed into an inverted microscope (Olympus IX71, Japan) where they were focused either using a 60X/1.2 numerical aperture (NA) objective (distribution/particle size measurements) or a 40X/0.9 NA objective (correlative CARS with SEM imaging). The backscattered CARS signal was collected by the focusing objective and detected with a photomultiplier tube (Hamamatsu, Japan). The CARS microscope system using the 60X objective had an axial spatial resolution of about 1 μm and a lateral spatial resolution of about 0.4 μm.

Hyperspectral imaging

Hyperspectral CARS imaging provides a rapid method to extract the CARS intensity profile (CARS spectra) over a range of wavenumbers allowing the identification of peaks suitable for recording chemically specific CARS images. The method for conducting hyperspectral imaging was published previously. Hyperspectral scans were recorded over the range of 2800–3100 cm⁻¹ using a step size of about 4 cm⁻¹ and taking about 4 min to collect.
Z-stacked imaging

Z-stacked imaging was conducted by stepping the microscope objective in the z direction in increments of 1 μm. Each z-stacked image (512 × 512 pixels) consists of about 20 slices and took about 1 min to record.

Particle size analysis using laser diffraction

The PSD of the primary budesonide particles was measured with a HELOS BF diffractometer using a RODOS dry disperser at 3 bar (Sympatec, Clausthal-Zellerfeld, Germany). The diffractometer was equipped with a 100-mm lens, and calculations were performed based on the Fraunhofer theory. Increasing the dispersion pressure to 5 bar did not affect the PSD of the drug, indicating that indeed the PSD of the primary particles was measured at 3 bar. To compare the RODOS data with the results from the analysis using Image J (see further), laser diffraction particle sizes were recalculated to projected surface areas according to $SA = \frac{1}{4} \pi d_{\text{drug}}^2$.

Particle size analysis using image J

Particle size analysis was performed using the particle analyser command available in the Image J software (http://rsbweb.nih.gov/ij/) according to the following steps. The image file was first opened by Image J, and the scale bar from the image was used to calibrate the software scale in Image J. The image was then converted to eight-bit type and converted to binary using the inbuilt automated routine based on the IsoData algorithm.[26] The scale bar was then removed from the image before setting the particle analyser command to analyse for particles between 0 and infinity μm².

Figure 1. CARS spectra extracted from hyperspectral data covering the CH stretching range from 2800 to 3100 cm⁻¹ for lactose (solid green), budesonide (dashed red), and salmeterol (dash-dot black).

Figure 2. Projected z-stack CARS images (A) budesonide (3046 cm⁻¹), (B) lactose (2888 cm⁻¹), (C) overlay image showing distribution of budesonide on the surface of a lactose carrier particle, and (D) transmission light image.
Comparative CARS and SEM method

Samples were mounted on a glass microscope slide using double-sided tape and suspended in the air above a 40X/0.9 NA objective. Carrier particles of interest were identified using the transmission signal. Z-stacked CARS images (512 × 512 pixels) were recorded for both the lactose carrier particle (2888 cm⁻¹) and the drug (budesonide, 3046 cm⁻¹, and salmeterol, 3050 cm⁻¹) loaded onto the surface. After CARS imaging, the glass microscope slide was removed from the CARS microscope and mounted on double-sided carbon tape and placed on the SEM sample holder. The samples were sputter coated with 20 nm of a gold/palladium alloy. SEM images were then obtained at an acceleration voltage of 3 kV (JSM-6301F, Japan Electron Optics Laboratory, Japan).

Results and discussion

CARS spectra

Hyperspectral CARS images of the pure chemical compounds were recorded to identify the key vibrational bands that would allow selective imaging of the adhesive mixtures. Hyperspectral scans covered the wavenumber region from 2800 to 3100 cm⁻¹, corresponding to the C–H stretch region. Figure 1 shows the CARS spectra extracted from the hyperspectral data for alpha-lactose monohydrate (green), budesonide (dashed red), and salmeterol (dash-dot black). The wavenumbers chosen for single-wavelength imaging were 2888 cm⁻¹ (lactose), 3046 cm⁻¹ (budesonide), and 3050 cm⁻¹ (salmeterol).

Drug distribution

CARS microscopy allows chemically selective imaging based on Raman vibrational resonances. This makes CARS ideal for studying the distribution of drug on the surface of lactose carrier particles because by only changing the wavelengths of the lasers, we can image different components of the same sample. Figure 2(A) shows a z-stacked CARS image (512 × 512 pixels) collected at 3046 cm⁻¹ that is selective for the budesonide. Figure 2(B) shows a z-stacked image (512 × 512 pixels) of the same area as Fig. 2(A) recorded at 2888 cm⁻¹ that is selective for lactose. Figure 2(C) shows the overlaid z-stacked images from Figs 2(A) and 2(B), while Fig. 2(D) shows the corresponding transmission light image. From Fig. 2(C), we can see that the budesonide (red) is distributed in clusters covering the surface of the lactose particles. Comparing the CARS image in Fig. 2(C) with the transmission image in Fig. 2(D) highlights the power of this technique, as the transmission image suggests little more than a rough surface and does not reveal the localised distribution of the drug on the surface.

CARS microscopy is inherently confocal with signal generated only within the focus of the objective; this feature provides the ability to record z-depth-stacked images to gain a greater understanding of the drug distribution on the rough surface of the lactose carrier particles. Additionally, z-stacked images can identify the penetration depth to which the drug reaches during the mixing process. Figure S1 (Supporting Information) is a video showing the individual slices from a z-stacked image (512 × 512 pixels) of a budesonide (red) loaded lactose carrier particle (green) starting from 0-μm depth and stepping every 1 μm until a depth of 21 μm. In addition to drug penetration depth, Figure S1 also provides information about surface curvature of the carrier particle because the focus can be seen shifting from the centre towards the edge of the particle as the z-stack progresses. This representation may offer the possibility to image changes in the depth of the drug in a multilayer of drug and added lactose fines when, for example, studying the effect of the order in which the different fine components are added to the blend.

Figure 3. (A) Original CARS image of a budesonide-coated lactose carrier particle, (B) binary representation of the CARS image containing the particles, (C) histogram showing pixel intensity with blue-dashed line indicating the threshold used for the binary conversion, and (D) particle size distribution from Image J analysis (striped black) and laser diffraction analysis (red).
Particle size analysis

The PSD is a critical parameter for the formulation of inhalation therapeutics. If the particles are too large, they will impact in the oropharynx and subsequently swallowed, while on the other hand, if the particles are too small, they are likely to be exhaled[27] It is generally accepted that the optimal aerodynamic diameter for particle inhalation roughly lies between 1 and 5 μm (depending on the precise target deposition site and the inhalation flow rate).[12,13] The Image J particle analysis command calculates the particle area by setting a threshold intensity that is then removed, and the remaining pixels are counted to give an area in μm². Figure 3(A) shows the original CARS image (reproduced from Fig. 2(A)) prior to particle size analysis. Figure 3(B) shows the background-removed image with only the drug particles (black) remaining. Figure 3(C) is a histogram representing the pixel intensity distribution for all of the pixels in Fig. 3(A) with a dashed blue line indicating the threshold used by the binary algorithm to remove background signal. From the histogram, it was calculated that about 30% of the total pixel intensity is retained in the binary image that contains only 6% of the number of pixels (Fig. 3(B)). It is expected that the majority of the pixels in Fig. 3(A) are background because the drug is only present in the sample at a concentration of 0.4% w/w. Figure 3(D) is a plot showing the distribution of particle size comparing the distributions determined using Image J particle analysis (striped black) and laser diffraction (solid red) methods. The PSD obtained with Image J analysis reports 68% of the particles to have a median projection area of 0.4 μm², while laser diffraction analysis results in a PSD with 94% of the primary particles within the same size class. The larger particle sizes measured with Image J analysis can be attributed to agglomeration of the primary particles during mixing.

Figure 4. (A + B) CARS, (C + D) SEM, and (E + F) correlative CARS and SEM images of salmeterol mixtures after 0.5 (left) and 600 min of mixing (right). Scale bars represent 20 μm.
Correlative CARS and SEM imaging

Combining SEM with CARS microscopy provides the advantages of the high resolution available with SEM and chemically specific imaging provided by CARS. With the combined techniques, we are therefore able to image the spatial distribution of the drug on the carrier surface and to relate this to morphological characteristics of the drug and carrier particles. This is particularly useful when the drug particles do not deviate in size or shape from fines originating from the excipient particles or when the mixing process causes the drug particles to change in such a way that they cannot be identified by comparison with the starting material. Figure S2 (Supporting Information) shows high-magnification SEM images of the pure starting materials prior to mixing. Figure S2(A) is a lactose carrier particle covered in fines, while Fig. S2(B) shows the plate-like shape of salmeterol.

Figure 4 shows the result of correlative CARS and SEM imaging of the salmeterol blends prepared by 0.5 and 600 min of mixing. After only 0.5 min of mixing, the characteristic plate-like shape of the salmeterol particles is still intact, allowing the identification of the drug particles on the carrier surface by only high-resolution SEM imaging. The seemingly random distribution of drug particles in CARS is confirmed by CARS. Interestingly, upon close examination, not all particles that may be identified as drug particles based on their plate-like shape by SEM imaging are identified as such by CARS. These particles could be lactose particles with a similar morphology as the drug particles, but it is also possible that because of high carrier surface roughness combined with a large z-stack step size (1 μm), CARS imaging missed some of the drug particles observed using SEM. Therefore, CARS imaging can possibly be further optimised by reducing the image scanning speed and by using smaller step size for z-stacked imaging, thereby improving the correlation with SEM imaging. As described previously,[28] prolonged mixing causes plastic deformation and aggregation of the drug particles, and they can therefore not be identified as drug particles with certainty by comparison with the drug starting material. CARS, in this case, confirms that the modified particles consist of salmeterol. It was also described that salmeterol forms a film on the carrier surface in addition to the spherical agglomerates after such long mixing times. However, this film formation is not observed with CARS, which may be because of the film thickness being below the resolution limit for CARS microscopy.

Conclusions

CARS microscopy provides chemical selective imaging and is able to rapidly image the distribution of micronised budesonide and salmeterol over the surface of lactose carrier particles. Particle size measurements using the particle size analysis tool from Image J software in this study calculated a slightly larger particle size than that determined using the laser diffraction technique. However, further work is required to validate the binary conversion algorithm to ensure that it is not rejecting small drug particles. Combining CARS and SEM is a promising tool for the study of adhesive mixtures for inhalation. However, a further optimisation and validation of the technique are required to ensure that all drug particles are observed.

Acknowledgements

AF is supported by the Dutch Technology Foundation Stichting Technische Wetenschappen (STW), which is the applied science division of Nederlandse Organisatie voor Wetenschappelijk Onderzoek, and the Technology Programme of the Ministry of Economic Affairs (STW OTP 11114).

References


Supporting information

Additional supporting information may be found in the online version of this article at the publisher’s web site.