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PORTRAYAL EXPLORATION OF PHARMACEUTICAL NANOPARTICLES

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ABSTRACT: Nanotechnology has found its way into most science fields at a tiny scale. Particle of this size and molecules were found to work differently and got diverse applications. Novel drug delivery system leads to the development of Nanocarriers, including Nanoparticles, Nanospheres, Nano capsules, Nano emulsion, liposomes, and Niosomes. The major area for research is to design Nanoparticles that can efficiently manage particle size, surface properties, and drug release. For this to get characterizations of Nanoparticles, it is critical to control their desired *in-vitro* and *in-vivo* behavior. Nanoparticles are characterized by their size, surface morphology, and surface charge, using highly advanced microscopic techniques. Colloidal stability is ascertained through zeta potential. Nanoparticles are also characterized by polymer and drug interaction. *In-vivo* characterization for binding and internalization of targeted carriers to the specific cell and bio distribution study of targeted Nanoparticles could also be confirmed using confocal microscopy. This review covers all the aspects related to the characterization of the pharmaceutical Nanoparticle.

INTRODUCTION: Nanotechnology is the science of small, very small particles. It uses the manipulation of matter at a tiny scale, and this size atom and molecule work differently and provides a variety of surprising interesting uses [1]. Pharmaceutical Nanoparticles are defined as a solid less than 100nm in diameter encapsulated using a drug carrier that may or may not be the biodegradable [2]. Delivering therapeutic compounds to desirable sites is a major task in treating many diseases.

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The conventional drug delivery system is characterized by its limited effectiveness, poor bioavailability, lack of selectivity, and side effects. Targeting a particular cell or tissue by means of an individually engineered carrier attached to a particular drug is found to be a more effective approach in a novel drug delivery system. This novel drug delivery system approach is known as specific tissue or cell targeting [3].

The composition of engineered Nanoparticles may vary. Source material like polymers may be of biological origins like phospholipid, chitosan, dextran, lactic acid, or various polymers like silica, carbon, and metals. Interaction with the cell for some biological components like phospholipid may differ from non-biological components such as metal [4].

Nanotechnology has effectively proven that Nanoparticles possess great potential as a drug carrier. Various size reduction methods and novel technologies found to yield a different type of Nanostructures that exhibit unique physical, chemical, biological properties. Because of the variety of these methods and technologies, Nanoparticles seem to be favorable for pharmaceutical and biomedical applicatios [2].

Advantages [5]:

1. Nanoparticles have a high drug-carrying capacity.
2. Shelf life and stability of drug increase for formulating it into nanoparticle.
3. They can be used to sustain and control the release pattern.
4. Nanoparticles can be used in therapy where two or more drugs can be co-delivered both hydrophilic and hydrophobic can be incorporated as per therapeutic needs.
5. Nanoparticle system increases the bio-availability of a drug.
6. Nanoparticles can be used for targeted drug delivery of a drug.

Disadvantages [5, 6, 7]:

1. The manufacturing cost of Nanoparticles is high, it results in an overall increase in product cost.
2. Solvents used in the formulation of nanoparticle are toxic and can alter the immune response and allergic nature.
3. Nanoparticles are difficult to handle in physical form because there may be particle-particle aggregation occurs due to their extremely small size and large surface area

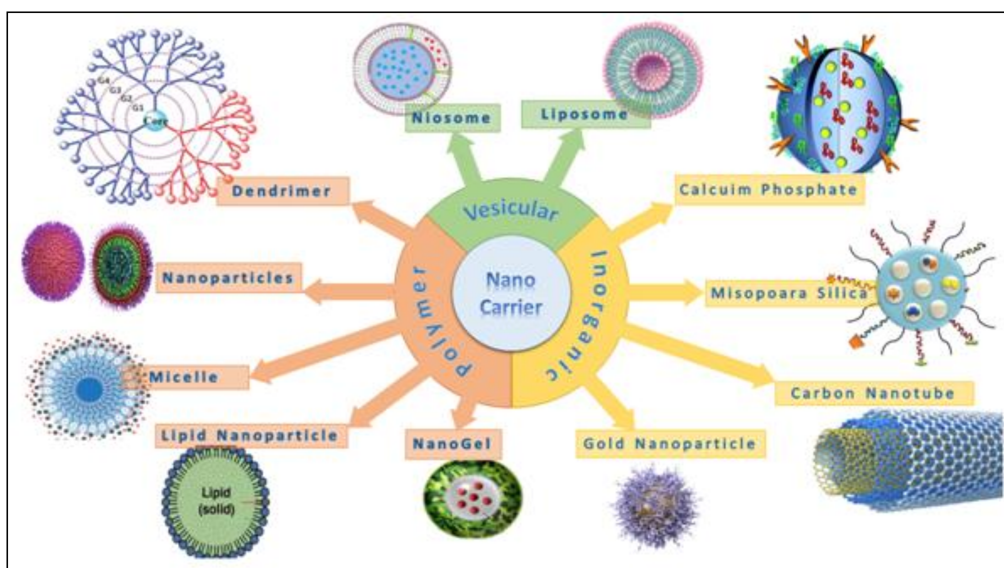

FIG. 1: CLASSIFICATION OF MOST COMMONLY USED NANOPARTICLES FOR DRUG DELIVERY

Nanoparticle construction intended for medical operations consists of a variety of material and because of their extremely small size unique physicochemical properties and enhanced biological activity often require modification of standard characterization technique. A rationale for nanoparticle includes physicochemical characterization, bio-distribution, sterility and pyrogenicity, and toxicity characterization, which include both *in-vitro* characterization and *in-vivo* animal studies. This review article will highlight methods that are uniquely useful for the characterization of nanoparticles or are indicative of their toxicity and efficacy [8].

Characterization of Nanoparticles: The key objective of designing nanoparticle as a novel drug delivery system is to manage or take care of particle size, surface properties as well as the release of drugs to achieve the specific objective. Hence characterization of the Nanoparticles is very critical to control them. It's behavior in *in-vitro* as

well as *in-vivo* conditions. Pharmaceutical Nanoparticles are characterized by various innovative, sophisticated techniques based on size, surface structure, and charge potential. The surface properties, along with the dimensions, may alter the nanoparticle's stability and *in-vivo* behavior of these Nanoparticles.

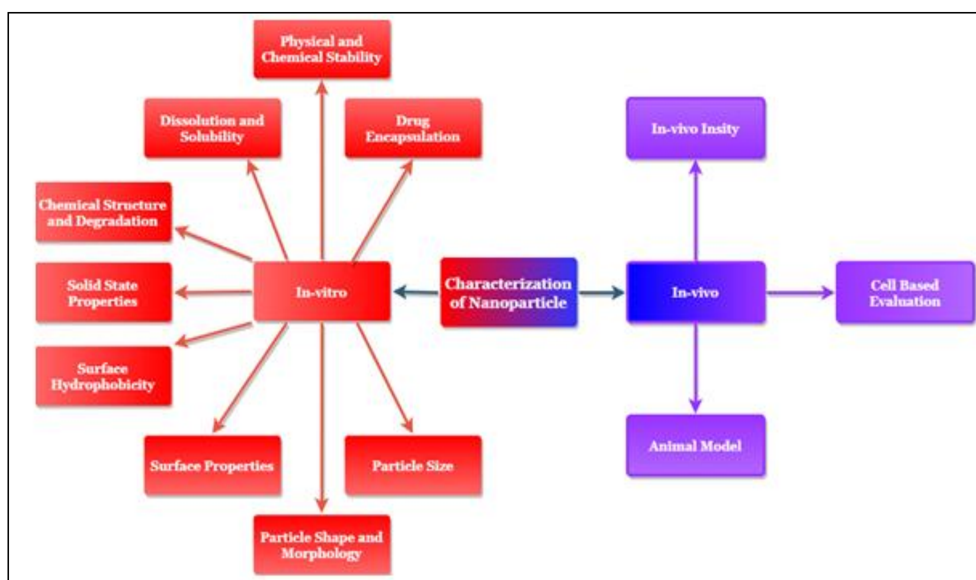

FIG. 2: CHARACTERIZATION OF NANOPARTICLES

TABLE 1: *IN-VITRO* CHARACTERIZATION OF NANOPARTICLE

Sr. no.	Quality	Method	References
1	Particle size	Imagine based technique: SEM, TEM, AFM	21-30
		Nonimaging-based techniques: light scattering-based techniques like DLS (dynamic light scattering), laser diffraction spectroscopy, Differential centrifugal sedimentation (DCS), Nanoparticle tracking analysis (NTA)	11-19
2	Particle shape and morphology	SEM, TEM, AFM	21-30
3	Surface properties	Zeta potential, DSC, SAXS	33-40
4	Surface hydrophobicity	Rose Bengal binding Water contact angle measurement X-ray photoelectron spectrum, Photon correlation spectroscopy	42 -43
5	Solid-state properties	FTIR, RAMAM, solid-state nuclear magnetic resonance, XRD, DSC, TGA	15-16,40
6	Chemical structure and degradation	HPLC, gel permeation and size exclusion chromatography Sorptometer	6,44
7	Dissolution and solubility testing	Intrinsic dissolution test, Dissolution, Drug release rate, Apparent solubility	45-46
8	Physical and chemical stability	Critical aggregation or micelle concentration, Low critical solution temperature, Foster resonance energy(free), TOF-SIMS	20
9	Drug encapsulation and loading capacity	Drug loading, Drug entrapment efficiency, Drug localization and drug release Drug recovery	45-46

TABLE 2: *IN-VIVO* CHARACTERIZATION OF NANOPARTICLES

Sr. no.	Quality	Method	References
1.	*In-vivo* testing	Phagocytic uptake, Complement activation, Protein adsorption	23,44,53
2.	Cell-based evaluation	Cellular uptake of Nanoparticle, Intracellular trafficking, 2 d monolayer, Bioactivity of Nanoparticle	44,55
3.	Animal model	Animal model of the tumor, The genetically engineered mouse model, Streptozotocin induce diabetic rabbit model	44,56

In-vitro Characterization:

Particle size: Size is an important parameter differentiating Nanoparticles from other drug delivery systems. There are a few numbers of methods available that can be used to determine the Nanoparticle size. Every method for size

determination has its merits and demerits; those should be looked at carefully while characterizingNanoparticle. The choices of a technique depend on various parameters, such as expected size, and population of the Nanoparticles [9].

Dynamic Light Scattering (DLS): It is the most frequently used method for particle size determination, it is a technique that determines particle size in suspension and is commonly referred to as photon correlation spectroscopy (pcs). When the size of the particles is less than the 1/10th of incident light wavelength, the scattered beam bears similar energy to the incident light and also its angle of independent [10]. When the diameter of particles crosses the limit of k/10, Rayleigh pattern is substituted by Mie scattering it means scattered beam does not carry the similar energy to the incident light and is angle-dependent. DLS is used to carry out the size analysis of nanoparticle having a size range of 1–500 nm. DLS is the most suitable technique to determine the size of unimodal Nanoparticles. DLS instrument detects scattered beams of laser light with a photon detector, and the intensity of the scattered light is proportional to the size of the monitored Nanoparticles. The latest DLS equipment is attached with APD (avalanche photodiode) detectors which have a limited quantum efficiency 65% for red wavelength hence mostly lasers of 633 nm are utilized. V. Filipe *et, al.,* used DLS (Malvern Zetasizer Nano ZS) and NTA to determine the particle size of polymer nanoparticles. The mean size obtained by NTA are smaller and closer to the expected values than the Z-average given by DLS, but all values are close to the bead size specify [11, 12].

Laser Diffraction Spectroscopy (LDS): Laser diffraction spectroscopy is a valuable technique that covers a much broader detection range (20–2000 lm). It is also known as laser light scattering, which can be used alone and in combination with pcs to obtain a total population size range from tiny to macro particles. Laser diffraction spectroscopy depends on the principle that once a laser beam is passed through a liquid where particles are suspended, the bigger particles scatter light at narrow angles, whereas the smaller ones scatter light at broader angles. The results produced by laser diffraction spectroscopy is utilized to estimate the correspondent spherical radius of particles as per the Mie scattering solution. Red lasers are responsible for estimating larger particles, whereas blue lasers are used to analyze tiny ones. However, it is not recommended for colloidal suspensions having notably lesser diameters than the laser wavelength. Although, laser diffractometer offers a fair estimation of the polydispersity of particles as it covers a broader size range from nanometer to 100 of a micron. However, its uses are limited to multi-component nanoparticles because knowledge of the refractive index at the measurement wavelength is critical as particle size distribution is extremely dependent on these optical parameters [13, 14].

Differential Centrifugal Sedimentation (DCS): The principle behind differential centrifugal sedimentation is that larger particles sediment faster than smaller particles if they have the same density. Nanoparticles do not settle under gravity itself, hence settling can be enforced by centrifugation. A hollow disc is introduced into an optically clear centrifuge tube with a whole in the middle of it and revolves at 600–24,000 rpm. The disc compartment is filled with fluid, which allows liquid rings to fall against density difference. The sample is injected through the central opening for measurement. DCS provides great resolution and various Nanoparticles having of <5% variation in size can be resolved fully. In recent times, alteration in the size of au Nanoparticles after surface modification usually is studied using DCS and found a swing of 0.5 nm in the size of the Nanoparticles after modification and this shift amounted to 2.1 nm after Nanoparticles modification with an entity having high molecular weight such as single-stranded DNA. The application of DCS in measuring the size of peg-alkane thiol-modified gold Nanoparticles was also reported by Krpetic *et al.* [2, 12, 15].

Fourier Transform Infrared Spectroscopy (FTIR): Fourier transferred spectra of a drug are used to characterize drug encapsulation in the polymer matrix as well as help to determine the compatibility of the drug with excipients. M. Brahmeshwar evaluated the nanoparticle by these methods, in which they have characterized the drug excipient compatibility as well as encapsulation of

drugs in Nanoparticles by using FTIR. Excipients and drug excipient blends were recorded on an FTIR spectrophotometer (Shimadzu 8400S, Kyoto, Japan). A mini-press potassium bromide disk was used to prepare a transparent disk of samples. The background spectrum was collected before running each sample. The samples were analyzed between wave numbers 4000 and 400 cm^{-1}. IR solution 1.10 software (Shimadzu, Kyoto, Japan) was used to analyze the FTIR spectra and record the data from the spectra. The major and important peaks were reported in cm^{-1} [16, 17].

Nanoparticle Tracking Analysis (NTA) Nanoparticle tracking analysis helps concurrent visual examination and poly-grade measurement of the Nanoparticles by co-relating the Nanoparticle diameter with the speed of Brownian motion. Nanoparticles are observed depending on the scattered light upon illumination by the laser beam. Nanoparticle tracking analysis has the unique capability to observe each and individual particle present in the dispersion by tracking each scattering independently. Measuring individual entities is particularly important when handling the dispersion of Nanoparticles. Impartial peak resolution of multimodal dispersion with Nanoparticle tracking analysis is very important and difficult to accomplish using differential centrifugal sedimentation [8, 19, 20].

M. Mellema *et al.* studied the urine samples to characterize calcifying nanoparticles using nanoparticle tracking analysis. For this study, they used the urine of nine healthy cats. Urine samples were collected, centrifuged at 1500×G for 5min, and filtered aseptically before storing at different storage conditions. Calcified nanoparticles were analyzed by Nano sight LH I0HS-48814TS instrument. The nanoparticle tracking analyzer identified a population of submicron-size particles with the calcifying nanoparticle size range [21].

Surface Morphology: Surface morphology is an important parameter differentiating Nanoparticles from other drug delivery systems. There are several methods available that can be used to determine the Nanoparticle surface morphology. Every method for surface morphology determination has merits and demerits; those should be considered carefully while characterizingNanoparticle.

Electron Microscopy: Morphology of Nanoparticles is ascertained using electron microscopy techniques which may establish their toxicity profile. An important application of pharmaceutical nanoparticles is the modification of drug release and drug targeting. Due to the low resolution of optical microscopy, it is very difficult to observe smaller particles with a diameter of less than 1 mm using normal light. Hence greater resolution is desired in the form of electromagnetic radiation of shorter wavelengths.

Scanning Electron Microscopy (SEM): Scanning electron microscope emits electromagnetic radiations of a short wavelength which provides great potential to determine size and morphology of Nanoparticles to study the Nanoparticles using scanning electron microscopy, they have to be first transformed to a dry powder, this powder sample is then sprinkled on a sample holder which is followed by coating using a conductive metal such as platinum, gold, iridium, tungsten, *etc*., using a sputter coater. A high-energy beam of electrons is passed to the sample to produce a diversity of signals on the surface of the specimens. Scanning electron microscopes mainly produce three principal images: backscattered electron images, external x-ray maps, and secondary electron images.

The secondary electrons emitted by the sample surface are used to acquire the surface properties of the particles. Nanoparticles must be capable of holding a vacuum, as the electron rays can destroy the particles. The only drawback of scanning electron microscope is that it leads to destructive sample preparation, limiting its analysis to other modalities [22]. An advancement in this technique is the environmental scanning electron microscope (ESEM), which allows the scanning of the samples in their natural state without sample preparation [23].

Because SEM has a sample compartment maintained at a low-pressure gaseous environment of 10–50 torr and coating particles with conductive substances is not required [24]. P. Varma *et, al.* prepared and characterized silver and selenium Nanoparticles. Silver and selenium nanoparticles were prepared by the chemical reduction method and characterized for its shape and size by scanning electron microscopy (JEOL JSM-64900LV, Japan).

SEM reveals the spherical shape of silver nanoparticles with the size of 80.32nm and selenium nanoparticles were found to be rod shape with the size of 74.29nm [25].

Transmission Electron Microscopy (TEM): Electrons transmitted through the sample is used for image formation in transmission electron microscopy. TEM has a greater resolution in contrast to SEM hence generally used to study Nanoparticles morphology. TEM gives direct images and provides chemical information of Nanoparticles at a spatial resolution down to atomic dimensions. The mode of action of TEM is that an incident beam of electrons is transmitted through a thin foil of the sample, which is transformed to unscattered electrons or elastically scattered electrons.

A line of electromagnetic lenses focuses and then project the scattered or unscattered electrons on a screen to generate the electron diffraction and amplitude-contrast image or a shadow image of altering darkness as per the density of unscattered electrons is produced also needs a vacuum of high level with that thin section specimen to penetrate electron-beam through the sample. TEM requires specimen treatment and drying, which might change the Nanoparticle's physicochemical condition. To withstand the high vacuum pressure of the microscope, the Nano carriers are preset utilizing a negative staining solution or derivative. After this, the particles are dried under a mercury lamp and observed under a monochromatic beam of electrons penetrating the sample and generating an image. Small particles can be observed, and the crystallographic structure of a sample can be imaged at an atomic scale [26, 27]. Lin-Al-Tai *et, al*. studied quantification of PEGylated gold nanoparticles (CPEG 5K-GNP) in the whole blood sample by using Transmission electron microscopy(TEM).the diameter of CPEG 5K-GNP was found to be 39.6nm with the particles numbers in four randomly chosen 2.0μm×2.7μm image zone determined to be 478, 467, 502,504 particle [28].

Field Emission Scanning Electron Microscope (FESEM): The surface morphology of PMMA/PEI Nanoparticles is determined by field emission scanning electron microscope (FESEM) and found that the Nanoparticles were evenly sphere-shaped and around 230 nm size in a dry state. Fabrication and characterization of solid-state Nano pores using a field emission scanning electron microscope studied by Hung chang and Samir M. In this study, Iqbal determined the surface defect by field emission scanning electron microscopy [12]. It also helps to study important morphological features of nanoparticles like porous pattern, topography, pore size, and shape [29]. R. Vishwanath *et al.* Characterization of Zns and Zns: y Nanoparticles by FESEM (FESEM-Carl Zeiss, Supra 40VP). Zns and Zns: y Nanoparticles were spherical in structure and agglomerated; individual particles were found to be 10-25nm in size when observed under FESEM [30].

Atomic Force Microscopy (AFM): Atomic force microscopy helps examine the properties of every particle and molecule under closer physiological conditions. Extremely high resolution can be achieved in particle size measurement using atomic force spectroscopy which physically scans the particles through a probe tip of the atomic scale. This instrument allows a topographical chart of objects depending on the force between the sample surface and tip. Scanning's are obtained in non-contact or contact mode depending on particle properties.

The advantage offered by atomic force microscopy over SEM or TEM is that it can scan non-conducting nanoparticle without any sample preparation because these delicate biological and polymeric nanostructure could be imaged. The AFM is suitable for the characterization of pharmaceutical Nanoparticles as it also provides 3D visualization with both quantitative and qualitative information about physical properties such as size, surface morphology, texture, and roughness. Also, the same scan can characterize a broad variety of particle sizes from 1 nm to 8 nm. Liquid dispersions AFM analysis can also be carried out in liquid and gas mediums. This ability of AFM can be highly beneficial for the characterization of Nanoparticles. Concerning SEM, the AFM scans are more time-consuming, but an entire measurement such as image acquisition, image acquisition, and image analysis consumes much less time. The advantages of AFM is that, AFM is a cost-effective instrument available for Nanoparticles imaging compared to other

electron microscope techniques, AFM requires smaller laboratory space than a TEM and SEM, and it is much simpler to operate compared to TEM and SEM, which requires a specially trained person to operate them [31, 32]. Klaptek *et al.* has analyzed the statistical parameters of nanoparticles using different data processing for AFM analysis of nanoparticles under non-ideal conditions [33].

Flow Cytometry: Flowcytometry is a powerful tool for characterizing nanoparticles. It allows simultaneous multipara-metric analysis of the physical and chemical characteristics of up to thousands of particles per second. This makes flow cytometry a rapid and quantitative method for the analysis of nanoparticles. R. M. Zucker *et, al.* studied the characterization, detection, and counting of metal Nanoparticles using flow cytometry. A stratedigm S1000 (San Jose, CA) flow cytometer containing two lasers was used. Silver nanoparticles coated with polyvinyl-pyrrolidone and gold nanoparticles were used for the study. The flow cytometer method accurately detected, counted and measured silver and gold nanoparticles [34].

Surface Charge: One of the properties of Nanoparticles is their surface properties. When a charged particle is present in a dispersed state, an electrical double layer builds up around the Nanoparticle's surface. The inner layer is composed of molecules or ions of opposite charge to that of the particle, which is the stern layer. Away from the stern layer, there is a decrease in electrostatic effects because of the surface charge associated with the particles, as per Debye's law. The redespersibilty and stability of the Nanoparticles dispersion and their *in-vivo* activity, depend on the surface charge of the Nanoparticles. The long-term storage stability of Nanoparticles is mainly related to the surface charge of Nanoparticles. Particle aggregation may take place slightly for charged particles because of electric repulsion between them [2].

Zeta Potential (F): Zeta potential (f) gives an idea about the net charge of Nanoparticles and hence can provide an idea of the electrical attraction or repulsion among them. Zeta potential is mostly determined by laser Doppler anemometry based on the Doppler shift principle. Zeta potential can be affected by a change in pH, ionic strength, and the various ions in the dispersion liquid. Zeta potential greater than ±30 mv stabilizes the Nanoparticle dispersion by arresting the contact between the particles because of electric repulsion. Zeta potential can be obtained by evaluating the potential difference between the outer plane and the shear surface.

Thus, the zeta potential of colloidal Nanoparticle-based dispersion assists indirectly in evaluating its storage stability. Zeta potential values, i.e., High zeta potential values, which can be either positive or negative, are achieved to ensure stability and avoid aggregation of the Nanoparticles. Zeta potential values can also be utilized to evaluate surface hydrophobicity and the nature of material encapsulated within the Nanoparticles or coated onto the surface [35, 36]. Rabab M. *et al.* studied Biosynthesis and silver nanoparticle characterization using *Trichoderma longibrachiatum* and Their effect on Phytopathogenic fungi. After doing a zeta analysis of nanoparticles, they found the zeta values of nanoparticles of -19.7MV [37].

Differential Scanning Calorimetry (DSC): Differential scanning colorimetry method based on the measurement of structural modifications of materials works by heat exchanges such as heat emission or heat uptake, where heat exchanges take place under controlled programmed temperatures and allow determination of the physical qualities of a material. DSC analysis is nothing but heating or cooling the sample at a programmed rate, and the heat emission or absorption is observed quantitatively. Mainly two types of DSC apparatus, namely power compensation DSC and heat flux DSC used for DSC investigations. Both these instruments contain two similar sample chambers, which are heated or cooled generally linearly.

One chamber holds a crucible containing a sample to be analyzed, the other holds a reference sample in a crucible and both are kept under symmetric conditions. Temperature variation is not observed between sample and reference until and unless the structural change occurs in the sample by increasing the temperature of both sample and reference crucibles at a similar rate. As soon as the sample exhibits a structural change, it absorbs

energy which is then supplied by the system in the form of heat. Then, the changes in the temperature are calculated and transformed into a heat flow signal via a calibration method. Not much variation has been found from the two different measurement DSC techniques. The only thing observed is that endothermic transitions are exhibited as an upward peak in power compensation DSC whereas a downwards peak is observed in heat flux DSC. The thermal stability and the quantity of the Nanoparticles and their conjugates can be calculated by various other thermal techniques [23]. H. Bunjes *et, al.* Studied characterization of lipid nanoparticles by DSC. They have used two different instruments, heat flux DSC and power consumption DSC. DSC heating curve of pure indium showed the influence of heating rate on the peak maximum temperature. The influence of extrapolated peaks onset temp was much smaller [38].

Mudasir A. *et al.* has studied and characterized HSP, WSP, AND LSP to represent starch nanoparticles, while HS, WS, AND LS represent native starch from horse chestnut, water chestnut and lotus stem, respectively by DSC, XRD, ATR-FTR *etc* [39].

Thermogravimetric Analysis (TGA): Nanoparticles, such as metallic Nanoparticles, consist of a surface coating or surface modification to aid in its dispersion and stability. This surface coating may affect the behavior of Nanoparticles in a biological environment; thus, it is important to measure [40]. Thermogravimetric analysis (TGA) can be used to determine the amount of coating on theNanoparticle's surface. TGA experiments run under inert atmosphere can also be used to determine residual metal content present in the sample. In this chapter, the TGA technique and experimental method are described [41]. The result of a thermogravimetric measurement is given as mass versus temperature curve or mass versus time curve, which is known as the Thermo Gravimetric (TGA) curve [42]. E. Verdonck, studied the quantification of nanoparticles coating by TGA. He studied gold nanoparticles coated with an organic self-assembled monolayer of thiols and protein a layer. The Q5000IR TGA from the TA instrument was used for this study. Temperature calibration of TGA was performed using the nickel Curie point standard. The resulting data demonstrated that coating on nanoparticles measures 9.4% of total mass, which can be accurately determined because of the observed flat baseline of TGA [43].

X-ray Diffraction (XRD): XRD is gaining more importance in nanotechnology to characterize crystalline size, shape, and lattice distortion of Nanoparticles [44]. This technique is based on the scattering of X-rays when monochromatic, radiation passes through a small gap with the same length as its wavelength. The diffraction of the X-Ray is elucidated as nanoparticles reflection of a collimated beam of x-rays incident on the crystalline planes of an object. Obtaining results from a single confirmation or binding state of material limits the use of XRD. Sample preparations require XRD of liquid samples, which is usually done by extracting out the solid Nanoparticles by centrifugation followed by drying Nanoparticles in an oven to eliminate all the moisture [23]. K. Walbruck *et. al*. Studied synthesis and characterization of PVP-stabilized palladium nanoparticles by XRD. Palladium nanoparticles were synthesized by the chemical reduction method. XRD analysis was carried out by Brucker D8 Discover diffractometer. The measurement was performed in the 2-Theta range from 37°-90° with an exposure time of 960 sec. Diffraction peaks positioned at Braggs angle (40.5°, 47°,68.5°,82.4°, and 87°) were observed [45].

Small Angle X-Ray Scattering (SAXS): It works opposite to XRD, which has limited application for crystalline structure; SAXS offers information about various properties by scanning either amorphous or crystalline materials from polymers, proteins to Nanoparticles [23]. The principle working of SAXS depends on producing a segment of an incident beam elastically scattered from the sample material, making a scattering pattern on a two-dimensional flat X-Ray detector in the perpendicular direction to the incident X-Ray beam. By determining the strength of the scattered beam within the scattering angle, SAXS can examine the size, shape, and size distribution of the Nanoparticle population and the structure of a diversity of polymers as well as of nanomaterial bio conjugates. SAXS does not require a crystallized structure hence of sample treatment is eliminated and it also serves as a non-destructive method. SAXS does not provide high resolution but it could

be achieved by attaching synchrotron as the high energy x-ray source [46]. K. Walbruck *et, al*. Studied synthesis and characterization of PVP-stabilized palladium nanoparticles by SAXS. Palladium nanoparticles were synthesized by chemical reduction method. SAXS analysis was carried out using Brucker D8 Discover diffractometer with cu microfocus X-ray source and a 2-Dimensional Vantec 500 detector. The measurements were performed in transmission mode with an exposure time of 90 sec. Around the beam stop in the center, an isotropic scattering was observed [45].

Surface Hydrophobicity: Surface hydrophobicity can be determined by various techniques such as rose Bengal binding, chromatography, hydrophobic interaction, adsorption of probes, biphasic partitioning, contact angle measurements, etc. Nowadays various sophisticated analytical techniques are reported in related literature for surface analysis of Nanoparticles. Identification of specific chemical groups on the surface of the Nanoparticles technique known as x-ray photon correlation spectroscopy is most widely used, which also permits the determination of hydrophobicity of Nanoparticle surface [47].

Photon Correlation Spectroscopy: Current research demands the fastest and most accurate method of determining particle size. Photon-correlation spectroscopy (PCS) is most widely used to determine the size of Nanoparticles in colloidal suspensions in the Nano and submicron sizes. This technique utilizes a solution of spherical particles in Brownian motion, which causes a Doppler shift when they are exposed to monochromatic light. This monochromatic light is the moving particle which changes the wavelength of the incoming light. The extent of this change in wavelength helps to determine the size of the particle. This parameter assists in the evaluation of the size distribution, particle’s motion in the medium, which may further assist in measuring the diffusion coefficient of the particle and ultimately helps to determine hydrophobicity of nanoparticles [3]. K. M. Ezealisji studied green synthesis and characterization of monodispersed silver nanoparticles using root bark extract of Annona muricata Linn and their antibacterial activity. The polydispersion index and zeta potential were determined using photon correlation spectroscopy (Mavern Nano ZS, ZS290, UK). Photon correlation spectroscopy analysis confirmed the existence of elemental silver and the shape of nanoparticles was spherical [48].

Solid-state Properties: Solid-state form of the material, such as hydrates, solvates crystal form, crystallinity, and amorphous form, can affect the physical property of the system, like solubility and dissolution rate. Hence knowledge of material properties is required to formulate Nanoparticles. When energy inputs are involved, like temperature changes or mechanical energy are involved, polymorphic changes or formation of amorphous material is possible and chemical degradation may take place. Solid-state analysis and interaction studies can be performed by thermal analysis, mostly differential scanning calorimetry, DSC, x-ray diffraction (XRD), infrared spectroscopy (IR), and Raman spectroscopy, and solid-state nuclear magnetic spectroscopy (SSNMR)

In drug Nanoparticle characterization studies, DSC and XRD have mostly used solid-state characterization techniques, butes like Raman spectroscopy have also beenalso The use of it in-drug Nanoparticle characterization studies is related to the recognition of interactions between the drug and excipient(s) in the system.

Chemical Structure and Degradation:

Chemical Stability: The most standard way to detect chemical purity or degradation of the rug is by using high-performance liquid chromatography.

High-performance Liquid Chromatography: In HPLC, impurities or degradation products are seen as new peaks, but determining side product softening requires other techniques like liquid chromatography-mass spectrometry, and LC-MS. Harsh process conditions can induce degradation of the drug, and hence exact knowledge of the chemical nature of the unwanted products is crucial to recognize the harmful process steps. One of the research studies showed naproxen degradation during Nano milling, which was studied with two stabilizers: hydroxyl propyl methylcellulose, HPMC, and tween 80. By using tween 80 stabilizers, resulted from nanocrystals were stable, but milling with HPMC as a stabilizer leads to molecular-level interactions between drug and stabilizer, which were analyzed by MT-DSC

analysis. Chemical degradation was confirmed in HPLC analysis when degradation peaks other than drug peaks were seen. The degradation product was recognized by LC-MS analyses. One more study confirms the chemical stability and analysis of the drug content of Itraconazole nanocrystals prepared by nanoprecipitation by HPLC analysis. The drug content had not changed significantly, and no degradation peaks noticed in the HPLC graph when these Nanoparticles were stored for 3 months [7].

Gel permeation Chromatography (GPC): Gel permeation chromatography can be used to determine the physical stability of Nanoparticles. This technique is based on the principle that it separates Nanoparticles from degraded Nanoparticles or their components. Nanoparticle stability can be estimated based on the elution times, given 39 for example, elusion time below 7, indicative of Nanoparticle aggregation due to hydrophobic interactions of the deionized polymer. These methods may be used for virtually any type of Nanoparticle system and can be performed with basic analytical equipment. In one of the studies of nanoparticles, this technique was also used to assess the formation and dissociation of insulin-hydrophobized pullulan Nanoparticle assemblies. The assemblies of particles showed high colloidal stability in water and buffer. Still, it was seen that insulin was released rapidly from the assemblies upon the addition of bovine serum albumin [49].

Physical and Chemical Stability: Maintaining Nanoparticle stability is a crucial requirement for successfully delivering drugs to targeted tissues. The fate of Nanoparticles *in-vivo* is largely determined by their ability to maintain their size, retain drug payload to the target tissues, and properly release drugs to the cells. Nanoparticles must remain stable i.e., to resist aggregation or degradation and retain the drug in the blood until it reaches the target sites. The instability of Nanoparticles leads to altered bio-distribution, bioavailability, and premature drug relultimately compromising the delivery system's efficacy Maintaining this evaluation of Nanoparticles stability is an important aspect of Nanoparticle characterization.

Critical Aggregation Concentration (CAC) or Critical Micelle Concentration (CMC): The critical association or aggregation concentration (CAC) or critical micelle concentration (CMC) can be used to evaluate the stability of Nanoparticle systems. The critical association or aggregation concentration (CAC) or critical micelle concentration (CMC) is the concentration at which a self-assembled particle or micelle associates or dissociates.

This value of CAC or CMC provides a quantitative measure of the physical stability of Nanoparticles. A comparatively low CAC or CMC indicates a more stable micelle system than one with a high CAC or CMC. This means Nanoparticles with a low CAC or CMC are more likely to resist dissociation upon dilution in the blood.

Critical micelle concentration can be measured using various methods, such as chromatography, conductivity, surface tension, fluorescent probes, and light scattering.

Another commonly used method to measure CAC or CMC is to utilize fluorescence probes, such as pyrene, which indicate micelle dissociation. Pyrene is a hydrophobic aromatic hydrocarbon, which leads to partitions in the hydrophobic domain of self-assembled Nanoparticles during 24 assemblies. When a Nanoparticles dissociates, pyrene is exposed to water, which shows a different fluorescence profile than in the hydrophobic domain of the Nanoparticles. Therefore, the CAC or CMC can be determined by monitoring the change in the fluorescence profile of pyrene and hence can be defined as the concentration at which a drastic band shift is observed. Also, light scattering is used to determine CAC or CMC. This technique measures the count rate i.e., the intensity of scattered light in DLS, which is proportional to the number of 30 Nanoparticles in solution when Nanoparticle size is constant. The count rate is plotted concerning Nanoparticle concentration, and here the CAC or CMC is defined as a concentration above which the count rate shows a linear increase with the concentration of the components of Nanoparticles.

The CAC or CMC measurement is a simple and sensitive method of characterization of stability. Still, the only disadvantage is that its application is limited to micelles and self-assembled

Nanoparticles whose formation is influenced by concentrations of the different components [20].

Low Critical Solution Temperature Temperature-sensitive Micelle Systems (LCST): Nanoparticles composed of co-polymer of the hydrophobic block and thermosensitive block can utilize lower critical solution temperature (LCST) to measure their stability. It is defined as the temperature at which the phase transition of a thermosensitive polymer occurs from hydrophilic to hydrophobic with an increase in temperature [31]. This phase change allows the system to release a drug in response to external thermal stimuli at a particular temperature. At temperatures below low critical solution temperature temperature-sensitive micelle systems (LCST), the polymer is amphiphilic and the drug remains encapsulated in micelles, but, at temperatures above LCST, the thermosensitive

The block becomes hydrophobic, destabilizing the micelle structure and releasing the drug. As an inherent property of a thermosensitive polymer, low critical solution temperature temperature-sensitive micelle systems can be utilized in comparing the stability of Nanoparticles based on such polymers. For example, LCST of poly (n-isopropyl acrylamide-co-maleic anhydride) copolymer increased from 31.1° to 45°c as the content of maleic anhydride and molecular weight increased. Whereas LCST of Pluronic and poly (n-isopropyl acrylamide) decreased when mixed with saccharides[20].

Forster Resonance Energy Transfer Technique (FRET): Forster resonance energy transfer (FRET) has been used to study the stability of Nanoparticles at the molecular level. In the study of cheng and colleagues, they encapsulated a FRET pair, consisting of hydrophobic fluorescent probes do as donor and dii as an acceptor, in a polymeric micelle to study the stability of micelles. The FRET pair retained in the hydrophobic core of the micelles shows a FRET signal due to their proximity to each other. In contrast, the encapsulated a FRET signal disappears as the micelles dissociate and release the dyes. Micelle stability during cellular uptake has been studied by the group using this phenomenon. The challenge that appears in using FRET analysis is the need for technical adjustment to avoid optical artifacts that may interfere with FRET detection.

For accurate assessment of Forster resonance energy transfer technique signals, various optical corrections need to be made to consider these issues. Furthermore, for Nanoparticle systems that require covalent labeling of nanoparticle -dye, this conjugation may affect the formation as well as the chemical conformation of the Nanoparticle, thus ultimately changing its properties [20].

Drug Encapsulation and Loading Capacity:

Drug Loading: Nanoparticle should have high encapsulation efficiency. In this manner, Nanoparticles decrease the amount of matrix polymers required for drug delivery. Drugs can be loaded to the Nanoparticles mainly in two ways. One is by absorbing the drug into the preformed Nanoparticles by saturating the Nanoparticles with a concentrated drug solution by adsorption or absorption; the other is, API is added at the time of Nanoparticles preparation, called as incorporation technique.

Encapsulation efficiency and % drug loading mainly depend on the molecular weight of the polymer with polymer composition, polymer interaction with the drug, and functional groups found on the outer polymer chain. Entrapment efficiency (EE) mainly depends on the method of preparation of nanoparticle used for that particular carrier and the properties of the drug as well as the properties of the polymer. The amount of encapsulated drugs into the nanoparticle influences the release kinetics; hence it is important to determine the encapsulation efficiency. The drug encapsulated per unit weight of nanoparticles can be quantified after removing the free drug. The free drug can be separated by extensive dialysis, ultracentrifugation, centrifugal ultrafiltration, ultrafiltration, gel filtration, or size exclusion chromatography. The amount of encapsulated drugs after separation of free drug is estimated using standard analytical techniques such as high-performance liquid chromatography (HPLC) or UV spectroscopy. Encapsulation efficiency can also be assessed by dissolving Nanoparticles into a suitable solvent and then analyzing those using standard analytical techniques.

The formula usually calculates loading efficiency, as the amount of the drug entrapped into the particles divided by the total amount present in the formulation, which is called as called encapsulation efficiency. Encapsulation efficiency is generally expressed in % [50].

Drug Entrapment Efficiency: The quantity of drug adhered per unit mass of matrix, that is, moles of drug per mg of matrix or mg drug per mg of the matrix is called entrapment efficiency of the Nanoparticles and expressed as a percentage relative to the polymer. Various drugs have been incorporated into the Nanoparticles used for drug and vaccine delivery. The various factors that affect the loading capacity of the drug in nanoparticle depend on the type of polymer, nanocarrier, and other aspects specific to that particular for Nanoparticles. In the case of solid lipid nanoparticle, the drug loading capacity depends upon the solubility of the drug into the melted lipid, drug melt, and lipid miscibility with this chemical and physical structure of solid lipid matrix, polymorphic state of lipid material.

To determine drug loading capacity, Nanoparticles are dissolved in an appropriate solvent followed by ultracentrifugation to separate the drug from the supernatant. A small volume of the supernatant is taken out, diluted with a compatible solvent, and absorbance is determined at the required wavelength spectrophotometrically against a blank, and the drug levels in the supernatant are determined with help of a standard curve which was earlier constructed for that particular drug.

The quantity of drug encapsulated in the nanoparticle is determined by reducing the amount of drug in the supernatant from the total amount of initially taken. Amar, S. K. Chandrashekar *et, al*. studied the formulation and characterization of Rivastigmine loaded solid lipid nanoparticles (SLNS). The Rivastigmine SLNS was prepared by the microemulsion method. The entrapment efficiency of Rivastigmine SLNS was estimated by the centrifugation method, at 1500 rpm for 30 minutes. The untrap Rivastigmine was determined by UV spectrophotometer (Shimadzu UV 1800) at 221nm and calculated by the following formula

% Entrapment efficiency = Total amount of drug – Free dissolved drug

Total Amount of Drug: % Entrapment efficiency of Rivastigmine SLNS was found to be 93.26-99.80% [51].

Drug Localization and Drug Release: A very important aspect of Nanoparticles as a carrier for drug delivery is to consider the pathway and amount to which the therapeutics molecules are released. Release profile studies are performed similarly encapsulation efficiency determination assays which are measured for a time interval to find out the release mechanism. Various techniques are reported to determine *in-vitro* release pattern of the encapsulated drug from Nanoparticles [52]. Some of them are as listed as follows:

- Stirring chased by centrifugation or ultracentrifugation
- Ultra-filtration or centrifugal filtration method
- Reverse dialysis bag method.
- Compartment diffusion cells with artificial or biological membranes.
- Diffusion through dialysis bag.

A drug release study is preferable to be carried out under controlled stirring and centrifugation. But, as the study takes a long time and has technical problems to be overcome in removing Nanoparticles from the release buffer, the dialysis method is mostly preferred for drug release studies.

Probable mechanisms for the release of drugs from Nanoparticles are listed as follows

- Combination of diffusion and erosion process.
- Diffusion through the polymer matrix,
- Membrane controlled diffusion
- The detachment of API adhered to the outer layer,
- Erosion of Nanoparticles matrix or

The release pattern of the drug depends on the solubility of the drug, diffusion through the matrix, and its biodegradation. The exact method to be utilized for drug release study will depend on the drug and Nanoparticle formulation. To determine

drug release from Nanoparticles, Nanoparticles are first lyophilized, weigheded, and then resuspended in a buffer followed by incubation on a water bath at 37 °C with slight stirring. An aliquot of dissolution medium at scheduled time intervals i.e., Between 15 min and 10 days, should be taken for quantification; the sealiquots are then replaced with fresh solvent to maintain sink conditions. The release of the drug can be quantified by determining the absorbance using UV spectrometry or HPLC to calculate drug release concerning time [53, 54]. The release patterns of the drug also vary as per the release conditions, *i.e.,* Sink or non-sink conditions and release medium *etc.* [21, 55, 56]. Amar, S. K. Chandrashekar *et. al*. studied the formulation and characterization of Rivastigmine loaded solid lipid nanoparticles (SLNS). *In-vitro* dissolution studies were carried out by USP apparatus type II, using 900ml phosphate buffer pH 7.4 and temperature 37±0.5°C. The rotation speed was 50rpm and samples were analyzed by UV spectrophotometer (Shimadzu UV 1800) [51].

***In-situ/Vivo* Characterization of Nanoparticles:** Nanoparticles can be evaluated in biological mediums, such as plasma cells, blood, or primary culture. Cho *et, al.* has reviewed various in-vitro and *in-vivo* characterization parameters of Nanoparticles in detail [57]. A few of them *in-vivo* tests which may be carried out are elaborated on in the following sections.

Phagocytic Uptake: Phagocytic uptake is the most widely used technique used to understand the *in-vivo* behavior of Nanoparticles, which is measured by calculating phagocytic uptake via macrophages of fluorescently-labeled Nanoparticles as a function of concertation and time [49, 58]. size and topical characteristics play a vital role in phagocytosis [58]. A study demonstrated that their morphology at the time of interaction with macrophages is important for the phagocytosis of microparticles. Because of their confrontation with phagocytosis, linear polymeric micelles circulated for a prolonged period compared to micelles of similar structural characteristics. Higher circulation time is required compared to spherical particles of the same composition after *in-vivo* injection to the mice for phagocytic uptake by macrophages of peg-coated rod-shaped gold nanorods were smaller. The main drawback of the macrophage uptake studies is that variations in the composition of cell culture media are comparable to biological fluids, which make phagocytic uptake studies unpredictable. Y. wang studied In Situ Evading of Phagocytic Uptake of Stealth Solid Lipid Nanoparticles by Mouse Peritoneal Macrophages. Stealth solid lipid nanoparticles (SSLN) were prepared by emulsion evaporation method and evaluated for phagocytic uptake by mouse peritoneal macrophages. Phagocytic uptake was determined by flow cytometer [59].

Complement Activation: The surface association of dissolved serum proteins called a complement system on the Nanoparticle outer layer starts a biochemical pathway that removes nanoparticle from the serum through complement-dependent receptor-mediated phagocytosis mechanism [60]. The complementary system can be monitored to estimate the capabilitynanoparticleicle to escape phagocytic removal. Nanoparticles could activate dissolved protein components, c_3, which cleave to the c_3b and c_3a. Hence, the relative proportion of c3b and c3a predict the degree of Nanoparticles mediated complement activation using immune electrophoresis of nanoparticle in plasma. The surface modification of Nanoparticle surface with a polymer manipulates the ability of plasma proteins to bind depending on the chain length of the polymer, electric potential, and its orientation [23].

Protein Adsorption: The immunogenicity of a Nanoparticle has only been detected by *in-vivo* studies; there has been increasing interest in predicting the immunological responses to Nanoparticles in earlier phases of Nanoparticle development, most probably by *in-vitro* studies. One of the basic techniques to study the properties of a Nanoparticle is to examine the extent of protein adsorption on the surface of theNanoparticle, the first step of phagocytic removal of nanoparticle is to assess the extent of protein adsorption, Nanoparticles having constant surface area are incubated in serum for a stated period and to remove proteins loosely adsorbed to the surface they are washed with water. The proteins bound to Nnanoparticle are desorbed with the help of surfactants like sodium dodecyl sulfate and subjected to gel electrophoresis or quantitative protein assay. In past decades attempts have been made to correlate protein adsorption to the

Nanoparticle surface and the in vivo fate of the Nanoparticles; for example, polystyrene Nanoparticles which are coated with an amphiphilic polyethylene oxide-polypropylene oxide block copolymers, showed much-reduced protein adsorption *in-vitro* and a prolonged circulation rate in rats. Protein adsorption alone can provide only a rough prediction of the potential immunogenicity, as it does not reflect the complex nature of subsequent immune reactions leading to the elimination of Nanoparticles [61].

Cell-based Evaluation:

Cellular Uptake of Nanoparticles: With the physical and chemical characterization of Nanoparticles, their biologic characterization is also measured in animal cell culture studies before investigating *in-vivo* administration. Animal cells are maintained in a cell culture flask and nutrients are supplied using a medium kept at 37 °C in a co_2 incubator. Monolayer cell culture model is generally used for Nanoparticle uptake study by the cells, and it is proved to be extremely beneficial for the study of translocation of Nanoparticles to the cells, the therapeutic potential of drug released throughnanoparticlee and side effects of the nanocarriers. Nanoparticle translocation is generally determined using confocal microscopy and flow cytometry whilst simple incubation is an easy and quick process buthas a risk of leaching out of lipophilic dye from the carrier which might lead to false-positive uptake by Nanoparticles. Confocal microscopy helps to determine the Nanoparticle localization inside the cells and the amount of nanoparticle translocation is measured using flow cytometry. Confocal microscopy has been used to investigate intracellular delivery of various detoxified pertussis toxoid to cells [61].

Mechanisms of Cellular Uptake: The mechanism behind the nanoparticle's entry into the cells is important as the successive pathways to be followed inside the cells depend on it. Physicochemical characteristics of nnanoparticle decide the mechanism of particle translocation such as macropinocytosis, phagocytosis, and clathrin- or caveolae-mediated endocytosis. Clathrin-mediated endocytosis occurs through clathrin proteins that cover the Nanoparticles inside a vesicle and are transfer to an endosome. Clathrin- or caveolae-mediated endocytosis involves cellular receptors for specific ligands such as folic acid, transferrin or albumin, which initiates endo or transcytosis of these molecules. Cells are subjected to inhibitors of particular internalization mechanisms to investigate the cellular uptake mechanisms before treatment with fluorescently tagged Nanoparticles. Chlorpromazine is used as an inhibitor of clathrin-mediated endocytosis, filipin and methyl-b-cyclodextrin are inhibitors of caveolae-mediated endocytosis.

Phagocytosis and macropinocytosis can be inhibited by pretreatment with amiloride or cytochalasin. Pretreated cells are then subjected to Nanoparticle internalization and subjected to either flow cytometry or confocal microscope equipped with software that will be able to support the quantitation of nanoparticle. Kb cells and a 549 lung cancer cells are treated with polylactide-co-glycolide Nanoparticles coated with folic acid, this showed that the particles are taken up in higher quantity by kb cells compared to a 549 cells.

To prove that the particle uptake is competitively inhibited cells are pretreated or co-incubated with a large excess of pure ligands. The size of the Nnanoparticleis critical for cellular uptake, receptor-mediated, hydrodynamic diameters of Nnanoparticle near to the vesicle size formed during clathrin- or caveolae-mediated endocytosis that is 100 or 60 nm. The particle size, particle shape, and surface charge also affect the cellular uptake of the nanoparticle [5].

Bioactivity of Nanoparticles: Care should be taken to retain a drug's strength, safety, and effectiveness to be encapsulated into the Nanoparticles.

Once an anti-cancer agent is targeted *via* nanoparticle, metabolic action or integrity of cells can be determined by using different techniques based on tetrazolium salts such as MTT, MTX, and XTT assay. Test based on colorimetry that is, MTT, MTS, and XTT assays, determine the capability of mitochondria of live cells to reduce into tetrazolium salts to deeply colored formazan dyes. Bioluminescence strength can be used to determine luciferase activity which is proportional to ATP generated by live-cell assays because of the conversion of luciferase into luciferin. The

presence of unviable cells can be detected by releasing lactate dehydrogenase, a constitutive cytoplasmic enzyme, when the cell membrane is disturbed [61].

Intracellular Trafficking: A Nanoparticle is internalized by cells, and its intracellular fate critically influences its therapeutic effect especially when the drug target is localized in a particular organelle or the drug is unstable in a specific intracellular environment, e.g., acidic pH to keep a track of the intracellular trafficking of the Nanoparticles, markers of intracellular organelles are localized with Nanoparticles and observed over a while. The organelles are located using labeled antibodies after fixation and also with the permeabilization of cells.

In the study of mesoporous silica and polystyrene Nanoparticles, the Nanoparticles were incubated with cells that were prelabelled with the help of lysotracker, a fluorescent probe that accumulates in acidic organelles. The mesoporous silica Nanoparticles and the lysotracker signals localized in 5 min, which indicatesthe residence of Silica Nanoparticles in lysosomal vesicles. With time the fluorescence of the silica Nanoparticles and lysotracker signals were separated, which suggested the escape of Nanoparticles from the acidic vesicles. Carboxyl-terminated polystyrene Nanoparticles did not show localization with lysotracker signals at any time, indicating their residence in recycling vesicles. This result is consistent with the limited intracellular accumulation of the polystyrene nanoparticles [61].

Confocal Laser Scanning Microscopy (CLSM): Confocal laser scanning microscopy (CLSM) is a widely used microscopic tool that provides morphological and functional information about nanoparticles within cells and tissues. Interaction of different types of and their interaction with skin detected by confocal laser scanning microscopy under various conditions. E. A. Gibbs *et. al*. studied confocal microscopy to detect nanoscale particle internalization by human lung cells. For this study, transformed human airway epithelial cells were used. Detection of nanoparticles using confocal microscopy proved robust in detecting several types of nanoparticles comprised of different materials [62].

***In-vivo* Evaluation of Nanoparticles using an Animal Model:**

Animal Models of Tumor: After Nanoparticles are studied for preliminary effectiveness *in-vitro*, these Nanoparticles are subjected to further evaluation regarding their toxicity profile and response in biological species. For these, a suitable animal model that can closely relate pathophysiology of the human disorder is a way to extrapolate healing potential in men. The selection of animal models and animal studies is highly specific and selected based on a drug under investigation and the proposed route of administration for the Nanoparticles. *In-vivo* evaluations of Nanoparticles might be carried out, such as dose–therapeutic response studies, the biodistribution of Nanoparticles among the various body organs, acute and multidose efficacy studies as well as safety and pharmacokinetic parameters, that is, absorption, distribution, metabolism, and excretion.

The goal of *in-vitro* and *in-vivo* evaluation is to correlate the physicochemical aspects of the Nanoparticles to their biological function. PEGylated doxorubicin liposomes were evaluated for pharmacodynamics response against cancer using animal species by subcutaneously injecting colon carcinoma cells into the c-26 mouse. The effect of the therapy was studied by determining the tumor size after the scheduled time and it was found that free doxorubicin was able to reduce the tumor size in a minute,. In contrast, liposomal doxorubicin reduced the size, which is not measurable [64]. Mean survival time was also more than 120 days compared to 50 and 49 days for plain doxorubicin and saline, respectively. The benefit of genetically engineered mouse models is that they truly produce tumors of a specific type and exactly represent tumor-host interactions; hence they have proven to be best for dissecting the roles of oncogenes. But genetically engineered models showed limited application compared to other *in-vivo* models in regular assessment of Nanoparticles because they are costly and violation of IPR [61].

Streptozotocin-induced Diabetes Animal Model: Diabetes is generally induced by using streptozotoc in (STZ) injected intraperitoneally into animals. STZ is glucosamine–nitrosourea compound derived from Streptomyceschromogens that are used as a

chemotherapeutic agent in treating pancreatic B cell carcinoma. STZ damages pancreatic b cells, resulting in hyperglycemia. STZ can induce diabetic state mainly in two ways, depending on the dose and animals showing blood glucose more than 250% *i.e.,* 200 mg/dl of fasting levels is used in the subsequent study. Vitamin b12-Nanoparticles are administered orally and plasma glucose levels were estimated to test the oral effectiveness of these Nanoparticles [61].

Alloxan-induced Diabetic Model: Diabetic control rabbits showed no significant changes in serum glucose levels (SGL) throughout experiments. However, as time progressed, a slight decrease in SGL was observed in diabetic control. The decrease may be attributed to the fasting effect on SGL, and Devarajan supported it. GB-treated rabbits showed tmin at 7.83 h with cmin 30.94% decrease in SGL of basal level. Nano suspension treated rabbits showed tmin 8.17 and cmin of 53.13% decrease in SGL of basal level, compared with plain GB. Nano suspension showed a very significant decrease ($p < 0.01$) in SGL, tmin, and AUC (area under the curve) when compared to GB-tested group [63].

CONCLUSION: Due to increased surface area at the nanometer level Nanoparticle can alter physical and chemical interaction from the molecular level to the systematic level. Which is creating the *in-vivo* delivery of nanoparticle as a fascinating research topic. The scope of nanoparticle has gone wider and wider in the last two decades. Nanoparticles are now made up of different types, depending on the type of matrix used, such as inorganic versus organic, with extraordinary control over drug encapsulation, particle diameter, particle morphology, surface characteristics, and its release. But it is being observed that their clinical transformation is comparatively slow, and only a few commercial products, such as micelles or liposomes, are on the market. Regulatory guidelines for robust technique Nanoparticle characterization are essential for assuring the safety of Nanoparticles. Nanoparticle, as a novel drug delivery system, is usually evaluated concerning ligand density and surface charge, which decide their interaction with the cell surface. In blood, and any other biological fluid, Nanoparticles are easily covered with protein aura, which eventually dictates therapeutic response and in vivo fates. To distinguish between in-vitro characteristics and *in-vivo* behaviors plenty of research groups started using models that can generate preliminary in-vivo data. Pharmaceutical researchers need to understand the challenges and restrictions of present technologies to characterize these Nanoparticles and discover a novel path for Nanoparticle evaluation that could forecast clinical findings of Nanoparticles at the initial level of product development with the highest reliability.

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