Chemical-selective imaging of adhesive mixtures for inhalation using coherent anti-Stokes Raman scattering (CARS) microscopy

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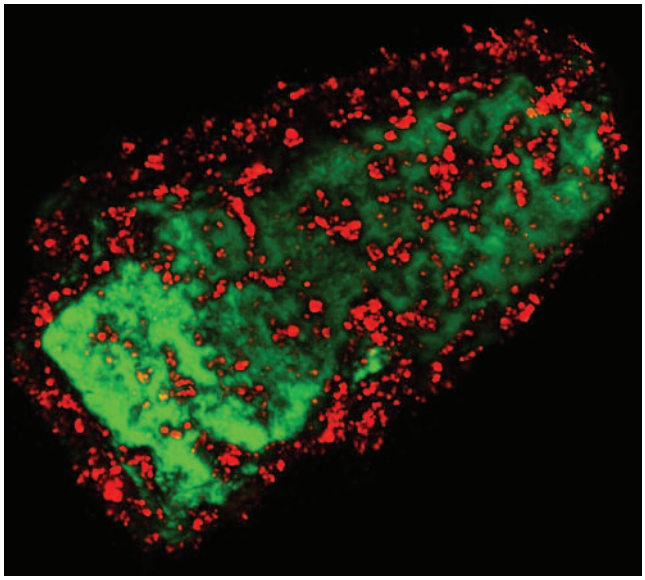
Abstract

Chemical-selective imaging may help to better understand and improve the dispersion performance of adhesive mixtures for dry powder inhalation. Coherent anti-Stokes Raman scattering (CARS) microscopy is a suitable alternative to other techniques for this, because it is fast and does not depend on differences in elemental composition of mixture components for contrast formation.

Adhesive mixtures

It may not seem rational to the novice inhalation technologist to formulate drug particles for inhalation into particles that are... well, simply too large for inhalation. Yet this is currently the most common practice in the development and manufacturing of dry powder inhalation products for the treatment of asthma and chronic obstructive pulmonary disease (COPD). Accurately and reproducibly dosing drugs against these diseases is very much complicated by their high potency and small particle size of roughly 1-5 µm. This results in the troublesome combination of a low required dose and a sticky, poorly flowing powder, respectively. To solve this problem, drug particles are often blended with a relatively coarse excipient, usually consisting of lactose monohydrate, into what is referred to as an adhesive mixture. Doing so dilutes the drug and improves the flowability of the powder, thereby enabling its reproducible dosing. During the mixing process, the drug particles will spontaneously adhere to the coarse lactose particles to form so-called "adhesive units." This usually results in a relatively high degree of homogeneity for adhesive mixtures,



which is desirable. But the fact that adhesive units are too large for inhalation deep into the human airways rather ruins the party.

Poor dispersion

As the adhesive units are too large to reach the lower airways, they have to be dispersed into the primary drug and lactose particles during inhalation with the aid of an inhaler for successful therapy. This requires a very delicate fine tuning of the adhesive forces between drug and lactose carrier particles. Forces need to be high enough to prevent segregation of the mixture during handling, but at the same time low enough to enable effective dispersion during inhalation. It is well known that variables of the formulation process such as mixture composition, mixing time and intensity, and characteristics of the starting materials affect drug/lactose adhesive forces, and with that the dispersion performance of adhesive mixtures. However, despite several decades of intensive research in this field, the exact mechanisms by which these variables affect mixture dispersion performance are, to a large extent, unknown. As a result, the optimization of adhesive mixtures for dry powder inhalation products is mostly an empirical undertaking, and for lactose-based inhalation products dispersion is generally very poor with inhalable drug fractions being only 10-

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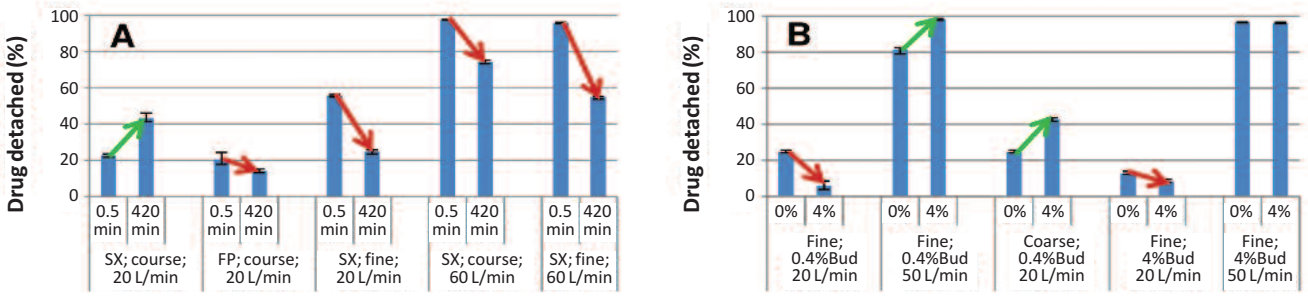
Figure 1

Interactions with the effect of mixing time (A) and the addition of 4% fine lactose particles (B) on the dispersion performance of adhesive mixtures. A: Prolonged mixing from 0.5 to 420 minutes may result in increased or decreased drug detachment from the lactose carrier during inhalation, depending on the type of drug (FP = fluticasone propionate; SX = salmeterol xinafoate), the carrier size fraction (coarse or fine) and the inhalation flow rate. B: The addition of 4% fine lactose particles to the mixture may result in decreased or increased drug detachment from the lactose carrier during inhalation, or no effect at all, depending on the carrier size fraction (fine or coarse), the drug content (0.4% or 4% of budesonide) and the inhalation flow rate.



50% of the nominal dose.[1] The remaining undispersed fraction of the dose will not reach the intended site of action to exert a therapeutic effect, but may contribute to adverse drug reactions due to deposition in the mouth and throat.

"It depends"

A fundamental understanding of the way formulation variables affect the dispersion performance of adhesive mixtures is crucial for the development of more efficient and safer lactose-based dry powder inhalation products. Obtaining such an understanding is, however, greatly complicated by the occurrence of interactions between many of the variables that are involved in the formulation and dispersion of adhesive mixtures.[2] Consequently, the question of the effect a certain formulation variable has on mixture dispersion performance can rarely be answered with anything other than "it depends." Namely, it depends on the choice of other formulation variables as well as on the choice of inhalation variables, such as the inhalation flow rate or the type of dry powder inhaler used. For example, prolonged mixing or the addition of fine lactose particles can increase dispersion performance under a certain set of conditions, but can also decrease it when the inhalation flow rate, the lactose carrier size fraction or the type of drug are chosen differently (Figure 1). In other words, interactions cause apparently conflicting test results and are, therefore, a major cause of confusion surrounding adhesive mixtures.

A different approach

Studies on adhesive mixtures have long focused on finding simple relationships between formulation variables and dispersion performance that, because of interactions, do not exist. Instead, an approach in which the interactions are better accounted for may be more successful for obtaining a fundamental understanding of adhesive mixtures. One such approach is to study the direct effects of formulation variables on physicochemical properties of the adhesive mixtures and their dependence on other formulation variables. Once these effects are better understood, the relationships between mixture properties and dispersion performance, and also their dependence on inhalation variables, can be studied to complete the picture. As may be clear, mixture properties play a very important role in such a different approach. The big question that then arises is: What mixture properties are relevant to dispersion performance and should, therefore, be studied?

Mixture properties

In the scientific literature on adhesive mixtures for inhalation, some mixture properties have been used time and time again to explain the effects of formulation variables on dispersion performance. Hypotheses referring to these mixture properties even carry their own names. The "agglomeration hypothesis," for example, refers to the particle size distribution of the drug on the lactose carrier surface, including any pure drug or composite drug/fine lactose agglomerates. The "active sites hypothesis" amongst other things refers to the spatial distribution of the drug over sites on the lactose carrier surface with different "activities." The degree of compression of drug particles onto the carrier surface during the mixing process is covered by the "press-on hypothesis." And a hypothesis referring to the tensile strength or fluidization behavior of the mixture? Indeed, the "fluidization hypothesis." Knowing the mixture properties likely to be relevant to the dispersion performance of adhesive mixtures is important, but in order to study them they also need to be characterized. Unfortunately, the ease of finding applicable names for the hypotheses is compensated by a difficulty to characterize the corresponding mixture properties.

Scanning electron microscopy

Imaging is an important tool used in the study of adhesive mixtures. It enables, to some extent, the characterization of the agglomeration behavior of the drug and its spatial distribution over the carrier surface. A technique that is routinely used for this is scanning electron

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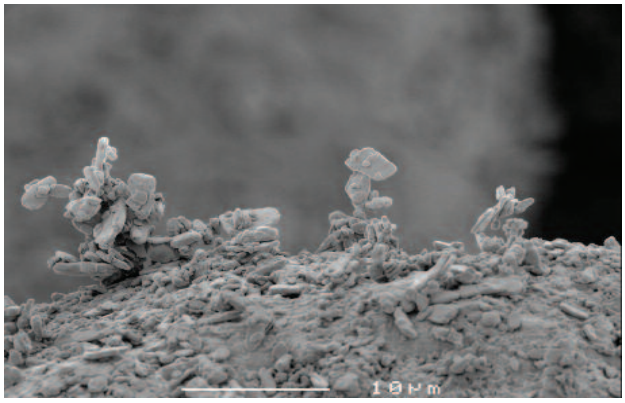
Figure 2

Scanning electron microscopy (SEM) image of micron-sized salmeterol xinafoate particles on a lactose carrier particle.



microscopy or SEM, and then specifically its secondary electron (SE) signal. SE-SEM is an imaging technique with a very high resolution that enables the imaging of adhesive mixtures into spectacular detail (Figure 2). Although this technique is tremendously helpful for studying adhesive mixtures, it does not offer the possibility to differentiate between mixture components, especially when the components are similar in size and shape. All images resulting from this technique appear in many shades of grey. Therefore, one often cannot say with certainty whether it is the drug component or perhaps the always-present fine lactose that is imaged. The difficulty becomes even greater when the mixture contains multiple drugs, such as with the increasingly popular combination preparations. For better determination of the drug particle size distribution in the mixture and its spatial distribution throughout it, some form of chemical-selective imaging would, therefore, be preferable.

Chemical-selective imaging

Several techniques exist that may be applied for the chemical-selective imaging of adhesive mixtures. Examples include backscatter electron imaging and energy-dispersive x-ray spectroscopy (EDX or EDS). Backscatter electron and x-ray signals are also produced during SEM, in addition to the secondary electron signal. Chemical-selective and topographical images of the same sample can, therefore, be easily obtained and combined with these techniques. However, contrast formation in backscatter electron and EDX imaging relies on differences in weighted-average atomic number or elemental composition of the sample material, respectively. As the drugs used in the treatment of asthma and COPD, and lactose, are all organic compounds, only a few drugs are distinguishable from lactose, based on these characteristics. A different principle, based on the inelastic scattering of monochromatic light, is used by Raman techniques.[3,4] They are highly chemical-specific, and therefore, more suitable for the imaging of adhesive mixtures for inhalation than the SEM imaging techniques mentioned.

Raman techniques

Raman imaging typically uses laser light to excite molecules in the specimen from the ground state to a virtual energy state, which is characterized by an oscillating polarization in the molecules. This virtual energy state is an intermediate state from which the molecule can pass into an excited vibrational state under the emission of a photon with a lower energy or longer wavelength than the incident photon (i.e., the incident light is red-shifted). This process is referred to as "Stokes scattering" (Figure 3) and it is the basis for spontaneous Raman techniques. The vibrational state is characterized, for example, by the stretching and bending of chemical bonds, and its energy level equals the energy difference between the incident and emitted photon. The transition from a ground state to a certain vibrational state via an intermediate virtual state is independent of the wavelength of the incident light. Therefore, a Raman spectrum, which is highly chemical-specific, shows the intensity of the scattered light as a function of the energy difference between incident

Figure 3

Energy diagrams representing Stokes, anti-Stokes and coherent anti-Stokes Raman scattering (CARS).

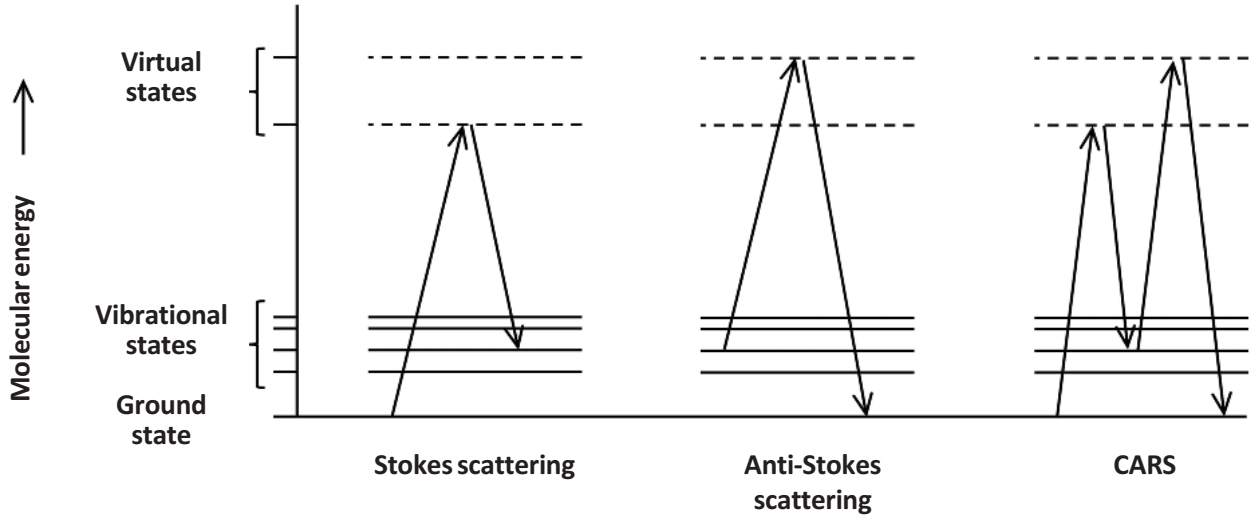


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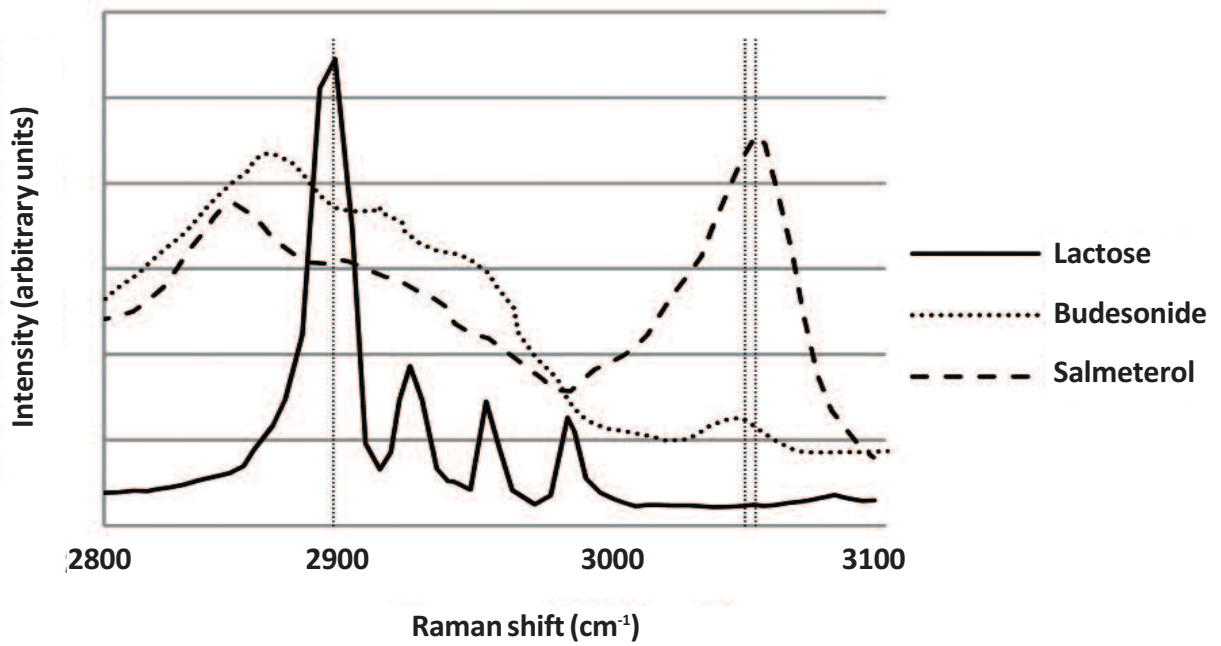
Figure 4

CARS spectra extracted from hyperspectral data covering the C-H stretching range from 2800-3100 cm^{-1} for lactose, budesonide and salmeterol xinafoate. Wavelengths chosen for imaging of these substances are represented by the vertical dotted lines from left to right, respectively.



and emitted photon, and thus the energy level of the vibrational state.

Coherent anti-Stokes Raman scattering (CARS)

In addition to the ground state, a vibrational state of molecules can be excited to a virtual energy state by incident light, after which the molecule can pass into the ground state under the emission of light with a higher energy or shorter wavelength (i.e., the incident light is blue-shifted). This process is referred to as "anti-Stokes scattering" (Figure 3) and is the basis for coherent anti-Stokes Raman scattering (CARS). In CARS, three photons are used to stimulate Stokes scattering, followed by anti-Stokes scattering (Figure 3). CARS differs from spontaneous Raman mapping techniques by an approximate 100 times faster imaging speed, which makes the acronym all the more fitting.[5] Furthermore, because CARS produces an anti-Stokes (blue) shifted signal, it is free from single photon fluorescence, which hampers spontaneous Raman measurements.

CARS setup

The Optical Sciences Group at the University of Twente in the Netherlands built a custom CARS microscope setup that was used for the imaging of adhesive mixtures. To an inhalation technologist not familiar with specialized imaging techniques, it is an impressive accumulation of green laser light, electronic equipment, an array of mirrors, beam splitters, lenses and an inverted microscope. To those who are familiar with specialized imaging techniques, it is simply a CARS microscope that is based on a picosecond pulsed laser that pumps an optical parametric oscillator, producing two lasers which are then combined and directed into a commercial microscope. Other scientifically meaningful details of the setup used are described elsewhere.[6] The CARS setup has a spatial or in-focal plane resolution of approximately 0.4 µm and an axial or "depth" resolution of approximately 1 µm. This is considerably lower than the resolution of a technique such as SEM, which restricts the amount of detail one can obtain with the CARS technique.

Imaging adhesive mixtures using CARS

The first step in imaging adhesive mixtures by CARS is the so-called "hyperspectral imaging" of the pure mixture components. In this step, it is determined at which wavelength or "Raman shift" the different components in the mixture can be imaged. Hyperspectral scans of lactose, budesonide and salmeterol xinafoate are presented in Figure 4. The next step is to image the sample at the determined wavelengths. CARS is an inherently confocal imaging technique, which means that a signal is only produced in the (very small) focal volume. Therefore, in order to obtain a three-dimensional image, several images of the sample at different "depths" or axial or z-directional distances have to be combined into one image. This is referred to as "z-stacking" of images. An example illustrating the confocal nature of CARS is shown in Figure 5.

Combining CARS and SEM

As CARS is, in principle, a non-destructive imaging

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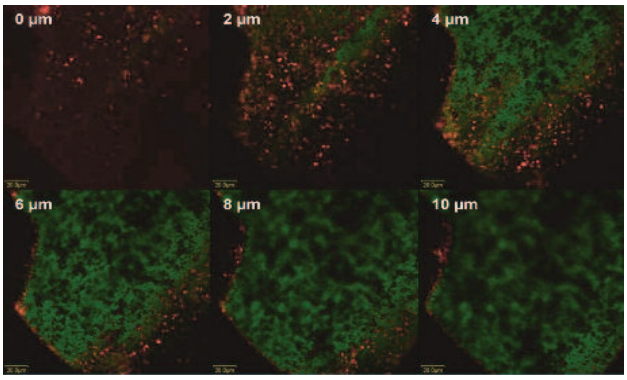
Figure 5

Frames from a z-stacked image of a lactose carrier particle (green) loaded with budesonide (red). Displayed in every frame is the axial position or "z-distance" in micrometers. Note that lactose ($C_{12}H_{22}O_{11} \bullet H_2O$) and budesonide ($C_{25}H_{34}O_6$) do not differ in their elemental composition. Because of this, a technique like EDX or EDS would not be able to distinguish between these components.



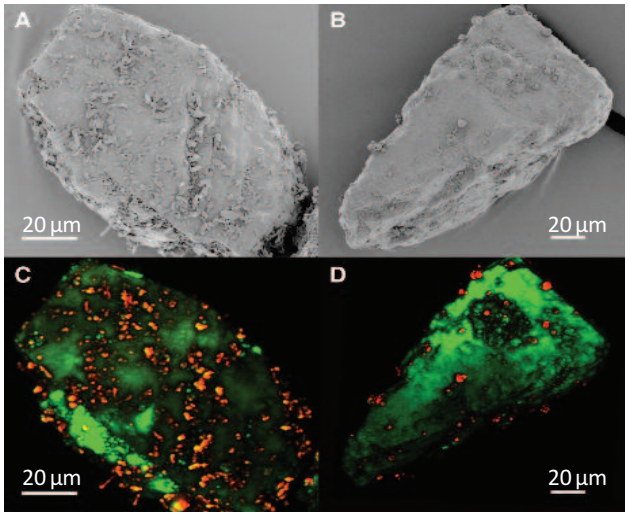
technique, samples can be imaged by CARS and subsequently by SEM to obtain the best of both worlds: chemical-selectivity as well as high resolution. One of the drawbacks of using high power lasers for imaging is sample heating. For CARS to be truly non-destructive to a sample, one therefore has to make sure that the sample mount is completely transparent. Otherwise, it is the authors' personal experience that the intensity of the laser light will set organic samples on fire! A thin piece of glass with transparent double-sided adhesive tape works quite well for this. Two CARS images of a lactose carrier particle with adhered salmeterol xinafoate particles and their corresponding SEM images are shown in Figure 6.

Future challenges

CARS provides a valuable addition to other techniques for the imaging of adhesive mixtures, with several advantages over more commonly used techniques including SEM and EDX. However, there are a number of limitations and challenges that would need to be overcome for it to be suitable as a routine technique. For example, the attainable field of view is currently limited to only one or several adhesive units, which makes it difficult to image a mixture in a representative way. Furthermore, some drug particles, especially the smaller ones, might not be imaged by CARS due to its relatively low resolution. This would affect the outcome of any software-aided post-imaging analysis, such as the determination of a particle size distribution of the drug component in the mixture.[6] Wang et al. explained that a larger field of view can be attained by using collection optics with a lower magnification factor.[7] Alternatively, they showed that repeated sampling will lead to a more representative image of (the drug content of) the bulk powder. Some drug material may not be imaged by CARS due to its amorphization from mechanical stress during mixing.[8] This may be confirmed by low frequency Raman spectroscopy, which can be used to assess the crystallinity in microparticles for inhalation.[9] Therefore, Raman techniques, in general, may aid in obtaining a more fundamental understanding of adhesive mixtures for inhalation in the future.

Figure 6

High resolution scanning electron microscopy images (A & B) and the corresponding CARS images (C & D) of adhesive mixtures containing salmeterol xinafoate that are mixed for 0.5 minutes (A & C) or 600 minutes (B & D). Note how the drug particles have changed in shape during prolonged mixing (A vs. B), and how drug particles can, therefore, only with certainty be identified after prolonged mixing by the chemical-selective imaging with CARS (D).



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