Chemical imaging of pharmaceutical granules by Raman global illumination and near-infrared mapping platforms

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Raman global illumination and near-infrared (NIR) mapping instruments were used to chemically image pharmaceutical granules obtained by the wet granulation process in order to determine whether the API was mixed with the major excipient or granulates on its own. The granules were randomly distributed onto a microscope slide and an average area of about 3.5 mm × 3.5 mm, covering 50–100 granules, was analyzed by both instruments. Light microscopy images of the separated granules were collected before the spectroscopic data acquisition. Both Raman and NIR signals of API and major excipient (mannitol) were easily detected by both techniques which allowed the chemical structure of the granules to be characterised. Most of the granules were found to contain both API and mannitol but pure mannitol and a few pure API granules were also identified. Raman global illumination was found to provide a comprehensive insight into chemical structure of the granules being able to more clearly determine the API in comparison with NIR mapping. Owing to the differences in shapes of the particles and reflection characteristics, visual microscopy and methods based on reflection can be potentially useful for analyzing this particular formulation.

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1. Introduction

Chemical imaging is a methodology that allows automated measurements of samples through the combination of microscopes with vibrational (e.g. near-infrared and Raman) spectrometers to produce a 2D image of the components in a sample [1–3]. At each position of the microscope’s XY(Z) stage either a spectrum of the analyzed sample is obtained or an image at a pre-defined wavenumber is obtained. If a spectrum is obtained, this is known as a mapping experiment; if an image is obtained as the raw data then this is a true imaging experiment. Chemical images are obtained from the mapping data through chemometric analysis of the three-dimensional array of spectra obtained during the data acquisition. Another important difference between the two approaches is in the mode of scanning. Global illumination is carried out on the entire sample observed in the microscope’s fields of view. In mapping experiments, line or point light sources are used to probe the sample at discrete points on the sample surface. The final result of a chemical imaging experiment, be it mapping or true imaging, is a chemical image in which (ideally) all the components of the analyzed sample are identified based on their chemical composition and their spatial position in the sample. This method allows the identification of chemical micro-structures in the sample that are not observed by bulk measurements of the sample. This can be particularly useful in pharmaceutical industry where seemingly non-informative surfaces of tablets, powders, beads, and various other materials can be analyzed and the observed chemical images a better understanding of the sample can be achieved. A number of publications on the applications of chemical mapping and imaging in the pharmaceutical industry have appeared recently [3–15]. Of these publications, some initial assessments of the functionalities of both Raman and near-infrared
(NIR) mapping and imaging in the pharmaceutical environment have been specifically made [7]. However, within the scientific community mapping techniques are more widely used to create chemical images. This is despite the fact that for imaging methods chemical images are immediately available as raw data and no additional data processing may be required to enhance the information content.

The objective of the work presented here is to generate chemical images and characterise pharmaceutical granules obtained during a wet granulation process in which granules are produced with the use of water. A thorough review of the literature does not reveal reports on any similar studies. This should be the first publication in which the analysis of pharmaceutical granules using Raman chemical imaging is reported.

There are a number of reasons for studying this formulation by imaging approaches: firstly, it is useful to gain knowledge on whether the applied wet granulation process has been successful, i.e. whether the API (active pharmaceutical ingredient) combines with other formulation excipients to form granules. Secondly, various physicochemical characteristics of the granules (such as dissolution) may be affected by their chemical structure (e.g. polymorphic or hydrate/solvate form) which will be determined by chemical imaging. Finally, the sizes of pure API granules can be estimated and correlated with the size of the particles in the blend.

Analysis of single granules that are spatially separated objects can be tackled by both mapping and imaging instruments. Because of the granules spatial separation the Raman global illumination method is particularly suitable to use. This is because the material on the sample slide is scanned via large fields of view, and data acquisition is not compromised by the void areas. This method is also known to be able to provide images of high quality. These results represent the first application of the global illumination method for imaging the granules. In addition to the global imaging, NIR mapping is also employed as a comparative method. The NIR mapping method is rather fast, the instruments are widely available, and produce high quality spectra. A potential disadvantage is in the fact that large areas on the slide contain no sample which leads to the lack of signal from the corresponding pixels. This may have negative consequences to the subsequent analysis of the mapping spectral data set.

2. Experimental

The dried granules were analyzed as obtained. No sample preparation of any sort was applied. Relatively large granules (having a dimension of least 200 μm) were easily separated by simply tapping the microscope slide (glass). The samples also contained smaller granules/particles which were not the focus of this study. For this reason no sampling methodology was developed to separate these particles.

The analyzed formulation contained relatively high concentration of API (20%, w/w). The major excipient was mannitol (a polyol, 65%, w/w) whose major role was to act as a diluent. Four more minor excipients existed in this formulation. The distribution of these components are not discussed as (i) the focus of this work is related to the distribution of the API in the granules, and (ii) for a number of reasons (e.g. having poor Raman scatter or at a concentration below the limit of detection) these excipients are not detected in the granules. The chemical structure of the API is not provided, as this has no relevance to the generation of its chemical image.

The global illumination Raman images were obtained on the ‘Falcon’ instrument (ChemImage Corp, Pittsburgh, PA). An average area of 3.5 mm × 3.5 mm was typically imaged. Since the granules were randomly distributed onto the microscope slide the total imaging area was chosen to cover the largest number of granules in a reasonable experimental time, typically less than 9h. The imaged area was therefore slightly different from one experiment to another. All the Raman images were preceded by the acquisition of white light images of the sample across the area selected for imaging.

Fortunately, the region of strong and non-overlapping Raman bands for mannitol and API were found to be next to each other in the region 1010–1080 cm⁻¹. This spectral region was employed for all the Raman measurements. The liquid crystal tunable filters (LCTFs) that select wavenumbers at which an image is to be obtained [2], were set to collect chemical images at 10 cm⁻¹ intervals covering the selected spectral region in 8 steps, generating a sample image at each step. The sample was excited by a laser line at 532 nm with 200 mW power typically being employed. No sample damage was detected in the experiments. The global illumination instruments are theoretically capable of excellent spatial resolutions (theoretically submicron) but in this case the spatial resolution of 15 μm was considered acceptable because of relatively large sizes of the granules imaged. For these studies the gross composition of the granules was required (i.e. API only, mannitol only, or an API/Mannitol mixture). This was achieved by significant binning of the pixels on the CCD detector which in turn led to stronger Raman signal on the CCD and consequently to shorter acquisition times. The acquisition time of 15 s per wavenumber was normally employed although satisfactory results were obtained with shorter acquisitions too. ×10 magnification was used in all experiments with the field of view (FOV) set at 190 μm. This means that the typical imaged area of ca. 10 mm² was scanned through stage shifts of 190 μm.

NIR mapping data were collected on a PerkinElmer Spotlight system. The maps were normally collected across 5 mm × 5 mm areas with a spatial resolution of 25 μm, also subject to satisfactory distribution of the granules. The spectra were collected with 16 scans and 16 cm⁻¹ wavenumber resolution which led to about 160 min of acquisition time. ×20 magnification was used in all experiments.

All the data analysis and chemical image production was carried out with ChemAnalyze software (ChemImage, Pittsburgh, PA). Although obtaining chemical images from the mapping data can require considerable computation including data pre-processing, the data analysis was quite simple for these data sets. Both Raman and NIR spectra have only been baseline corrected. All the chemical images were produced using univariate analysis. The multivariate images were also produced for the NIR data but these were not found to be noticeably better than the univariate ones.
3. Results

A (white) light microscopy composite image of a typical sample prior to mapping or imaging analysis is shown in Fig. 1. Each sample was independently measured on an unmarked slide meaning that in no case exactly the same area on a sample was imaged on both platforms. An exact comparison between the two employed techniques is thus unattainable as the granules are not fixed to the microscope slide.

It should be noted that in addition to the large separated particles, there is relatively fine material in the upper right corner in Fig. 1 that cannot be easily separated by tapping. This situation is repeated in a number of samples that were imaged leading to conclusion that the large granules can very easily be prepared for chemical imaging while the smaller granules (or particles) of the analyzed formulation tend to stay closely agglomerated so that more complex separation must be applied to prepare them for chemical imaging. The distinction between the ‘big’ and ‘small’ granules is completely subjective in this case.

3.1. Global Raman imaging

The original, unprocessed Raman image at 1050 cm\(^{-1}\), where a strong API peak resides, is shown in Fig. 2. Analysis of this image reveals that some particles reflect the laser beam better than the other. For example, there are several strongly reflecting particles (that thus appear white due to higher intensities obtained from them) in the middle of the image as opposed to a few particles in the bottom left corner the contours of which are recognizable but their poor reflection renders them dark in comparison with the before-mentioned particles.

Fig. 1 – The white light image (3.5 mm × 3.5 mm) obtained on the global illumination Raman imaging instrument. The emphasis of the analysis is on the large, distinguishable granules and not on the fine material gathered in the upper right corner that cannot be easily separated into individual granules/particles.

Fig. 3A shows several granules of pure mannitol that are confirmed through the appearance of the mannitol specific peak at 1030 cm\(^{-1}\) shown in Fig. 3B. The particles from which those Raman bands are obtained are circled in Fig. 3A. Similarly, Fig. 3C and D, respectively, display the images and spectra for the API. The presence of API is proven via its strong peak at 1050 cm\(^{-1}\). As expected, there are more pure mannitol particles present although a few pure API particles are found too. The rest of the granules exhibited the spectra with various ratios between the bands at 1030 and 1050 cm\(^{-1}\) which is consistent with the granules containing both mannitol and API. This conclusion also draws from the white light and unprocessed Raman images which will be commented on later. Using the white light image (Fig. 1) and the chemical images of API and mannitol (Fig. 3A and C, respectively) it can be determined which granules are pure mannitol, pure API or mixtures of the two components.

The obtained chemical images show that the granulation produced a large number of granules that contain both mannitol and API and a smaller number of pure mannitol and pure API granules. It needs to be mentioned here that there is some arbitrariness with regard to distinguishing what is a granule of pure material because the quality of the Raman spectra is not so good to make it entirely clear that there are no indications of API in a spectrum from the granule dominated by mannitol and vice versa. The spectra in Fig. 3 are the best examples of the separation.

It is also important to note that the comparison between the original Raman chemical images (Fig. 2) with those that are baseline corrected (Fig. 3C) reveals that all the weakly reflecting particles are those of mannitol while the efficient reflection comes from the API-rich granules. This demonstrates that there is greater sensitivity for API detection in these samples.

Finally, valuable information can be obtained from the white light images too. A careful inspection of those images reveals that the API-rich granules tend to be prominently...
Fig. 2 – The original Raman image of the sample obtained at 1010 cm\(^{-1}\) above (left) and the subsequent spectra that correspond to some of the granules (right). The spectra demonstrate different reflection ability of the granules through broadly varying baseline. The spectrum with the weakest baseline is obtained from the granule on the left, the one in the middle from the granule in the middle while the most intense spectrum is obtained from the intense granule at the bottom.

Fig. 3 – The Raman chemical image of mannitol at 1030 cm\(^{-1}\) (A). The circled granules are believed to be pure mannitol on the basis of the spectra in (B) that feature only the mannitol band at 1030 cm\(^{-1}\). The Raman chemical image of API at 1050 cm\(^{-1}\) (C). The circled granules are assigned to the pure API on the basis of the presence of only the strong API peak at 1050 cm\(^{-1}\) (D). These images have been produced from the baseline corrected data.
white while the mannitol-dominated granules are mostly of rectangular shape and with smoother surfaces if compared with mixed granules and those with more (or of pure) API.

3.2. Near-infrared mapping

The white light images on the NIR mapping platform (Fig. 4) are not as clear and are thus less informative than those from the Raman global illumination platform. This is in part due to the objectives used. Raman microscope systems use glass objectives which give good visual images. The FT–NIR microscope system uses a Cassegrainian objective which is designed for Infrared performance rather than white light performance and leads to a poorer visual image. The rectangular shapes of the pure mannitol granules can be recognized but the pure API granules cannot be easily identified.

The reflectance ability of the granules is different for the laser light at 532 nm than for the range of wavelengths in the NIR region so that original, unprocessed NIR data were also found not as informative as the Raman ones (not shown here).

The NIR spectra of the API and mannitol are shown in Fig. 5. The overlap between the two spectra is significant and the API spectrum does not appear particularly rich in features but the region around 4500 cm$^{-1}$ is certainly suitable for producing univariate images. Mannitol can be univariately imaged at a number of wavenumbers, with 4800 cm$^{-1}$ being chosen here as the most representative. There is an API band close to the NIR band used to create the mannitol image. However, the relative NIR response for mannitol is significantly larger than the API response so there is no API contribution in the mannitol chemical image. In order to accentuate the difference between the two spectra in the region <5000 cm$^{-1}$, the scattering effect is eliminated by the second degree polynomial baseline correction instead of multiplicative scatter correction that is more often used as a pre-processing step in analyses of NIR data. The univariate API image at 4500 cm$^{-1}$ obtained from the baseline corrected spectra is shown in Fig. 6A with the accompanying spectra given in Fig. 6B. The weakness of the NIR spectra from the API and the ensuing consequences regarding chemical images are illustrated in these two figures. The differences between the spectra from the granules that are believed to have more API and those having more mannitol are visible but are not very pronounced. In addition, the strength of the NIR signal is proven to be a function of the size of the granules so the mannitol-rich granules tend to have stronger signal than those assigned to the pure (predominant) API.

An additional problem encountered here refers to the link between the thresholding of the grey-scale images and the sample preparation. The separation of the granules leaves a significant part of the slide uncovered and apparently there is no NIR signal from those positions. The absence of NIR response from such areas leads to the threshold for grey-scale images being set unreasonably low, at zero counts. As a consequence, the contrast between the API- and mannitol-rich granules is poor. This results in chemical images that are somewhat difficult to interpret. Normally the intensity of image pixels is proportional to the amount of a component present. In this case the bright white pixels in Fig. 6A are due to the API, whilst dull/grey pixels are due to mannitol. These identities are confirmed through the spectra associated with each pixel (Fig. 6B). The lower threshold for the image at 4500 cm$^{-1}$ is not set at a certain number of counts that would reflect the presence of mannitol but it is set at zero and therefore the obtained chemical image (Fig. 6A) only partially reflects the API/mannitol differences in the content of the granules. This is an interesting thresholding problem for which no reference is found in the chemical imaging literature. It can be eliminated by uniformly and completely covering the slide. However, whenever physically separated material is analyzed by mapping technique, this difficulty is likely to occur.

One way to alleviate this issue was found in univariate imaging at ∼5200 cm$^{-1}$ where only the weak signal from API can be found. The overall response at ∼5200 cm$^{-1}$ effectively starts from zero counts so that the problem of low threshold from uncovered positions disappears and the grey-scale image is truly based on the recorded NIR signal. The uni-
Fig. 6 – The NIR chemical image of API at 4500 cm$^{-1}$ (A). The two smaller circled granules are believed to be pure API and their spectra are shown in (B, thin lines) while the bigger granule is that of mannitol, its spectrum is also shown in (B, thick line). The chemical image in (C) is obtained at 5200 cm$^{-1}$ where mannitol has no signal and thus pure mannitol granules are missing there, while the image in (D) is obtained at the position of the mannitol peak at 4800 cm$^{-1}$. Mannitol appears ubiquitous except in somewhat shaded granules that contain API predominantly (compare with A).

The results presented prove that chemical imaging is very useful for determining chemical nature of the pharmaceutical granules. This is believed to be the first application of chemical imaging for identifying chemical identity of the real-world pharmaceutical granules. Both applied mapping and imaging techniques clearly recognize the granules consisting only of the major excipient (mannitol) or the API. The majority of the granules are found to be mixtures of API and mannitol providing strong evidence that the granulation process...
was successful. However, the Raman imaging approach produced API images in which there was better confidence in the domains which contained API. In addition, although the emphasis here was in collecting and analyzing the spectra and images from the ‘big’ granules, it should be mentioned that smaller granules or particles (less than 100 μm) are also clearly visualized and can be chemically imaged.

Technically, the global Raman imaging was more successful for analyzing this particular formulation. Other formulations may have APIs with significantly different spectroscopic features and thus no generalization can be made. The advantages observed in this series of experiments included (i) informative white light images obtained on the Raman instrument used and (ii) the ability to clearly differentiate between the granules of the APIs and mannitol in respective chemical images. There are three types of images (API, mannitol and white light) that can easily be produced on this instrument and then mutually correlated. The results show these images are complementary to each other providing confidence in the conclusions made from the experimental data.

A couple of issues were spotted with chemical mapping using the NIR platform. The first was found to be less useful white light images compared to those acquired on the Raman instrument. Secondly, and more importantly, the contrast for univariate chemical images was also poorer. Mapping the API proved to be difficult with the need to additionally check the identity of the granules believed to be the pure API which is carried out by assessing the spectra from pertinent pixels. Pure mannitol granules were most convincingly imaged at a position with quite a weak overall signal which may cast shadow on the reliability of the image. The positive element in favour of NIR chemical mapping is in the spectrally rich datasets which allow positive identification (or absence) of components compared to those obtained on the global illumination platform. Given NIR mapping data sets provide a number of full-length spectra, various multivariate methods can be employed to extract most of the vast amount of data collected. This was not proven to be beneficial here but this option may be substantially useful in other cases as multivariate analysis may be significantly more effective than the univariate analysis where there are many overlapping bands [7].

An important point for consideration for both platforms used was the length of acquisition. This element is intrinsically linked with the spatial resolution of the images, particularly for the global illumination Raman instrument. For the experiments described here, typical acquisition on the NIR platform took about 3 h while Raman experiments took no less than 9 h despite the short wavenumber range employed. Further refinement of the experimental conditions could have probably led to shorter acquisitions on both platforms. For example, if the CCD pixels on the Raman global illumination system are more heavily binned, the resulting Raman signal would be stronger and thus shorter acquisition times would be employed. This would obviously compromise the spatial resolution but given that quite large granules were analyzed, it is unlikely that the visual appearance of the chemical images would have been noticeably worse.

An interesting observation is that reflection-based techniques and visual microscopy might be suitable for chemical identification of the granules. While only chemical imaging can provide categorical evidence of the chemical nature of granules, the mannitol and API can be characterised using their visual appearance and reflection coefficients. The high API content (20%, w/w) makes this analysis possible. Of course, this conclusion holds for the formulation analyzed here and there is no guarantee that this observation is applicable to other formulations. Visual microscopy and reflection-based techniques are faster and less expensive techniques than chemical imaging and thus may be preferable choices for assessing chemical nature of the granules. However, the information content is much higher in chemical mapping and imaging measurements. This makes them indispensable in preliminary experiments for determining the nature of the granules. Once the chemical identity is reliably assigned to defined granule shapes or reflection ability, the chemical imaging may not be as important for characterisation of the sample.

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