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IN VITRO EVALUATION AND CHARACTERIZATION METHODS FOR SUB-MICRON PARTICULATE DRUG DELIVERY SYSTEMS

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Abstract: Formulation of nanoparticle drug delivery has proven to offer distinct advantages over other formulations. Some of them include increased solubility, better bio-distribution, better subcellular penetration, better pharmacokinetic profile, active and passive drug targeting in cancer therapy. In vitro evaluation and characterization of any type of formulations is an important area to understand, predict and correlate the in vivo performance of these formulations. Understanding of how different the nanoparticle formulations are from the conventional sized formulations is a necessity to understand the minutiae of the evaluation and characterization techniques used for nanoparticles. Different parameters of evaluation for nanoparticles include particle size, morphology, zeta potential, polymorphism, stability, intermolecular interactions, in vitro drug release etc. This review aims at discussing the most important and commonly used techniques of evaluation and characterization for the nanoparticles which include Dynamic light scattering, Scanning electron microscopy, Transmission electron microscopy, Atomic force microscopy, Field flow fractionation, Laser Doppler electrophoresis, X-ray diffraction, Differential Scanning calorimetry, Infra-red spectroscopy. Understanding the principles and applications of these methods shall help in selection of evaluation methods and interpretation of data from the results.

Keywords: Dynamic light scattering, Electron microscopy, Field flow fractionation, Laser Doppler electrophoresis, X-ray diffraction, Differential Scanning calorimetry

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INTRODUCTION

Nanoparticles are ultrafine sized substances which have particle size defined in terms of nanometers generally 1 to 1000 nm. The specification of the particle size of the nanoparticles changes according to the materials and field of application. The pharmaceutical nanocarriers are colloidal sized particles lying in submicron range.

In terms of physicochemical properties, nano sizing has effect on the fundamental properties of the bulk substances. Particle size is inversely proportional to the surface area. So, particle size reduction causes increase in surface area and thus, increase in surface exposure of the molecules of the substances. This in turn influences reaction rates, melting point, solubility etc. [1]. From the physiological point of view nanoparticles are the most suitable form of drug delivery to reach subcellular levels. Nanoparticles can reach deep into tissues through blood capillaries and through fenestrations of the vascular bed. For efficient transport through the blood vessels the recommended size for nanoparticles is below 300 nm. The nanoparticles do not block the blood vessel capillaries as the narrowest diameter of the smallest capillaries is 2000 nm. Most of the sites of action of the body are accessible through the microcirculation of the blood capillaries, apertures/pores and gates present at the cellular and subcellular levels [2,3].

Various forms of Pharmaceutical nanoparticles are formulated which include Polymeric nanoparticles and nanocapsules, metal nanoparticles, solid lipid nanoparticles, niosomes, liposomes, micelles, dendrimers, quantum dots, magnetic nanoparticles [1,4]. These nanoparticles are formulated to impart properties into them which would control their biological behavior in a desirable fashion and make them perform various diagnostic and therapeutic functions simultaneously. The use of nanoparticles formulations aims at minimizing the drug degradation before and after administration, minimize the unintended side effects, and improve the bio-distribution and bioavailability of the drug. The prominent outcomes of nanoparticles formulations include increased circulation time, desired bio-distribution, active or passive targeting, response to physiological or other stimuli, contrast serving ability in different imaging modalities like magnetic resonance imaging, gamma-scintigraphy [5–7]. In the last couple of decades nanocarriers have emerged as platform for cancer drug therapy. Studies have been done and are being done to explore the use of nanocarriers in active or passive targeting of the tumor cells. The passive targeting utilizes the enhanced permeability and retention effect of the tumor vasculature for macromolecules in which the particles greater than 50 kD molecular weight selectively accumulate in the tumor tissues because of the vascular hyper permeability and poor vascular drainage [8]. The active targeting involves use of some affinity ligands conjugated to the nanocarriers with chemotherapeutic agent, which when administered, bind to the receptors, antigens or other cellular targets specific to the tumor

cells; thus, delivering the drug selectively to the tumor cells [9]. Targeting to other regions or tissues can also be achieved by using bioadhesive materials, thermally responsive materials, magnetic materials etc. in the nanoparticle formulations [10–12].

A successful formulation development is always a key prerequisite to achieve the objective of the drug delivery systems. So, evaluation and characterization of the formulations becomes very important part of the formulation development process. Before thinking about the technical issues in the evaluation of nanoparticles, understanding how these are different from conventional suspensions and powders will help define the task of evaluation and characterization. So, the first and foremost thing comes out is the colloidal particle size. Because of the submicron size range the methods used for conventional suspensions and powders cannot be used for nanoparticle formulations. To complement this statement, the light scattering methods used for submicron sized particles are not useful for larger sized particles. A second difference becomes evident; both the behavior and potential uses of nanoparticle systems vary from those of the conventionally sized particle systems. Because of the low mass of the nanoparticles their kinetic energy essentially becomes to the order of those of inter-particulate interactions, hence they behave differently [5]. These remain suspended in conditions in which the conventional sized systems would undergo sedimentation. Because of their particle size the applications of nanoparticles differ from those of conventional suspensions e.g. these can be given intravenously. Hence evaluation of the upper limit of the particle size distribution becomes critical in such cases because of the potential for embolism. In some cases, however, the characterization techniques used for the nanoparticles are no different than those used for the macro-analytes e.g. X-ray diffraction and thermal methods used for analysis of physical form [13,14].

Different evaluation parameters for nanoparticle formulations include the particle size, size distribution and polydispersity, morphology, stability, crystallinity, drug release, drug content and entrapment efficiency etc. This review aims at studying the most common and important evaluation and characterization techniques of nanoparticle formulations. The understanding of the insights of the evaluation and characterization techniques would be helpful in selection of appropriate techniques and would aid in better interpretation of the data. Most commonly used evaluation and characterization techniques for nanoparticles are depicted in table no. 1.

Table no. 1 – Most commonly used evaluation and characterization techniques for nanoparticles.

Sr. No.	Parameter	Technique	References
1	Particle size	Dynamic light scattering Scanning electron microscopy Transmission electron microscopy Atomic force microscopy Field flow fractionation	16,20,22,25,29
2	Morphology	Scanning electron microscopy Transmission electron microscopy Atomic force microscopy	20,22,25
3	Zeta potential	Laser Doppler electrophoresis	10,34
4.	Physical state (polymorphism) and intermolecular interactions	Powder X-ray diffraction Small angle neutron diffraction Small angle X-ray scattering Electron diffraction Differential scanning calorimetry	6,21,31,33.35
5.	*In Vitro* drug release	Diffusion cells, Dialysis sacs	6,38

Particle size, Size distribution, Polydispersity & Morphology

Particle size is the most important parameter for nanoparticle formulations as it governs most of the salient features of this form of drug delivery. A particle is three-dimensional structure and may have different shapes. “Particle size” is a term that represents the three-dimensional particle in one-dimensional scalar value [1]. The bio-distribution and elimination of particles is governed by their particle size. Soo Choi et al defined the requirements for renal filtration and urinary excretion of inorganic, metal-containing nanoparticles. Quantum dots having hydrodynamic diameter less than 5.5 nm resulted in efficient urinary excretion and elimination from the body [15]. These quantum dots were intended for fluorescence imaging studies. This suggests that evaluation of the particle size of nanoparticles is important to predict their bio-distribution and *in vivo* fate. In continuation of these studies Soo Choi et al investigated the number of high affinity ligands that can be conjugated to these quantum dots such that the hydrodynamic diameter remains below 5.5 nm. They found the upper limit of 5–10 ligands per quantum dot for renal clearance [2]. Similarly, particles can accumulate in different organs like liver, kidney, spleen, stomach according to their particle size and blood flow to the organs [3].

Thus formulation of nanoparticles with different particle sizes of the same formula may result in different bio-distribution and accumulation *in vivo*.

Dynamic light scattering (DLS)

Dynamic light scattering is the most commonly used method of determining the mean particle size, size distribution and polydispersity. DLS is also referred as Photon correlation spectroscopy or Quasi elastic light scattering. The technique is type of laser light diffraction method based on the Brownian movement of the particles in the sample which diffract light when illuminated. The phenomenon is called as Tyndal effect. This is an indirect method of particle size measurement, which gives mean diameter of a sphere, called hydrodynamic diameter; having the same translational diffusion coefficient as that of the particles in the sample. The detector which measures the change in frequency of diffracted light is usually at 90^0 to the incident laser beam [16]. Smaller the particle size, greater is the Brownian motion and thus grater the change in the frequency of the diffracted light. The size of the particle is calculated from the Translational diffusion coefficient by using Stokes - Einstein equation.

Polydispersity is derived from the particle size distribution. The polydispersity index which rates the sample from 0 to 1, gives an indication of homogeneity in the particle size distribution. A value of less than 0.1 indicates high particle size homogeneity in the sample while value greater than that indicates a wide distribution of particle size in the sample [3].

Gaumet et al have emphasized the importance of sample parameters such as viscosity or pH of the medium, temperature, concentration and particle sedimentation; as change in these parameters give artifacts in the particle size measurement. Aggregation of particles and adsorption of water or other solvent molecules may also give artifacts in particle size. The authors found that particle size measurements by light scattering and electron microscopic methods differed a lot [3]. Thus, interpretation of particle size should be done with caution when only one method of particle size determination is used. For accurate determination of particle size or to avoid errors in the interpretation of results, use of at least two methods with different principle of working is suggested.

Nanoparticles can be formulated in different shapes and structures according to their methods of preparation viz. core shell, dispersion matrix, agglomerates, nanoparticle coatings, hollow / porous structures, regular or irregular crystals. The role of particle size in bio-distribution has been studied extensively; but, the impact of particle shape in bio-distribution is being explored in recent studies. The role of particle geometry in the process of phagocytosis has been discussed by Champion et al [17]. They used polystyrene particles of various sizes and shapes, to study their phagocytosis by alveolar macrophages. The interesting finding of this research was

that the particle shape, not the size played a dominant role in phagocytosis. Devarajan et al reported particle shape as a new design parameter for passive targeting of nanocarriers in splenotropic drug delivery [18]. They found that irregular shaped polymer lipid nanoparticles localized well in the spleen evading the uptake by kupffer cells of liver compared to the spherical polymer lipid nanoparticles. Tao et al have reviewed the opportunities and challenges in shape specific nanomedicine [19]. Recent advances in fabrication of shape specific nanoparticles and their unique biological and pharmacological properties have been discussed by the authors. All these studies indicate important role of particle shape along with the particle size in the *in vivo* fate of nanoparticle formulations. Thus, evaluation of particle shape may be considered as an important parameter for prediction of in vivo performance of nanoparticle formulations.

Electron microscopy

The electron microscopic techniques use electron beam produced by an electron gun as a source of illuminating the sample. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) are the techniques which allow direct measurement of particle size [20,21]. These techniques are also useful in morphological evaluation of the nanoparticle formulations [22].

In SEM, The electron beam travels through electromagnetic lenses and is held through vacuum. The sample needs to be dry as water may evaporate in vacuum. For making the sample conductive, a coating of gold or platinum is done using a "sputter coater". Coating may not be required for metallic samples. When the electron beam hits the sample, electrons and X-rays are produced due to interactions with sample surface. The detector(s) collect these backscattered electrons and X-rays; and convert them into signal which is then processed as an image [23].

The TEM works like a slide projector, in which the electron beam hits the sample and is partially transmitted through the sample. The transmitted portion of the electron beam is then focused by the objective lens into an image. The sample preparation includes drying, staining and then deposition on copper grids before subject to illumination [24]. TEM has been useful in investigating hollow / porous, crystal structures of nanoparticles [22,24].

Electron microscopic methods have wide application in topographic evaluation of different array of samples. A direct imaging and measurement of particle size and analysis of shape is allowed by these techniques. Similar to the dynamic light scattering, impact of sample preparation conditions shall be taken into consideration while interpreting the results of electron microscopic analysis. For the electron microscopic analysis, the samples are required

to be dried before analysis which can cause changes in the actual size and morphology of the original samples. Thus, data analysis and interpretation shall be done with caution.

Elemental analysis of metal nanoparticles can be performed when electron microscopes are equipped with an assembly that analyses the X rays emitted by metal atom present in the sample. This analysis is called Energy dispersive X ray analysis. The emitted rays are characteristic to the metal present in the sample. This technique is useful in evaluating Encapsulation efficiency of the metal nanoparticles [11].

Atomic force microscopy (AFM)

Atomic force microscopy is also known as scanning force microscopy, has been widely used to evaluate particle size and morphology of nanoparticle formulations [25,26]. The particle size and morphological information of the sample is gathered by feeling the surface of the sample with a mechanical probe. The mechanical probe has atomic scale sharpness and is rastered through or over the sample to produce a topological map of the sample surface. The analysis can be done in contact mode or non-contact mode with the sample. Sample preparation generally involves evaporation on a smooth mica surface. A very high resolution of up to fractions of nanometers can be obtained with AFM [27]. Shahgaldian et al studied imaging of polymeric nanostructures using AFM. The nanoparticles were subjected to DLS and AFM. The results were interpreted in terms of formation of nanocapsules that collapsed on deposition and drying in the sample preparation for AFM, as the particle size was smaller for the AFM and the shapes were condensed in the same [26]. AFM is also useful for studying inter-particulate forces. Ducker et al described AFM as a direct measurement technique for colloidal forces [28]. Hence, atomic force microscopy is not only useful in evaluating particle size and shape, but also the inter-particulate interactions of the sample.

Field flow fractionation (FFF)

Field flow fractionation is a group of techniques used for characterization of colloids, macromolecules and particulate material; primarily by size and by composition in some cases [29]. The difference between chromatography and FFF is the method used to achieve differential migration of the components in a mixture. In FFF the separation of the sample analytes is achieved though the interaction of the sample with a perpendicular physical external field, rather than by a stationary column or phase. Interactions with the force field reduce the rate of movement of the analytes in the flow stream [29,30]. The sample is driven through a ribbon like thin channel with the mobile phase. A field force in the form of heat, centrifugation, cross-flow with other solvent, sedimentation is applied perpendicular to the sample path. As a result, particles are driven towards the top or bottom wall of the thin channel. The eluent flow

becomes parabolic so that the particles with least interaction with the field remain at the center and elute first. Use of multi angle light scattering (MALS) as online detector with FFF allows the determination of particle size (hydrodynamic diameter) and size distribution [31]. Fraunhofer et al have discussed the pharmaceutical and biopharmaceutical applications of FFF [30]. The methodology and interpretation of data are more complex for FFF as compared to chromatography; but, it provides a wide range of information about the sample and hence is considered more useful for evaluation of preparative nanoparticles.

Zeta potential

The zeta potential is a physical property shown by dispersions i.e. suspensions and emulsions. Zeta potential is the potential difference between the stern layer and the diffuse layer of charged surfaces. Assessment of zeta potential helps in predicting short term and long term stability of formulations. It is also useful in formulating stable products. Zeta potential controls the interactions due to charge and not the charge at the surface of the particles. Particles having high zeta potential values with same sign tend to repel each other and thus render the formulation stable. Conventionally a high zeta potential value is considered to be greater than +30 mV and smaller than -30 mV [32]. A similar value for zeta potential is an indicative of similar surface properties [33].

Laser Doppler electrophoresis (LDE)

Laser Doppler electrophoresis, also called as Laser Doppler anemometry is the most common technique used for determination of zeta potential [10,34]. Commercially the instrument is available integrated with the DLS. In this method electric field is applied across the dispersion sample. The particles start moving under the electric field, towards the electrode with opposite charge with a velocity commensurate with the zeta potential. The velocity under the electric field called as the electrophoretic mobility is measured from the frequency shift or phase shift of the incident laser beam caused by the movement of the particles. This electrophoretic mobility is then converted in to zeta potential by the application of Smoluchowski or Huckel theories [32].

Matrix state, polymorphism, phase behavior and intermolecular interactions

Analysis of the physical form of drug, whether crystalline or amorphous, is an important parameter of evaluation in nanoparticles. As mentioned earlier a change in fundamental properties such as melting point, crystallinity is observed on particle size reduction. Study of these changes becomes necessary to correlate them with the performance of the size reduced formulations. These studies also help in evaluating the anticipated particle structure depending

on the method of preparation or ingredients, which may have special role in their bio-distribution or biological function. The main techniques used for these studies include Differential scanning Calorimetry, infra red spectroscopy, X-Ray Diffraction which includes Power X-ray Diffraction, Small-Angle Neutron Scattering, and electron scattering.

X-ray Diffraction (Power X-ray diffraction, small-angle neutron scattering, small-angle X-ray scattering, electron scattering)

Illumination of crystalline substance (phase) gives a specific diffraction pattern which can be used as a fingerprint for that specific substance. The X-ray scattering techniques are based on recording the scattered intensity of an X-ray beam hitting a sample as a function of incident and scattered angle, polarization, and wavelength or energy. Here, the geometric scattering of the incident radiation from the crystal planes of the sample or the absence of the crystal planes is determined, thus estimating the degree of crystallinity of the sample. Generally, for particles less than 100 nm, broadening of the diffraction peaks is observed [5]. For nanoparticles of the order of smaller scale the investigation can be done by reducing the wavelength and angle of radiation [35]. This is usually achieved by using small angle X-ray scattering, small angle neutron scattering and electron scattering [31,35].

Bunjes and Unruh have reviewed the use of DSC, X- ray and neutron scattering for characterization of lipid nanoparticles. Basics of small angle X-ray and neutron scattering have been explained which are promising methods for characterization of nanoparticles [35].

Differential scanning calorimetry (DSC)

Differential scanning calorimetry is another most widely used method for studying the physical state and the phase behaviors [14,33]. It involves application of programmed heating or cooling to the sample and subsequent measurement of temperature and energy associated with various thermal events such as melting, crystallization, glass transitions, and decomposition reactions [36]. The enthalpies associated with these events are more than useful to study and differentiate these events in different physical forms of the sample. The DSC and X-ray diffraction are many times used as complementary techniques to provide structural information of the dispersed particles [35]. Both of these techniques have also been used to study ageing phenomenon and to evaluate the stability of the formulations.

Kheradmandnia et al investigated effect of increasing concentrations of carnauba wax on crystalline form of solid lipid nanoparticles. They concluded that the increasing proportions of carnauba wax in the lipid core of the nanoparticles resulted in increased melting enthalpy and lipid crystallization [33].

Infra red (IR) spectroscopy

IR spectroscopy has been commonly used to characterize polymeric nanoparticles to investigate physico-chemical interactions between the drug and polymer [14,21]. Exposure to IR radiations causes vibrations (stretching or bending) in the chemical bonds of molecules; and each type of bond has specific resonance frequency according to the bond strength. So, the IR spectrum of the sample can be used for structural elucidation. The chemical and physical interactions can be investigated as the change in chemical bonds and molecular environment causes change in the IR spectrum [37].

In vitro drug release

Dissolution of the active drug at the site of action or site of drug release is the ultimate objective of the formulation intended for therapeutic purpose (This may not be the case for nanoparticles used in imaging studies). The mechanism of drug release would differ according to the type and composition of the nanoparticle formulation. The aspect which is different from the regular in vitro drug release studies is the differentiation of the solubilized form from the non-solubilized form of the active drug. The conventional micron sized filters are generally not used for sampling, as they may not provide the required filter size and get clogged easily. For this, dialysis sacs are most commonly used as they are less prone to blockage and provide the required pore size. The nanoparticles can be placed inside or outside the dialysis sac and the sample can be taken vice versa [38]. Another approach is to use centrifugation of the sample and then perform the analysis [39]. Diffusion cells are also useful to conduct the in vitro drug release studies for nanoparticles [5].

Summary & Conclusions

Here some of the important evaluation and characterization techniques for nanoparticle formulations were discussed with their current applications in the nanoparticle formulations. Understanding of how different the nanoparticles are from the conventional powders and dispersions shall be taken into account before going into the technical details of the evaluation and characterization techniques. Understanding of the principles behind the techniques will help plan and select the evaluation and characterization experiments, which would ultimately help in data interpretation of these experiments.

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