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Emerging analytical techniques used for nanocrystal and nanosuspension

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Introduction

Since last decade, many nanoformulations like; nanosuspension and nanocrystal have received the significant attention as a approachable tool to deliver the drug at the specific site and diagnosis. Drug nanocrystals are the nanoparticles, which are present in solid state. They are stabilized with using the polymers and/or surfactants by providing the significant barrier against the particles aggregation [1-4]. However, sometimes there are no uses of stabilizers [5]. The development of barrier can be dependent upon one material either polymers or surfactants, but many times mixture of polymers and surfactants are used [6]. Nanocrystals get dissolved very quickly, when come into the contact of fluids in-vivo. When the rate of release and dissolution are not important factor in certain preparations like; in intravenous preparation or implants, the size and shape of particles, surface charge and some other properties of product plays an important role [7]. Thus, in drug nanocrystal system it is necessary to recognize the application of such system that whether particles are meant to be dissolved quickly or having the aim to release the drug in controlled manner [8-10].

A suspension of drug nanoparticles in a liquid is called a nanosuspension. Nanosuspensions are sub-micron colloidal dispersion of pure particles of drug, which are stabilized by surfactants. Pharmaceutical nanosuspensions are usually very finely dispersed solid drug particles in an aqueous vehicle. The main difference from conventional suspension is that the particle size distribution in nanosuspension is less than 1 µm with an average particle size range between 200-600 nm. [11-13]. Nowadays, nanosuspension is a very fast accelerated approach for different pharmaceutical product development due to having the various advantages such as improved bioavailability; targeted delivery of drugs to the specific site to minimize toxicity; improved stability of drugs against enzymatic degradation; improved dissolution rate of the drug, reduced dosing frequency; improved patient compliance and the ease of administering through various routes [14-16].

The production of stable nanosuspensions in a safe, fast and predictable manner would be highly advantageous. Thus, production of stabilized pharmaceutical nanosuspensions have been a recent focus of the pharmaceutical industry where it has been shown that nanosuspensions of poorly water-soluble drugs exhibit a greatly increased dissolution rate, and as a consequence, an increased in vivo bioavailability [15, 17]. Despite the several advantages, some drawbacks are also related to the nanosuspension and stability problem is one of them. Stability is an important concern that ensures the product safety and efficacy such as; particle agglomeration could create a serious is-

sue in pulmonary drug delivery because it affects the deposition amount and finally drug safety and efficacy. Similarly, nanosuspension having particle size more than 5 µm can lead to capillary blockage and obstruct the blood flow [18, 19]. Thus particle size and size distribution should be monitored carefully during storage. Sometime stability issues arise since manufacturing because high pressure and temperature could produce the crystalline changes in the drug particles [1, 19, 20].

The stabilizing agents used in the production of pharmaceutical nanosuspensions may have a significant effect on the size and morphology of the resulting particles, and as a result, can affect various chemicals, mechanical and physical properties of a substance, as well as many processing parameters. Furthermore, stabilizing agents used in crystalline particle formation can significantly alter the nucleation, growth, and morphology of crystals by the exploitation of the solvent/stabilizer's intrinsic chemical properties [15, 21].

Nowadays, different in-vitro and in-vivo techniques are used to characterize a new developed nano-formulation or new discovered molecules and predict its clinical efficacy. These techniques are so efficient that it can facilitate the product and process optimization. Due to inadequate application of these characterization techniques, we consider their limitation as a prediction tool, only [22]. This article provides a critical review on the most important characterization techniques used for drug nanocrystal and nanosuspension. The characterization techniques (Table1) used to determine the physical stability of nanosuspension and nanocrystal like; particle size and solid state form are discussed in detail in this article. Sizing techniques may be categorized into imaging and non-imaging techniques.

Non-imaging techniques

The selection of measurement techniques are directly depends upon their purpose and this involves the research purpose, quality control purpose, process improvement purpose and measurement time purpose. Nano-sizing techniques can be categorized into imaging and non-imaging techniques. In non-imaging technique, particle size, size deviation and some degree of aggregation can be visualized [23]. The most utilized non-imaging techniques are the light scattering and electron microscope based techniques [6, 24]. In general, combination of two or more than two techniques are uses for research purposes, and for new sample evaluation like combination of light scattering and electron microscopy method.

Table 1: Characterization techniques with relevant to characteristics of nanosuspension and nanocrystal

Characterization technique	Characteristics attributes	Comment
Imaging techniques; Scanning lectron microscopy, Transmission electron microscopy	Particle size, Particle shape, Particle morphology	Time consuming technique, sample used in dried form, only part of the sample is analyzed, drying and gold or platinum coating may change the surface property of the sample
Imaging techniques; X-ray diffractometry, Differential Scanning Calorimetery	Solid state properties	Used in solid state characterization , interaction studies and in chemical structure determination
Non-imaging techniques; Laser Light Scattering	Particle size, sample homogeneity, particle aggregation	Fast and repeatable technique, measurement in the form of suspension, shape information cannot be produce
Non-imaging techniques; Laser Doppler Electrophoresis	Particle's electrophoretic mobility, particle surface charge/Zeta potential	Measure the speed of moving particles, very fast and accurate technique, work easily at high concentration of drug, repeatable technique

Laser light scattering

Laser light scattering is the alteration of the direction and intensity of a laser light beam that strikes an object and caused by the combined effects of reflection, refraction and diffraction [25]. For this, samples may be solid particles suspended in liquid, solid particles or liquid particles suspended in a gaseous media (aerosols). There are two types of different laser light scattering techniques are available which can be used to analyze the particle size distribution of solid liquid dispersions. These techniques include; Static laser light scattering and Dynamic light scattering.

Static laser light scattering

This technique is also known as "Rayleigh" scattering, "Laser diffraction", Low-Angle Laser Light Scattering (LALLS) or Fraunhofer diffraction. Measurement of particle size and distribution using laser diffraction has become preferred choice for wide range of samples [26, 27].

The interaction of laser light with particles leads a scattering pattern due to reflection, refraction and/or diffraction phenomena, as shown in Figure **1**. The assumption behind the laser diffraction, during determining particle size, is based on the concept that when the particles move through a laser beam it scatter the light at fixed angle. This is based on Refractive Index (RI) and particle size of the material which undergone for their study. The intensity of the scattered light may be affected by particle size, refractive index, number of particles, angle of observation and the wavelength of light. The higher the refractive index of the particles relative to the medium exhibited the more light that will be scattered. For larger particles, the scattering intensity will be high at narrow angles and for smaller particles; the intensity will be low and isotropic at wider angles.

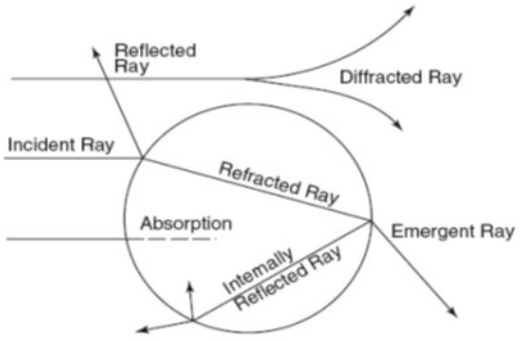

Figure 1: Interaction between light and particles [28]

Two different optical models are used to calculate the particle size distribution for laser light scattering experiments: Mie Theory [29] and the Fraunhofer Approximation [30, 31]. The Mie theory is a tool used to calculate size distribution based on refractive index differences between the particles and dispersion medium. This theory holds true for spherical, isotropic particles illuminated by monochromatic light. The Fraunhofer approximation assumes the particles are opaque, two dimensional, large circular discs and describes light scattering from the edges of an object.

Such technique has wide flexibility to different sample types; wide size range; rapidity; and high precision. It shows the wide range of particle size distribution during the presence of non-spherical particles [26].

Dynamic light scattering (DLS)

DLS is also known as photon correlation spectroscopy, Quasi-elastic Light Scattering, and diffusing Wave Spectroscopy [28]. It is generally used to determine the particle size and their distribution. Polydispersity index of various types of samples including nanoparticles, colloids, gels, emulsions, pigments, liquid crystals, DNA, polymers and proteins can measure using this technique [32].

In this technique, the temporal fluctuation in intensity of scattered laser light (in microseconds or milliseconds) by the particles present in solution or suspension is processed with the auto-correlation function. PCS gives the information about the particle size distribution, particle motion in the medium and the dynamics of the dispersed particles. In this technique, diffusion co-efficient is converted into the particle size by using the Stokes-Einstein equation with considering the assumption that the particles are available in spherical shape having no interparticulate interaction. The PCS technique gives particle size as a hydrodynamic diameter while particle size distribution is based on scattering intensities. The fluctuation in intensity of scattered light at a given scattering angle (usually 900C) is due to particle movement generated from the Brownian motion [33]. The lower size limit of detection with this instrument depends on differences in refractive index between the particles and the medium, experimental noise (arising from electronic noise as well as temperature fluctuations, and environmental disturbances). Similarly, upper size limit detected with medium viscosity and density of materials.

When a particle denote his probability density function by (d) for the Brownian motion and diffusion coefficient by (D) then relationship between them can be expressed as [34]:

$$P(d,t|0,0)=(4\pi Dt)-3/2 \exp(- d2\ 4Dt)$$

DLS provides three types of diameters of samples such as Z-average diameter, number mean diameter, and volume mean diameter etc. Z-average diameter shows the mean diameter based of laser scattering intensity, obtained from an exponential fit. The Polydispersity Index (PDI) can also be measured using DLS (Dynamic light scattering) instruments. Samples present in Monodispersion form exhibit the lower PDI value. The higher value of PDI shows a broad particle size distribution and the polydisperse nature of the sample. PDI can be calculated by the following equation as follows [35]:

$$PDI = \Delta d/davg$$

Where, (Δd) is the standard deviation of particle size and (davg) is the average particle size. The usual range of PDI values is:

0 - 0.05: for monodisperse standard

0.05 - 0.08: for nearly monodisperse

0.08 - 0.7: for mid range polydispersity

> 0.7: for very polydisperse

DLS method is a non-destructive, noninvasive technique. There are no need of calibration for this technique and required small volume of sample. It also doesn't require extensive sample preparation and required short analysis time, and modest development costs. Particle size, size distribution and polydisperisity index of various systems such as polymers, proteins micelles, carbohydrates, nanoparticles, emulsions and microemulsions, can be measured using this technique [36].

Laser doppler electrophoresis

Laser Doppler Electrophoresis (LDE) combines the principles of Laser Doppler Anemometry (LDA) and electrophoresis [37]. This technique used to measure the speed of moving particles taking into account the Doppler shift of scattered light by particles in motion. It is the most widely used technique to determine electrophoretic mobility, particle surface charge or zeta potential for colloidal dispersions including nanoparticles [38], biological cells and bacteria [39-42].

The motion of the charged colloidal particles, relative to the liquid, under the influence of an electric field is known as electrophoresis [43, 44]. The positively charged particles move to the cathode and negatively charged particles migrate towards the anode. There are three ways by which a solid particle (colloid) dispersed in a liquid media can acquire a surface charge. First, by the adsorption of ions, present in the solution and second one by ionization of functional groups on the particle's surface. Third one, due to the difference present in dielectric constant between the particle and medium. The zeta Potential is defined as the difference in potential between the surface of the tightly shear plane and the electro-neutral region of the solution (Figure 2) [45].

The zeta potential can be calculated with the help of some theoretical models and the data obtained from electrophoretic mobility, experimentally. The most widely-used theory for calculating zeta potential is the Smoluchowski theory [46]. The theory is depend upon on electrophoresis and can be denoted as:

$$\mu = \zeta\varepsilon/\eta$$

Where, (μ) and (ε) represent the electrophoretic mobility and electric permittivity of the liquid, respectively. (η) and (ζ) denotes the viscosity and zeta potential, respectively. The instrument measures the electrophoretic mobility *Ue* of the introduced sample and converts it into the ζ potential with the help of Henry's equation

$$Ue = \frac{2\ E\ \zeta\ f\ (\kappa a)}{3\ \eta}$$

Where, *Ue* and *E* denotes the electrophoretic mobility and dielectric constant of the dispersion medium, respectively. Similarly, ζ and *f (κa)* represent the zeta potential (mV) and Henry's function, respectively, and η is the viscosity of the solvent medium. The ζ potential is an important parameter to determine electrostatic colloidal dispersion stability [47- 49].

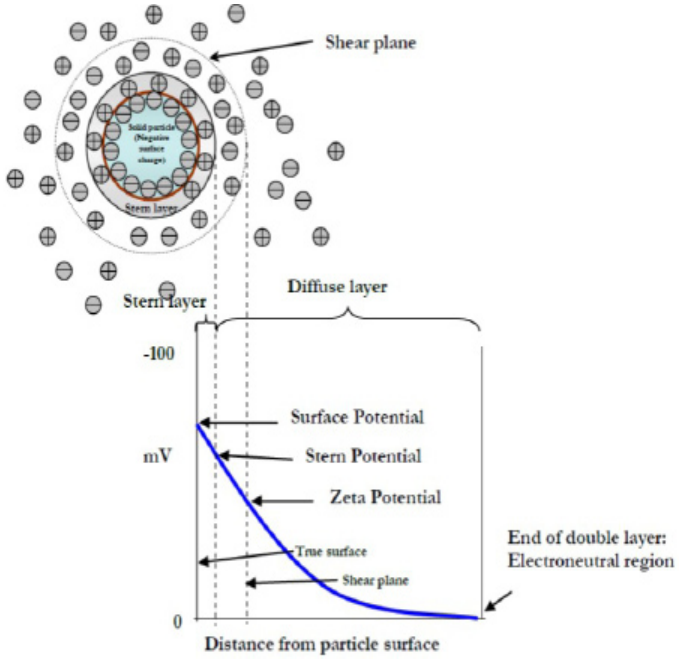

Figure 2: Schematic representation of various potential gradients on the surface of a particle [47-49]

Laser Doppler Electrophoresis (LDE) is based on the combination of electrophoresis and Laser Doppler Anemometry (LDA) as described before. This technique is widely used to measure velocities and thereby zeta potential of colloid particles. It is based on the measurement of light scattering to the determine particle size for diluted dispersions or suspensions when particles flow through a series of interference fringes. When an electric field voltage is applied across the liquid dispersion juncture, particles which were migrate towards either electrode depending on their magnitude and sign of charge. The higher the velocity of the particle shows the higher zeta potential. The velocity of the particle is measured by the Laser Doppler Anemometry (LDA) technique and converted to zeta potential using Smoluchowski equation [50].

Laser Doppler Anemometry (LDE) is a very fast and accurate technique which can measure the zeta potential in few seconds. With the help of this technique, the Wide range of particle concentration and solution containing high concentration of salts can be measured. With the help of this instrument any small value related to electrophoretic mobility can also be detected very easily. Typical applications are in the formulation of particulate dispersions such as suspension. Most widely used instrument available is Zetasizer® with standard cell [51].

Imaging techniques

With the help of imaging techniques some kind of visualization of the particles can be performed, and in-spite of particle size and size deviation, some additional information like particle morphology, shape, that are not available with the non-imaging techniques, can also be determined. In particle size analysis, the imaging techniques takes the too much time and more laborious for exact particle size determination. However, non-imaging techniques like light scattering based techniques are faster, less laborious and thus, should be the first choice for particle size determination. The light scattering measurements itself are enough for quality control purposes, but for research purposes, the combination of techniques, like electron microscopy with light scattering techniques is more preferable, especially, in involvement of new or unknown sample [52].

Electron microscopy

Electron microscopy techniques, like Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM) can be used for particle size analysis and their morphology. Such technique gives the information about the particle size and morphology at the same time [53, 54]. SEM requires clean, dry and electrically conductive samples. Thus, sample need to be coated with platinum or gold, before the measurement. If the sample is present in the liquid state then it is necessary to dried it before going to measurement. Sometimes this method can alter the s ample properties, also.

Scanning electron microscopy

The Scanning Electron Microscope (SEM) is one of the most important instruments, applied to surface microstructure imaging [55]. It is a type of electron microscopy that produces the images of the sample surface using a focused beam of high-energy electrons.

SEM working based on the signals obtained from elastic and inelastic interactions between the electron beam and the specimen surface. When the primary electron beam bombards on the sample, electrons penetrate into the sample surface and form an excitation zone. It is known as the interaction volume. The accelerating voltage and atomic number are responsible for the shape and size of the interaction volume. In this process, the electrons lose their energy due to repeated random scattering. Elastic scattering occurs due to deflection of incident electrons by the atomic nucleus or outer shell electrons. This is responsible for the negligible energy loss and produces backscattered electrons at an angle of more than 90^0. In inelastic scattering, the secondry electrons are emitted due to the transfer of substantial amounts of energy. Electronic amplifiers amplify the signal and cathode ray tube scans the surface. The images are captured by photography or digitally and displayed on computer monitor.

SEM is one of the widely used instruments in a variety of research area. It is used for combination of large depth of field, high resolution, high magnification, minimal sample preparation, easy sample observation, and rapid data acquisition. Generally, SEM is used to analyzing the shapes and surface topography of samples but sometime also used in analyzing the spatial variation in different chemical compositions using the elemental maps, and spot chemical analysis. It is also widely used to identify the crystalline orientation of materials and different type fabric [56].

Transmission electron microscopy

Transmission Electron Microscopy (TEM) is a very effective tool that provides direct visualization of the particle morphology and ultra-structure of colloidal system by giving the information of particle size, shape, and ultra-structure [57, 58]. This instrument also provides the contrast images of fairly good resolution which is obtained by staining with contrast agents such as uranyl acetate [57, 59]. TEM perform their working due to using of electron beam. In this technique, the image is created due to the interaction of the sample specimen with electron beams.

TEM image is based on the interaction of an electron beam with the object using various electron scattering mechanisms, illumination conditions, and also on the action of objective lens and arranged apertures [60]. Various contrast modes are available in this technique for bettering the image quality. These

modes includes: Bright and dark field; diffraction contrast; and phase contrast [61]. Bright and dark field mode is based on the occlusion and absorption of electrons in the sample. The diffraction contrast mode is used to study the crystalline structure. Using selecting Bragg scattering, sharp spots can be visualized. In phase contrast mode, scattered electrons are allowed to recombine with un-scattered electrons to form the image.

It is the most widely-used technique for the structural elucidation of SLN dispersions, nanosuspensions and other nanostructures. TEM is generally used to obtain the information such like composition, shape and size of the various materials. Recently, High Resolution TEM (HRTEM) has been used to obtain a resolution of 0.2 nm [62].

Solid state properties and chemical stability

The different solid state forms of the material like amorphous, solvates, hydrates, crystal form, and degree of crystallinity directly affected the physical performance of the system such as solubility and dissolution rate. In general thermodynamically stable form shows the lower solubility. When temperature changes or mechanical energy are generated during the mechanical process, can lead the polymorphic changes or formation of amorphous material. Sometime these mechanical processes also degraded the material. Such polymorphic changes, formation of amorphous form or any other changes in the material can be studied using the solid state analysis. Such analysis includes the thermal analysis such as Differential Scanning Calorimetry (DSC) and different spectroscopic techniques like powder x-ray diffraction [52].

Powder x-ray diffraction analysis

X-ray diffraction is also an important characterization tool used in solid state chemistry and materials science, widely [63-67]. X-rays are electromagnetic radiation exhibit the wavelength about 1 Å (10^{-10} m), which is about the same size as an atom. This technique was discovered by WC Roentgen in 1895. It was efficiently used in the field of Crystallography to probe the crystalline structure at the atomic level. All the crystalline materials show their own fingerprints [68]. Some applications for X-ray powder diffraction are as follows:

- Single-phase material's identification
- Identification of multiple phases in microcrystalline mixtures
- Determination of the crystalline structure of different materials
- Structural analysis and identification of clay minerals
- Recognition of amorphous materials

The three-dimensional structure of any crystalline compound is depicted by regular, repeating planes of atoms that form a lattice. When a collimated beam of monochromatic X-ray impinges with these planes of atoms, the proportion of the beam is diffracted in various directions. Depending on the arrangement of atoms in the crystal lattice, the identified material would produce different patterns of diffraction. Thus, it is possible to measure the distances between the planes of the atoms by applying Bragg's Law [69]. Bragg's law states that diffraction occurs only when the distance traveled by the rays reflected from successive planes differs by a complete number (n) of wavelengths.

Thus, for a monochromatic X-ray beam with wavelength λ (lambda) is projected onto a crystalline material at an angle (θ) (theta), Bragg's Law states that:

$$n\lambda = 2d \sin \theta$$

Where, the integer (n) is the order of the diffracted beam, (d) is the distance between adjacent planes of atoms (the d-spacing). The characteristic set of *d*-spacing generated in a typical X-ray scan provides a unique "fingerprint" for the sample. When properly interpreted, by comparison with standard reference patterns and measurements, this "fingerprint" allows for identification of the material.

The advantages associated with the PXRD analysis are as follows

1. Beneficial for fast measurement
2. Non-destructive analysis of multi-component mixtures
3. There are no need for extensive sample preparation
4. Relatively straightforward and significant data interpretation
5. This instrument can be used for analysis of a variety of transformations obtained during different manufacturing processing and storage such as: Polymorphic transformations; alterations in crystallinity; changes in state; and degree of hydration [70].

Thermal analysis

Thermal analysis broadly covers various techniques that measure chemical or physical changes that occur when a known quantity of a material is subjected to a controlled temperature program over time.

Differential scanning calorimetry (DSC)

Differential Scanning Calorimetry (DSC) is one such technique that uses to measure the heat exchange (*e.g.* heat uptake during melting or heat release during crystallization) during the structural changes of a material, introduced into the instrument [71, 72].

DSC measures the enthalpy dependence of a sample related to a reference. There are some thermal phases that can be detected with the help of DSC. These thermal phases include; endothermic phase, exothermic phase and change in heat capacity of the introduced sample (e.g. glass transition) [73-75]. DSC instruments may be of two types. First one is the power compensated DSC and another type is heat flux DSC. In power compensated DSC, the different pans like sample and reference pan are heated from different sources. The differential temperature between the sample and the reference is maintained zero while the power to maintain the same temperature in the two pans is measured [76]. However, in the heat flux DSC both pan are heated with the same source and the differential temperature is measured. The DSC curve is displayed as a function of time or temperature on the abscissa and the rate of energy absorption (heat flow) on the ordinate axis. Transitions accompanying changes in specific heat are demonstrated as peaks with areas proportional to the total enthalpy changes.

DSC uses to determine the heat capacity and heat of reaction. Such instrument also uses in determination of glass transition state, Degree of crystallinity and Crystallization tempera-

ture. Stability studies, porosity measurement and polymorphic transition state can also be estimated with the help of this instrument.

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