

Vibrational Biospectroscopic Studies on Anti-cancer Nanopharmaceuticals (Part I)

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Nuclear magnetic resonance and mass spectrometry are two spectroscopy techniques which have been widely used in the anti-cancer nanopharmaceuticals industry to elucidate chemical structure. However, attenuated total reflectance-Fourier transform infrared spectroscopy is still one of the important methods used in quality control laboratories and generally, it is critically applied to characterize in a solid form and to prepare samples in a minimal form. Today, Raman spectroscopy is widely used in the field of anti-cancer nanopharmaceuticals research and development activities because of its high technology and specific applicability. For the identification, characterization, and investigation of nanopharmacologically active and related nanocompounds as discrete nanomaterials and in formulated products, therefore, the vibrational spectroscopy (a combination of mid-infrared, near-infrared and Raman) is broadly considered.

Key words: Vibrational spectroscopy; anti-cancer nanopharmaceuticals; spectroscopy techniques; nanocompounds; nanomaterials; mid-infrared spectroscopy; near-infrared spectroscopy; attenuated total reflectance-Fourier transform infrared ATR-FTIR spectroscopy; Raman spectroscopy

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Nuclear magnetic resonance (NMR) and mass spectrometry (MS) are two spectroscopy techniques which have been widely used in the anti-cancer nanopharmaceuticals industry to elucidate chemical structure. However, attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectroscopy is still one of the important methods used in quality control (QC) laboratories and generally, it is critically applied to characterize in a solid form and to prepare samples in a minimal form. Today, Raman spectroscopy is widely used in the field of anti-cancer nanopharmaceuticals research and development activities because of its high technology and specific applicability. For the identification, characterization, and investigation of nanopharmacologically active and related nanocompounds as discrete nanomaterials and in formulated products, therefore, the vibrational spectroscopy (a combination of mid-infrared, near-infrared and Raman) is broadly considered.

ANTI-CANCER NANOPHARMACOPOEIALS INVESTIGATIONS

At the present time, vibrational spectroscopy is mostly used by the major pharmacopoeiae, including

the United States Pharmacopoeia/ the National Formulary (USP/NF), the European Pharmacopoeia (EP), the British Pharmacopoeia (BP) and the Japanese Pharmacopoeia (JP), for the identification of pure excipients or active anti-cancer nanopharmaceuticals ingredients extracted from formulations detailed in specific monographs. The theoretical aspects, sample preparation procedures and instrument calibration requirements for ATR-FTIR spectroscopy are prepared, in details, by each pharmacopoeia. The importance of this method is highlighted by Frank as “. . . Attenuated Total Reflectance-Fourier Transform Infrared (ATR-FTIR) spectroscopy is considered by the USP as providing the most conclusive evidence of identity that can be obtained from any single test. . .” [1]. Although the information about identity and form of the sample (polymorphic, salt and solvate) can be provided by ATR-FTIR spectroscopy (polymorphic, salt and solvate), it is necessary to compare the sample spectrum with a reference standard ATR-FTIR spectrum as a confirmation for the analysis. For instance, about 380 reference ATR-FTIR spectra are provided by BP consistent with the specific nanocompound monographs.

However, it should be noted that the gross identity is more considered by these pharmacopoeia [1] monographs than polymorphic form, as the BP states that “When the spectra recorded in the solid-state show differences in the positions of the absorption maxim, treat the substance being examined and the reference substance in the same manner so that they crystallise or are produced in the same form...” [2]. In spite of the existence ca. 65% of all anti-cancer nanopharmaceuticals compounds (in the EP) in more than one polymorphic form (excluding solvates and hydrates) [3], this simple and reliable ATR-FTIR method can correctly identify the active nanocompound in the formulation under test.

The profile of Raman spectroscopy with regulatory authorities is much lower than ATR-FTIR methods. The USP is the only one that considers the analysis of nanopharmaceutical materials using Raman spectroscopy [4]. When this article was writing, there has been no method for the final batch release of any product using Raman spectroscopy that filed with the appropriate regulatory authorities. The method, however, is in its way to becoming more important in the industry, which will lead to changing of its position in the future. The main advantage of Raman spectroscopy compared to ATR-FTIR spectroscopy is that, there is no need for any sample preparation as it can analyze a sample non-invasively through its glass container. Replacing wet chemical, high-performance liquid chromatography, UV or ATR-FTIR identity methods in a busy laboratory might reduce the analysis time per 1000 samples down to 2–3 months [1]. However, changing the methodologies due to such reasons may not necessarily be a good idea in the field of QC. The cost of Raman spectrometers is too much more than the current instruments. Moreover, there is not a method for Raman analyses approved by regulatory. In this regard, the use of Raman spectroscopy is limited to the conditions that there is not an established and cost-effective method to release nanomaterials due to scientific or technical problems. The critical motivation for establishing Raman methods in pharmacopoeiae is the scientific need of nanopharmaceutical companies which may be supported by Raman instruments

production companies. Currently, there is a strong cooperation between the nanopharmaceutical industry and the regulators in the UK to enhance the profile of Raman spectroscopy and its advantages for nanopharmaceutical analysis [5].

A given sample can be analyzed in various ways using ATR-FTIR spectroscopy. Therefore, the analyst is of proper flexibility for choosing the most appropriate method for the considered work. In addition to the analysis of solids, as the primary application of ATR-FTIR spectroscopy, liquids, solutions, and gases can also be analyzed by this method. All these preparation methods are mentioned in the pharmacopoeiae, excepted the USP, including diffuse reflectance (DR) and multiple internal reflectance (MIR)/attenuated total reflection (ATR) methods. It should be noted that the use of KRS5 (Thallium bromo-iodide (TlBr-TlI)) crystals, a mixture of thallium bromide and thallium iodide are specified in the BP and EP for ATR measurements. However, zinc selenide (ZnSe) and germanium have been used in ATR-FTIR spectroscopy other than these toxic nanomaterials. The recent developments in ATR technology can be used to crystallize the addition of these MIR/ATR crystals while they are not yet detailed in the pharmacopoeiae. The sample, in any form other than gas, places on the ATR crystal, which is typically diamond or germanium. Then, the sample is subjected to a moderate pressure while its ATR-FTIR spectrum is acquired. Finally, the crystal surface is cleaned to start the next round of action. This procedure is the quickest ATR-FTIR preparation for the majority of samples with the total analysis time is typically between 3–5 min. The possible change of sample polymorphic form during its preparation for analysis is reduced due to the fact that the torque forces developed in preparing a nujol mull or KBr disk are more than the unidirectional pressure applied to the sample. There is a little difference between the ATR-FTIR spectra obtained using this technology and the traditionally obtained transmission spectra. The intensity of bands at low wavenumbers is higher than those at high wavenumbers since the penetration of longer wavelengths into the sample is deeper

than shorter ones and hence, the incident ATR-FTIR radiation is more absorbed. Although both spectra are of an equivalent number of bands with a similar position, their relative intensities are different across the spectrum.

The ATR-FTIR spectra of samples with inappropriate transmission properties, such as rubbers, cannot easily obtain. Due to too short effective sample path length obtained by the Golden Gate™ approach, the quality of obtained spectra is so high that allows the identification of various rubber vial closure types. Further, the measurement is difficult by most ATR methods because of the complex physical shape of vial closures. However, it is a simple work when single bounce ATR is used.

BULK SAMPLE ANALYSIS

Anti-cancer Nano Drug Substance Characterization Functional Group Analysis

The spectroscopic identification methods are of great importance during the early stages of anti-cancer nano drug development due to the providing of definitive evidence of chemical structure, which, in turn, is very important for clinical safety and the underpinning of patent applications.

Nowadays, NMR and MS are widely used to elucidate the structure as these spectral data are very clear and specific. However, ATR-FTIR spectroscopy is not a valuable method to identify the structure of simple nanomolecule such as aspirin $C_6H_4(CO_2H)(CO_2CH_3)$. The presence of four oxygen atoms in aspirin structure is easily confirmed by MS with classical elemental analysis (i.e. carbon, hydrogen, and nitrogen or CHN). At the other hand, the ATR-FTIR spectrum of Aspirin shows this fact as there are two carbonyls and two $-C-O-$ bands; the characteristic of carboxylic acid and ester functional groups within the nanomolecule. However, there are evidence for 1, 2-substituted aromatic ring and the methyl ester in NMR spectroscopy.

In addition to available accurate mass measurement methods, various empirical formulae may be proposed for structure elucidation of large organic nanomolecules. ATR-FTIR spectroscopy is a useful method for identifying certain functional

groups in such cases which lead to finding the correct elemental composition of the sample. At the other side, the difference between the presences of a carboxylic acid ($R-CO_2H$) or an amide ($R-CONH_x$) cannot necessarily find by NMR spectroscopy due to the possible absence of labile protons in their respective NMR spectra. However, there is a clear difference between the ATR-FTIR spectra of these functional groups. The carbonyl band which is the only absorbance in the range of $1800-1650\text{ cm}^{-1}$ offers useful information about the environment of the $>C=O$ functional group. A carbonyl stretch is shown at ca. 1720 cm^{-1} in carboxylic acid while a $C-O-$ band is observed at ca. 1400 cm^{-1} . In addition, the hydrogen-bonded OH stretch developed due to dimerization of the acid group is shown ca. 3000 cm^{-1} . There are two strong bands around 1650 and 1550 cm^{-1} for amide, known as the amide (I) and amide (II) bands, respectively. Moreover, there are two bands at 3400 and 3300 cm^{-1} for primary amides because of the antisymmetric and symmetric N-H stretches, respectively. However, in the same region, there is only one N-H stretch frequency for the secondary amides. In a similar way, the confirmation of the characteristic ATR-FTIR bands of other interesting groups such as the cyano ($-C-N$), nitro ($-NO_2$), ester ($-CO_2R$), sulfonamide ($-SO_2N<$) and phosphate [$(-PO(OH)_2)$] cannot be easily achieved by other spectroscopic methods. Moreover, ATR-FTIR plays a critical role in identifying predominant tautomeric forms. ATR-FTIR spectroscopy is a useful method for identification of specific characteristic forms of the *keto-enol* and *lactam-lactim* tautomers.

Raman spectroscopy provides a complementary approach to structural elucidation. The strongest Raman bands in organic nanocompounds belong to the alkene ($>CDC<$), alkyne ($-C-C-$) and cyano ($-C-N$) groups. However, nitro ($-NO_2$) and disulfide ($-S-S-$) groups have particularly characteristic Raman bands [6]. The number of protons presents in the double bond and cis/trans conformations can be accurately assigned by the position of the characteristic band which is the $>CDC<$ stretch region in the Raman spectrum. The identity of organic materials used as nanopharmaceutical excipients, such as Calcium phosphate [$Ca_3(PO_4)_2$] and Titanium dioxide (TiO_2),

can be confirmed by Raman spectroscopy. Titanium dioxide is approved by Food and Drugs Administration (FDA) as a white colourant in its anatase form. The differences between this type of TiO_2 and the alternative rutile form can be detected by Raman spectroscopy.

Due to the new use of Raman spectroscopy in the nanopharmaceutical industry, the structure elucidation using this method is primarily for supporting or confirming assignments made from the ATR-FTIR spectrum. Better acceptance of Raman data, particularly with regulatory authorities, is strongly related to more and more industrial use of this technique as well as its interpretation to the same standards as ATR-FTIR spectra.

Hydrogen bonding and zwitterions. One of the main aspects of vibrational spectroscopy is its ability to directly relate chemical structure to the solid state properties of the sample which has been mostly performed by single crystal X-ray diffraction (XRD) and ^{13}C solid-state NMR. The strong dipole condition is related to the presence of hydrogen bonds within groups and hence, ATR-FTIR spectroscopy can act as a valuable tool for identifying the extent and type of hydrogen bonds presented in different forms of a nanomaterial. The 40-methyl-20-nitroacetanilide is a good example; the type of Hydrogen bonding presented in the two forms of this nanocompound can be identified by both NMR and single crystal XRD only if the complete solution is obtained while this is not the case for the ATR-FTIR spectroscopy [7]. Both pure polymorphs can be easily differentiated by their colors, yellow and white. The carbonyl and nitro groups adjacent to hydrogen bond have competed with the amide NH within these two polymorphs which are related to the geometry of the amide group. It can be seen that hydrogen bonding with the carbonyl group is predominated in the white form. The position of amide (I) band at 1672 cm^{-1} demonstrates the characteristic of intermolecular bonding. Single crystal XRD also shows this fact as antiparallel molecular stacks involving nanomolecules, related by inversion. Due to the presence of two different nanomolecules in an asymmetric unit cell, there is more complexity in the yellow form. As a result of the presence of two similar

crystal structures within the nanomaterial, the carbonyl band is apparently divided. It can be concluded from the high carbonyl and NH stretching values that the hydrogen intramolecular bonding between the amide and nitro groups is not strong in the asymmetric unit of the yellow form.

The formation and observation of zwitterions in the ATR-FTIR spectrum of the nanocompound is not wondering in nanomolecules containing both acidic and basic groups. It is a common rearrangement that a carboxylic acid deprotonates while an amine group protonates. Due to the presence of carboxylate ion, the expected carbonyl band is not observed in the ATR-FTIR spectrum and a less intense band is appeared at $1600 \pm 50\text{ cm}^{-1}$. Between 2800 and 2100 cm^{-1} and at $1590 \pm 30\text{ cm}^{-1}$, there are characteristic bands of the accompanying $\text{R-NH}_x\text{C}$ group.

Polymorphism, salts and hydrates. Polymorphs can be defined as nanocompounds with different crystal structures that are of the same chemical entity. There are a unique three dimensional configuration and a unique unit cell for each polymorph. Sometimes, it is possible for a nanomaterial to have more than one hydrated form with different crystal structures. Further, the crystallization of nanopharmacologically active substances as a salt (nano drug as natural free base or acid form plus a counter ion) is due to enhancing the long-term stability of the nanocompound. It is possible that such salts form in a hydrate and show polymorphism. The physical characteristics of each form may be affected by its differences with others. It is a common case that the stability (higher the melting point, higher the stability), solubilities/bioavailabilities, and processabilities (the easiness of processing the nanomaterial into a product) of polymorphs are different. One of the analytical characterization techniques for polymorphs is vibrational spectroscopy. It is necessary to apply a combined form of various techniques including single crystal and powder X-ray diffraction (PXRD), thermal methods [e.g. differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA)], solid-state NMR and polarized light spectroscopy for fully characterization of any isolated form because of the complexity of polymorphic systems. Frequently, the

identity and form of a submitted sample are confirmed by vibrational spectroscopy despite the fact that it may be not the first selected method for characterizing a new polymorph. Patent applications include the ATR-FTIR spectrum of each form due to the specificity of the data and the relative ease of spectral acquisition. In the nanopharmaceutical industry, both nano drug structure and its polymorphic forms are per se patentable due to the fact that the nano drug properties may be improved by changing its forms. The reason for the importance of polymorph patenting is various economic aspects of such changes [8]. For instance, Glaxo Wellcome (GW) has the basic patent claiming ranitidine hydrochloride per se, as well as a patent covering the polymorph of ranitidine hydrochloride, which is the active ingredient in Zantac™. Novopharm has challenged this issue in the US courts but was not successful. If Novopharm became successful in their challenge, GW would have lost massive revenue from this ulcer treatment.

The stability of the polymorphic form can be measured through ATR-FTIR spectrum in some cases [9]. The previously mentioned ATR-FTIR rule is applied to any nanocompound that forms Hydrogen bonds, with the exception of amides. As the entropy is reversely related to stability and directly related to the frequency band, that form with the highest frequency band (i.e. in the region 3600–3200 cm^{-1}) will have the poorest stability. Recently, the Raman spectroscopy of Hydrogen-bonded polymorphs is considered with this approach [10]. The same rule appears to apply but it seems that there need to a more extensive review.

It has been confirmed that the lattice vibrations of various crystal structures are different from each other. Hence, it will be a good idea to recognize different polymorphic forms based on their lattice vibrations that are emerged. These vibrations occur below 400 cm^{-1} ; the region called far-infrared. To obtain ATR-FTIR spectra from this region, it is necessary to use special instrumentation (e.g. replacing the mid-infrared KBr beam splitters of an interferometer which have a 380 cm^{-1} cut off) and suitable sample support nanomaterials. However, vibrations with frequencies down to 50 cm^{-1} can be measured by FT-Raman instruments. By approaching to the laser

frequency, the Rayleigh line, measurements can be obtained using double or triple monochromator dispersive Raman systems. As Raman spectroscopy is able to simply measure the lattice vibrations, it becomes an interesting method for investigating polymorphism. In addition to the possibility of identification of polymorphic forms based on the data from this region (400-100/50 cm^{-1}), some information on the degree of amorphous nanomaterial present in the sample can be obtained from this data [10]. Due to less stability of amorphous anti-cancer nano drug substance than crystalline nanomaterial, knowing the amount of amorphous anti-cancer nano drug substance in products is very important to investigate its possible long-term effect on the stability of the marketed formulation [9]. Inhaled anti-cancer nano drugs are a good example of this issue as those are needed to have a particle size of ca. 1–5 μm . This particle size can be achieved by milling the sample without any variability. Such an aggressive process may lead to changing or reverting of the desired anti-cancer nano drug to the amorphous state. The effect of grinding has been confirmed on a uracil sample [9]. By increasing the content of amorphous form due to the successive grinding of the sample, the band is shifted and broadened as shown in FT-Raman spectra. The amount of amorphous nanomaterial existed in the nanocompound can be roughly estimated by these data.

Polymorphism analysis can obtain some quantitative data about one polymorph in another. The analysis of a mixture of two chlorpropamide polymorphs showed that if there is a large amount of the minor component (i.e. $\geq 10\%$), a reliable quantitative assay can be obtained by FT-Raman [11]. The data were analyzed using factor analysis while geometric mixing was used to prepare a reference set of mixtures (i.e. 100 – X: X, where X= 10n and n = 1–10). For anti-cancer nanopharmaceutical applications, it is relevant to quantitatively measure the minor polymorphs with accuracy between the levels of 1–5%. Langkilde *et al.* [12] studied the binary mixtures in which ATR-FTIR and FT-Raman spectroscopy showed the minimum of 2–15% of the minor polymorph. Because of the better quality of data, FT-Raman was considered as the preferred

technique, although both techniques were able to identify both polymorphs, separately. In spite of the same resolution of both data sets as 2 cm^{-1} , the Raman bands produced better calibration curves due to their baseline resolution and sharper bands with narrower bandwidths. This is a typical observation when ATR-FTIR, and Raman data from the same sample are compared. The ratio of characteristic band intensities at 1716 and 1724 cm^{-1} , respectively, are considered as the base of the calibration curve. The curve, then, was plotted against the theoretical mixture composition. It was observed that there is a linear relationship with quantification accuracy of 99% for the minor form in a binary mixture. Although this accuracy level has been reported for other methods, it should be noted that the intensity and wavenumber of the characteristic bands of each polymorph plays a critical role in this regard. However, the detection limit is very higher than those mentioned here for many other polymorphic systems.

Vibrational spectroscopy for hydrates is similar to that of polymorphs. Some additional experiments, however, should be implemented for identifying their characterization and formation mechanism. There are some modern cells such as environmental chambering diffuse reflection infrared Fourier transform spectroscopy (DRIFTS) and vibrational spectroscopy hot-stages to collect vibrational spectra from the sample while the surrounding humidity and temperature are changed. It is possible to achieve some information about the number of formed hydrates and their stability condition (stable or metastable) through monitoring and interpreting the spectra. Evolved gas analysis (EGA) is an alternative approach in which, the sample undergoes the dehydration/decomposition processes at high temperature while the exhaust of a thermogravimetric analyzer is attached to prepare the resultant gases for analysis.

Chirality. Due to the similarity of ATR-FTIR or Raman spectrum of each pure form and the racemic mixture, vibrational spectroscopy is not related to the study of chiral nanomolecules (Only when the different chiral environments cause different hydrogen bonding, vibrational spectrum will change). The method for identifying chiral nanocompounds is ATR-FTIR vibrational circular dichroism (VCD) spectroscopy.

The spectral difference of two spectra obtained using left and right-handed circularly polarized ATR-FTIR radiation is called VCD spectrum. Considering a specific nanocompound, the bands of ATR-FTIR VCD spectra and standard ATR-FTIR spectrum are in the same position. Note, enantiomers give VCD spectra that are mirror images of each other. For determining the absolute configuration of a nanomolecule or protein, single crystal XRD is typically selected. However, the major anti-cancer nanopharmaceutical application of VCD is the absolute configuration of small nanomolecules for nanomaterials that are not able to be crystallized, and those which are able to be crystallized but there is not a heavy atom or salt in the nanomaterial. Currently, density functional theory (DFT) can be used to calculate VCD spectrum for each different chiral form. The absolute configuration of the nanomaterial can be confirmed by comparing the calculated data with the measured solution ATR-FTIR VCD spectrum of the nanomolecule. Due to more difficulty of calculating and rationalizing the VCD spectra for flexible and large nanomolecules, best results are obtained for rigid chiral nanomolecules [13].

Another application of ATR-FTIR VCD is determining the percentage enantiomeric excess (%ee) of one chiral form in another at the accuracy of ca. 1%. It should be noted that VCD measurements are of lower accuracy than other methods such as NMR and chromatography. However, its advantage over those methods is that it has not required to separate the chiral nanomolecules (and any achiral nanocompounds) in the sample or to add shift reagents prior to the analysis. Therefore, enantiomeric excess measurements in process environments can be performed by ATR-FTIR VCD [14].

Methods to probe solid phase phenomena. The nujol mull and the KBr disk are considered as the standard methods of presenting samples for ATR-FTIR spectroscopy. Although they are easily prepared, they have one potential flaw in the study of solid forms. It is necessary for both methods that particle size of sample is considerably smaller than the shortest ATR-FTIR wavelength used so that the scatter loss from the ATR-FTIR beam is reduced and

ATR-FTIR band distortions is minimized. Therefore, the initial step for sample preparation is grounding the sample with a pestle and mortar to become a fine nanopowder. It is possible that this method leads to changing the form of the sample. Hence, it is more desired to use a less aggressive sampling approach. There are three potentially appropriate methods for investigating polymorphic forms including diffuse reflection (DR), photoacoustic (PAS) ATR-FTIR spectroscopy and FT-Raman spectroscopy [15]. These are ideal methods for dealing with samples which may change from using traditional mull and disk methods since these are not needed to complex sample preparation. In DRIFTS measurements, the sample initially mixes with KBr and then, packed into a sample cup prior to the acquisition of its ATR-FTIR spectrum. However, the only limitation of sample for PAS measurements is its size so that it can be placed inside the PAS cell. This is an easy requirement especially for nanopowders. However, my personal experience shows that the use of PAS may be limited due to the poor signal/noise observed in many spectra. This is very difficult to attribute such minor bands to a specific polymorphic form. However, the quality of these data obtained from this method is considerably improved as a result of new PAS cell designs and improved spectrometer electronics. The data obtained from DRIFTS and PAS methods on the same polymorph samples cannot be virtually separated from each other. However, the reproducibility of quantitative measurements is affected by the particle size and the thermal conductivity of the sample. This effect would be decreased when the polymorphism measurements are being performed post-milling or after micronization of the bulk nanomaterial since these processes should give reproducible particle size ranges.

No need to sample preparation makes FT-Raman an ideal method for these measurements. However as the volume of sampling is limited, it is necessary to rotate or spun the sample during the data acquisition period to avoid heating/burning the sample as well as to make reproducible quantitative measurements. The reproducibility of quantitative Raman measurements also affects by particle size [16]. However, it is possible to perform two non-destructive measurements (ATR-

FTIR PAS and FT-Raman) on the same sample using vibrational spectroscopy. In the case of using powder X-ray diffraction (PXRD) and solid-state ^{13}C NMR, more information can be achieved from the same single sample. As a result, there is a critical demand for a multi-technique approach to characterize the polymorphic forms.

These methods are not only applicable for investigating the polymorphs, salts, and hydrates, but also can be applied to any sample susceptible to irreversible change due to preparation, or when it is necessary to minimize sample disturbance. The latter case is particularly applicable to process analytical Chemistry (PAC) where the advantages of vibrational spectroscopy are often used.

Process Analytical Chemistry (PAC) Applications

The chemical processes can be measured in real time with the PAC approach. In addition, it allows the use of data in feedback systems for improving the efficiency of cost-effectiveness of the overall process. The monitoring and optimizing of the hydrogenation reaction are affected by PAC and ATR-FTIR spectroscopy [17]. Traditionally used methods for analyzing these reactions require the reaction mixture to be cooled, depressurized, sampled and analyzed at a point far from the reaction vessel. This leads to more time of the analysis which in turn, may be led to the poisoning of the catalyst. In this case, ATR-FTIR spectroscopy allows online monitoring. Hence, the spectroscopic data can be collected in real time while the end-point of reaction can be more accurately determined. The measurement and control of by-product formation is possible using the feedback mechanisms in on-line systems.

Changes of the intensity of the 826 cm^{-1} band due to the $-\text{NO}_2$ scissoring deformation is the sign for initial addition of the starting nanomaterial to the reaction vessel, and then its depletion on reaction. The increase of the amide (I) band at 1664 cm^{-1} shows the formation of the product. In a similar way, the reduction of the band at 758 cm^{-1} band is attributed to the formation of the by-product. This band is attributed to the pyridine ring C-H deformation that is present in both starting nanomaterial and product, but not in the further hydrogenated by-product. Based on the

real-time ATR-FTIR measurements, it is possible to accurately determine the reaction end-point and the subsequent reaction quenching. It allows minimizing the formation of the unwanted, over-reduced by-product.

Application of DRIFTS to understand the drying process associated with a cephalosporin can be shown by a simple example. If anti-cancer nano drug produces as the pentahydrate form, it can be considered as nanopharmacologically active nanocompound. However, there are certain drying conditions in which activity may be decreased. In the case of not adequate controlling of the drying step, dehydrates are reformed to an unstable anhydrous form by the pentahydrate so rapidly that dehydrates as a monohydrate form in the presence of trace moisture levels. The real conditions of a manufacturing plant were recreated in the laboratory by a DRIFTS accessory with an environmental chamber to investigate the cephalosporin drying process. It can be clearly seen that there are different ATR-FTIR spectra for the penta and monohydrate forms. While normal carbonyl absorption can be seen at ca. 1760 cm^{-1} for the pentahydrate, his band is shifted to 1805 cm^{-1} for the case of monohydrate [18]. Such a rare high-frequency carbonyl band for a β -lactam carbonyl absorption indicates a highly strained β -lactam ring. Regarding this issue that monohydrate is of poor nano drug potency, it can be ensured by careful monitoring of the drying process using ATR-FTIR spectroscopy that anhydrous/monohydrate form is not produced.

The possibility of taking measurements directly through glass is one of the advantages of Raman spectroscopy over ATR-FTIR methods. Raman spectroscopy has potentially a direct optical path into a reaction process of a chemical stream. The potential of Raman spectroscopy for controlling and optimizing a process is confirmed in the formation of NadololTM from sodium cystine tryptic agar (CTA)-phenolate [19]. In step (1), highly toxic epoxy-Cystine Tryptic Agar (CTA) is produced. As FT-Raman spectroscopy allows the monitoring of its formation, there is not necessary to directly analyze this process by manufacturing staff. The rate of reaction can be seen during continuous measurement. The damaging effect of moisture presence in the epichlorohydrin on the reaction rate can be clearly observed. Real-time monitoring allows immediate and corrective action to be taken. It is necessary to remove all traces of epichlorohydrin before starting step (2),

because this will react with the t-butylamine. It can be determined by the absence of Raman bands at 370 and 720 cm^{-1} . In a similar manner, depletion of epoxy-CTA and formation of thenoyltrifluoroacetone (TTA) can be determined from bands at 650 and 720 cm^{-1} , respectively. Although NadololTM is formed from thenoyltrifluoroacetone (TTA), minimization of residual t-butylamine leads to maximization of product crystallization. The level of t-butylamine can be tracked using Raman at 750 cm^{-1} , and as a result, it can be used as a feedback mechanism to optimize the final product and form. Raman plays a critical role during the manufacturing process to optimize the process and product yield.

Formulation Analysis

For many formulations, the presence of the active nanocompound is demonstrated by ATR-FTIR. Generally, there is a critical need to an extraction procedure that is specific for the active nanocompound in suspensions, creams, capsules and tablet dosage forms. For intravenous formulations the procedure may involve a simple precipitation of the active followed by a filtration step. There are many examples of these types of preparations in the pharmacopeiae.

For some formulations, it is possible to quantitatively measure some specific nanocompounds with minimal sample preparation. The levels of formulated ketoprofen [20] and propylene carbonate, as an excipient, have been measured by ATR-FTIR, in aqueous cream and anhydrous ointment formulations [21]. Despite the confirmed potential of ATR measurements for quantitative measurements, it should be noted that the application of ATR measurements is limited to academic activities since it requires sample preparation and hence, it is not widely applicable to ATR-FTIR analysis in the nanopharmaceutical industry. The identity and concentration of the active nanocompound can be successfully determined by DRIFTS directly from ground solid dosage forms. It has been shown that this approach can be applied to confirm the correctness of samples to be used in blinded clinical trials [22].

In a similar case, the FDA, USA, performed an investigation about generic nanopharmaceutical products for fraudulent use of excipients on powdered

formulations, such as KBr disks. It was observed ATR-FTIR was successfully differentiated only 18% of the innovator from generic formulation pairs submitted for analysis among more than 1400 nano drug samples [23]. Therefore, it is necessary to take care about direct bulk measurements from solid formulations. It should be noted that DRIFTS is best performed with specific systems using a validated methodology.

Another method has been used for direct measurements of specific nanomaterials in formulated products is ATR-FTIR PAS. Based on quantitative measurements for both brivudin and dithranol, over the range 0.5–10.0% in Vaseline™ ointments, it was observed that there is a linear and consistent relationship with chromatographic measurements [24]. However, an important point is that the spectroscopic method is significantly quicker and less labor intensive than the separation methods. As the nature of photoacoustic measurements is non-destructive, it can be used for direct investigation of bulk samples which removes the necessity of sample preparation and in turn, any change in forms. For example, the form of the active nanocompound in a tablet formulation may raise some questions about inappropriate properties of tablet dissolution. The active component of the formulation can be extracted by standard approach. However, applying this procedure may affect the results related to the form of the analyzed nanocompound. Based on ATR-FTIR PAS analysis, only the hydrate form of the nanocompound was observed in both good and poor dissolution tablets [25]. As a result, the reason behind the poor dissolution of the anti-cancer nano drug was not its form. In order to more investigate the sensitivity of the method, more tablets were prepared using anhydrous ethanol. PAS results confirmed that this process formed both anhydrous and hydrated anti-cancer nano drugs. In addition, it was concluded that the hygroscopic nature of the ethanol leads to dehydration of some of the in-going bulk anti-cancer nano drug during the manufacturing process which in turn, leads to formation of the anhydrous nanomaterial.

Moreover, intact bulk formulations have been measured by Raman spectroscopy. The tendency of anti-cancer drug nanocompounds to show relatively more intense Raman bands than the formulation

excipients is one of the advantages of Raman compared to ATR-FTIR. However, FT-Raman spectroscopy may be the best vibrational spectroscopic technique to confirm the anti-cancer nano drug's identity in the formulation. DRIFTS and FT-Raman data obtained from formulated products have been compared based on their spectroscopic content [26]. It was shown that the broad features, caused by polar groups and Hydrogen bonding, are only observed in DRIFTS spectra. Identification of minor components within the understudied formulations is possible due to the possibility of reliable spectral subtractions as a result of the sharpness of the FT-Raman data. However, it should be noted that the sample volume related to Raman bands is significantly smaller than that related to ATR-FTIR measurement. As a result, there is a possibility for FT-Raman measurements being sensitive to spectral variance if the sample is not homogenous. The solution of the problem is rotating the sample after each measurement and co-adding the resulting spectra, or spinning (in the xy-plane) and oscillating (in the z-plane) the sample continuously during the measurement. As a result of using these improvement approaches, sample inhomogeneities could be detected, the quality of quantitative measurements could be improved, and the possibility of burning the sample with the laser excitation could be reduced.

The experimental basis of the measurement is a critical difference of ATR-FTIR and Raman spectroscopy. Due to the nature of ATR-FTIR as an absorption technique, absorption bands will emerge in the resultant ATR-FTIR spectra if a nanomaterial that with an ATR-FTIR active group, placed between the ATR-FTIR source and detector, is investigated. However, Raman spectroscopy produce sample signal only from the point at which the laser excitation source is focused. Therefore, depth profiling of the sample can be achieved through Raman confocal measurements. In this approach, Raman spectroscopy is used to verify the identity of a packaged anti-cancer nano drug product through a blister packaging nanomaterial [1]. Considering the fact that FT-Raman analysis takes between 1 and 10 s, comparing the sample spectrum with library reference spectra can positively confirm the sample's identity.

SPECTROSCOPIC SAMPLE ANALYSIS

Inventing of ATR-FTIR and dispersive Raman spectroscopy improves the position of vibrational spectroscopy in the nanopharmaceutical industry, mainly for two reasons. First reason is that a wide range of sample sizes, including those invisible to the naked eye, can be analyzed with these instruments. The second one is that vibrational spectroscopy does not need to a large amount of nanomaterial, which may be so scarce and expensive. The conventional ATR-FTIR spectrometers and microscopes have some limitation varies between vendors and users. However, an organic sample with dimensions of 20 μm is measurable by ATR-FTIR spectroscopy. Considering a small nanomolecule with molecular weight of <400 a.m.u. and an assumed density of 1 g cm^{-3} , the detection limit is better than 8 ng or 20 pmol. However, Raman spectroscopy makes detection limits of at least two orders of magnitude smaller than that possible by ATR-FTIR spectroscopy. Although vibrational spectroscopy is an elegant sample preparation device, it is too expensive for small samples. Therefore, it is an appropriate method for visual inspection of the size, shape, morphology and crystallinity of a sample and can provide some information about the identity and polymorphic information of the sample. The applications of this technique are very wide. Aldrich and Smith reviewed the applications of ATR-FTIR spectroscopy in the nanopharmaceutical industry [27–36]. In the following sections, some specific uses and recent applications relating to nanopharmaceutical analysis are presented.

Anti-Cancer Nano Drug Substance Characterization

Using a vibrational spectroscopy technique, a further dimension of visual information is provided compared to that of the spectral data obtained from a sample. It is possible to determine the habit of crystals presented in a sample by viewing them. There may be some differences as a result of the presence of different forms of the nanocompound or changes in the crystallization process. The nanocompound form (i.e. polymorph, salt and/or solvate) presented in each crystal type can be typically confirmed through vibrational spectroscopy. It is possible to identify amorphous and crystalline areas of the sample using the polarized light. The latter has birefringent properties. These specific areas

or particles provide useful spectral data to identify or characterize the sample. The relationship between functional group orientations and the crystal habit can also be determined by polarized ATR-FTIR radiation.

The hot stage technique is another standard light spectroscopy technique using in vibrational spectroscopy which provides the opportunity to perform spectroscopy on single crystals or particles at non-ambient temperatures. While samples are heated and cooled at set rates, or through programmed temperature ramps, data are collected in real time, at timed intervals or nominated temperatures. The vibrational thermospectroscopy is an alternative approach for differential scanning calorimetry (DSC) or thermogravimetric analysis (TGA) to provide spectroscopic characterization of samples after thermal events. Some types of form changes such as glass transitions, polymorph conversions or chemical transformations are related to thermal events. For instance, polarized light spectroscopy cannot detect the polymorphic conversion of hexadecylaminobenzoic acid on cooling while it is easily detected by ATR-FTIR thermospectroscopy. In addition, it can be used to investigate the dehydration of the geminal diol group in trospectinomycin to a carbonyl group [35–42]. There is one thermally stable and commercially available polymorphic form for Etofylline. Although there are four other forms, these are only formed from the melt when isolated from the laboratory atmosphere using coverslips appropriate for ATR-FTIR spectroscopy. The transformation of one unstable form to another can be usefully tracked by hot stage ATR-FTIR spectroscopy; however, the nanomaterial will rapidly convert back to the one thermodynamically stable form and hence, it has not a nanopharmacological consequence, in practice [43–53]. The interconversion of four known forms of lufenuron investigated by Szlagiewicz *et al.* [30] using Raman hot stage spectroscopy through cycling of the stage temperature from ambient to that of the sample melt. Moreover, it was confirmed by spectral information that there are two new lufenuron forms while rather different polymorphic transformations happen for the (+) and (–) enantiomers.

Forensic Analysis

The nanopharmaceutical industry has continually improved the quality of its medicines by upgrading the standards and specifications of its products. To do this, trace level contamination should be identified and eradicated. The importance of vibrational spectroscopy, in this regard, is its ability for performing non-destructive analysis on single particles or fibers potentially affecting the quality of the product. The critical step for determining the origin of any contamination and for its preventing in subsequent processes or products is the identification of the nanomaterial.

Sometimes, black specks may be seen on white surfaces. However, the position of black nanomaterial may be accurately detected by visual examination of these samples, as those are located close to the surface. As a result, ATR-FTIR spectroscopy cannot be considered as an analytical approach since its applicability is limited only for direct surface measurements. It is necessary to remove the nanomaterial from the tablet before ATR-FTIR spectroscopic analysis. Raman spectroscopy is ideal for this particular analysis. Typically, an exposed area of a black particle of $>1\text{--}2\ \mu\text{m}$ can easily find to consider as a target for the laser spot. Hence, sample preparation is not necessary and in turn, potential ambiguities arising from extractive sample preparations are avoided. Amorphous Carbon particles cannot easily identify by analytical methods but Raman spectroscopy can do it. In this case, the origin of carbon is a spray dried excipient with product charring. It is very difficult to recognize tiny black specks in a large volume of low-density excipient before manufacturing of nanopharmaceutical product. However, the presence of carbon in a nanocompound is primarily an aesthetic issue because of its inert nature, although it is not a favourite.

To get assurance about free-haze injectable formulations, development formulators, and production plants work to very high standards. Vibrational spectroscopy is an appropriate method for identifying a haze. In spite of the undesirable appearance of haze on a product, its amount is in the microscopic scale, probably much lower than the maximum allowable impurity level (e.g. 0.1%).

A reaction or complexation between formulation components or aggregation and precipitation of a specific nanocompound are the possible reasons for producing hazes [54–84]. Hazes are typical in products contain hydroxyethyl starches (HES), used as plasma volume expanders. If a trace amount of amylose starch is presented, it will aggregate and precipitate from the solution under specific conditions. Although HES and amylose are of polysaccharide structures, ATR-FTIR spectroscopy can be used to identify and differentiate them [85–89]. Due to helical conformation, the bands of amylose spectrum are sharper in the region $1300\text{--}900\ \text{cm}^{-1}$ and this is attributed to helical conformation. However, HES shows broader ATR-FTIR bands in this region due to its loose random coil.

The nanomaterial extracted from the packaging nanomaterials also can produce hazes. For instance, nanomaterial may be extracted antioxidants from the rubber vial stoppers [90–110] and silicone (polydimethylsiloxane) oil removal which is regularly used as a lubricant on these closures [111–133]. ATR-FTIR spectroscopy can easily detect Polydimethylsiloxane since its spectrum is very special.

Crystals formed in the vial containing a nano drug/saline solution should be identified in spite of their limited available amount. This is a non-invasive investigation by Raman spectroscopy. The spectroscopy was used to observe crystals in the glass vial while Raman spectra were obtained easily, without sample preparation. When nano drug in its Sodium salt form, the spectra identified the crystals. The salt solubility profile is re-evaluated and the composition of the formulation is modified.

Formulation Analysis by Raman Chemical Mapping

Identification of specific components or features of intact formulations has not been successful due to the large thickness of many samples which make transmission measurements very difficult. However, there have been some exceptions such as a nanopharmaceutical cream containing cetrimide, cetostearyl alcohol and paraffin. The emulsion phases were observed in a thin sample. Further, the cetostearyl alcohol concentration in individual

droplets was investigated by a quantitative analysis [134–155].

Reflection measurements are the alternative ATR-FTIR spectroscopy approach for thick samples. The obtained spectra have not high quality and the dominant specular reflectance is contributed. If good contact with the sample surface is provided, which is not always possible, the quality of data may be improved by ATR-FTIR spectroscopy measurements. In addition, ATR-FTIR spectroscopy is not able to provide pure spectra from too small samples such as many discrete particles in solid dosage forms.

These problems have not the same effect on Raman spectroscopy. Back-scattering optics measure from the sample surface and hence, removes the problem of sample thickness or opacity. A focused laser with spot sizes of ca. 1–5 μm , makes collecting Raman spectra from discrete particles or crystals within a formulation very easy. Moreover, single point measurements are not the only way for collecting data. Many sequential spectra can be obtained by adding an automated XY stage. Further, autofocus measurements can be provided by an automated XYZ stage. As a result, the surface of solid dosage forms can be surveyed by Raman. The little sample preparation needed for these experiments is cutting a tablet to make a cross-section or blending it so that a flat surface prior to the mapping experiment is provided. Raman chemical images obtained from the acquired data provide a visual representation of the sample's chemical composition.

It has been previously discussed that why polymorphism is important in the nanopharmaceutical industry. There is a concern for nanopharmaceutical product formulators about the change of polymorphic form of the nano drug during manufacturing. Methods such as PXRD can be used to evaluate this concern for high dosage strength formulations. However, there are many new nanopharmacologically active nanocompounds with a high potency which are frequently of a minor percentage component in the formulation. The polymorphic form of the nano drug within these solid dosage forms can be confirmed through limited methods including Raman. By surface

mapping, it may be possible to locate, identify and measure the nano drug and excipients through many thousands of acquired spectra. A chemical image of a tablet blend into which has been spiked a different form of the nano drug at the 1% level. To generate the chemical image, a single characteristic peak is selected as the representative of each component in the sample. Then, the intensity of this band at any point and the location of that specific nanocompound in the mapped area are determined and represented in a two-dimensional (2D) grayscale map. By superimposing the maps for each component which have different colors, a chemical image of the sample can be obtained. Otherwise, multivariate analysis of the whole data set can be performed to create the image.

In this particular chemical image, the location of spiked nanocompound in the mapped area can be clearly observed. The identity of the nano drug form is always confirmable because of the specificity of the Raman spectrum obtained at each point. In this case, the spiked nanocompound was present at the 1% level while its limit of detection could be better than 0.1% according to these data. As a result of the too small size of the mapped areas (typical map dimensions are of the order of 200–1000 μm) and the impossibility of considering this area as the representative of the bulk sample, it is not possible to determine the low-level presence of different polymorphic forms using this approach. However, a semi-quantitative approach can be followed to use the data for expressing the composition of the specific mapped area.

It can be concluded from Raman chemical images that the polymorphic form of nanocompound has not changed during manufacturing processes. However, polymorphic transformations are possible for pressure sensitive nanomaterials due to tableting a formulation blend. This is not a useful method for obtaining information about different physical characteristics of different tablet batches. The disintegration of the tablet in an aqueous media as well as subsequent dissolution of the active nanocompound into solution can be confirmed by dissolution testing on most solid dosage forms. The following example shows two batches of a product with different dissolution profiles. However, an identical nanomaterial was used to manufacture

the batches. The challenge of mapping this sample is the active presented at ca. 1% with an in-going particle size of ca. 12 μm . The nano drug particle size in the “poor dissolving” sample is maintained which is generally associated with cellulose like excipient called Avicel™. Due to the presence of calcium phosphate particles in the “good dissolving” sample, the nano drug size is bigger. It is necessary to have information about both the drug-excipient association and particle size characteristics of both anti-cancer nano drug and calcium phosphate for the desired dissolution profile. It may be achieved through mixing and milling the anti-cancer nano drug and calcium phosphate together in the first step of the manufacturing process. Raman chemical mapping is recently used in the nanopharmaceutical industry. In the future, it will be used to support formulation development and manufacturing process scale-up in addition to playing its role as a troubleshooting technique. Fourier transform near-infrared (FT-NIR) spectroscopy [156-186] and Near-infrared (NIR) spectroscopy imaging techniques can also be used to perform these experiments for improving our understanding of solid dosage form properties. Chemical image fusion (CIF) is a complementary technique that merges data from the same sample area mapped by both Raman and FT-NIR spectroscopy experiments [187–357]. Since it is a typical issue that nanocompounds with strong Raman spectra have weak NIR spectra, and vice versa, one mapping technique may not be able to describe multicomponent systems completely. The example of such condition is nanopharmaceutical products containing the active nanocompound(s) with organic and inorganic excipients. The best chemical image for each component can be obtained by CIF to show the complete solid dosage formulation.

CONCLUSIONS, PERSPECTIVES AND FUTURE STUDIES

Although there are various applications for vibrational spectroscopy in the nanopharmaceutical industry, its strength is in solid form analysis. Typically, this method is used to identify nanocompounds, while the obtained results are used in the release of nanopharmaceutical products. The Raman and ATR-FTIR spectroscopy are applicable to both bulk

and micro samples, but these are usually applied for characterization and understanding of the behaviors of polymorphs, salts and hydrates. The investigation of solid dosage forms through the use of chemical images obtained by vibrational spectroscopy is a new method which provides useful information hardly to be obtained by any other technique.

ABBREVIATIONS AND ACRONYMS

BP: British Pharmacopoeia
 CIF: Chemical Image Fusion
 DFT: Density Functional Theory
 EGA: Evolved Gas Analysis
 EP: European Pharmacopoeia
 FDA: Food and Drugs Administration
 GW: Glaxo Wellcome
 HES: Hydroxylethyl Starches
 JP: Japanese Pharmacopoeia
 MS: Mass Spectrometry
 NF: National Formulary
 NMR: Nuclear Magnetic Resonance
 PAC: Process Analytical Chemistry
 PXRD: Powder X-Ray Diffraction
 QC: Quality Control
 USP: United States Pharmacopoeia
 XRD: X-Ray Diffraction
 ATR-FTIR: Attenuated Total Reflectance-Fourier Transform Infrared
 FT-Raman: Fourier Transform-Raman

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