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Review

Advanced Characterization and Sample Preparation Strategies for Nanoformulations

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Abstract: The escalating impact and remarkable progress of nanotechnology have shifted the paradigms of medicine and the healthcare system. Nanosystems have emerged, extensively holding the potential to advance disease diagnosis and treatment specificity. The extraordinary attributes imparted by nano-systems have helped in overcoming the limitations of conventional interventions to an extent and led to targeted therapy, to name one. The role of nanotechnology in diagnosis is another breakthrough in its appellation. This article aims to address the current characterization and sample preparation techniques for the analysis of nanosystems and provide insights into novel methodologies and in situ instrumentation that have eased sampling procedures.

Keywords: nanoformulations; characterization; sample preparation; nanomedicine

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1. Introduction

Nanotechnology has revolutionized materials science and biotechnology by enabling the precise manipulation of matter at the nanoscale. At the forefront of this transformative field are nanoformulations, characterized by their dimensions typically ranging from 1 to 100 nanometers [1]. These nanoformulations play a crucial role in biomedical and pharmaceutical sciences, promising enhanced drug delivery, improved therapeutic efficacy, and reduced adverse effects [2–5] (Figure 1). Central to the advancement of nanoformulations are functionalization and encapsulation techniques, which significantly enhance their versatility in biomedical applications. Functionalization involves modifying the surface of nanoparticles with targeting ligands, antibodies, or biomolecules, allowing for the precise targeting of specific cells or tissues, thereby improving therapeutic efficacy, and reducing off-target effects [6–8]. Encapsulation, on the other hand, ensures the efficient loading and release of drugs from nanoparticles, optimizing their pharmacokinetics and therapeutic index [9–11].

Advanced characterization techniques and sophisticated sample preparation strategies are essential for understanding and enhancing the physicochemical properties of nanostructures, ensuring their stability, functionality, and safety in healthcare applications. As per the Gartner Hype Cycle model, the progression of a conventional technology does not fit in nanotechnology owing to the predictable depression of visibility after the maximum expectations being extremely low in this instance, and the plateau of productivity has been reached very swiftly in the past few years [12,13]. From the standpoint of formulation development and drug delivery, the robust analysis of the developed nanosystem plays a crucial role in the developmental process. Certain analytical techniques remain indispensable when dealing with the characterization of nanomaterials, and sample preparation remains

the main stronghold in the analytical science. Sample preparation is a multistep process employed for sample analysis which has a major impact on the accuracy and precision of results [14].

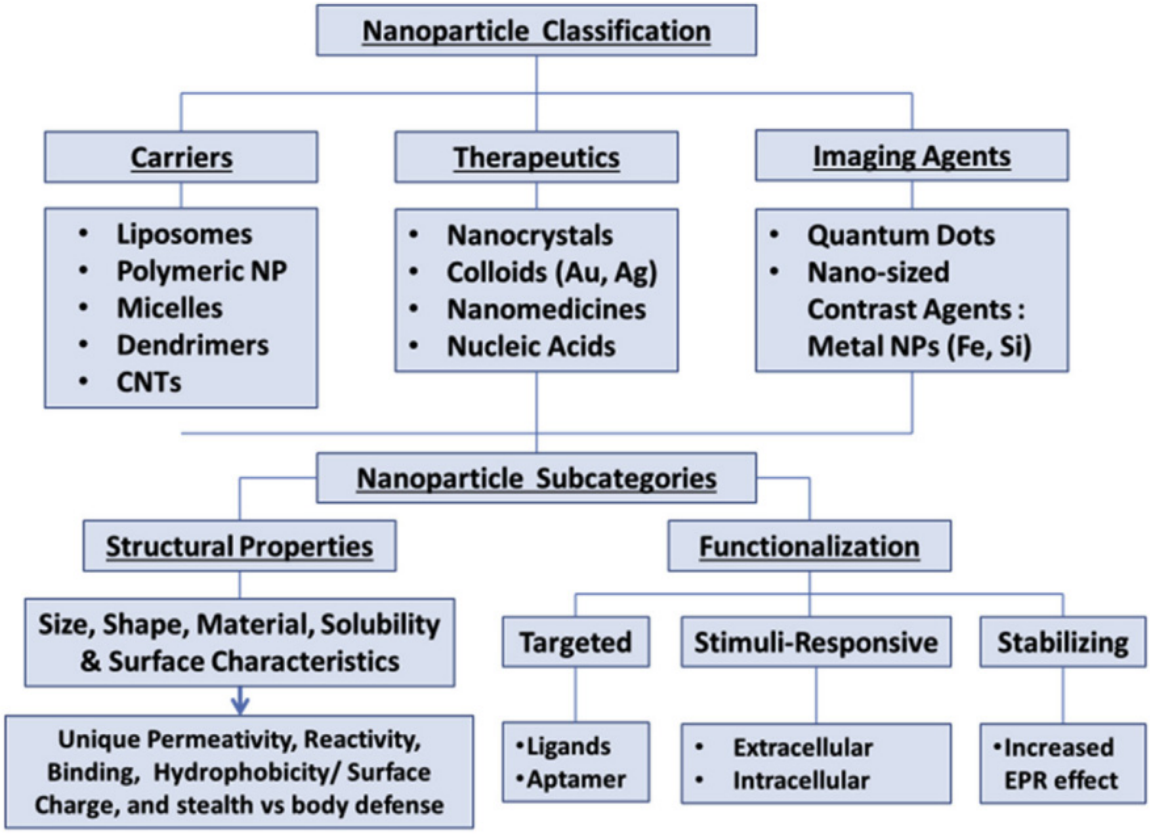

Figure 1. Nanoparticle classification and their subcategories. Reproduced with permission from reference [15]. 2019, Elsevier.

Accurate sample preparation protocols can eliminate the possibility of errors, provide a higher accuracy, shorten the analysis time, and minimize the cost. Sample preparation is an exhaustive process and starts from specimen collection up to sample measurement. Thus, it is important that the original physicochemical properties of the sample should not be altered and the conversion of the sample into a form suitable for measurement should not cause sample loss and hamper the sample integrity. This article aims to address the current sample preparation techniques for the analysis of nanosystems and provide insights into the novel methodologies and in situ instrumentation that have eased sampling procedures. Table 1 summarizes different analytical techniques used for nanoformulation characterizations.

Table 1. Comparison of analytical techniques for nanoformulation characterization.

Technique	Advantages	Disadvantages	Resolution	Information Provided	References
Dynamic Light Scattering (DLS)	- Measures size, hydrodynamic diameter, and state of aggregation of nanoparticles - Suitable for colloidal dispersions, nanoparticles, polymers, and proteins	- Highly concentrated samples require dilution - Cannot measure dry powders directly - Electrostatic interactions can affect results in deionized water	Sub-micron scale	- Size and size distribution - Aggregation state - Hydrodynamic diameter	[16,17]

Table 1. *Cont.*

Technique	Advantages	Disadvantages	Resolution	Information Provided	References
Scanning Electron Microscopy (SEM)	- High-resolution imaging - New developments like ESEM allow imaging without drying	- Requires conductive coating for non-conductive samples - Liquids or wetted objects cannot be observed as high vacuum is used	Sub-nanometer scale	- Surface morphology - Structural details	[18,19]
Cryo-SEM	- High-resolution imaging of hydrated samples without distortion - Preserves natural state of samples	- Requires advanced equipment - Sample preparation can be complex	Sub-nanometer scale	- Hydrated state imaging - Structural details	[20]
X-ray Diffraction (XRD)	- Identification of phases - Detection of elements	- Sample preparation can be time-consuming - Requires precise control of sample conditions	Angstrom scale	- Crystal structure - Phase identification	[21]
Transmission Electron Microscopy (TEM)	- High-resolution images of thin materials - Can utilize automated sample preparation	- Sample preparation is time-consuming - Dependent on operator skill	Sub-nanometer scale	- Internal structure - Micrography of biological cells and viruses	[19]
Atomic Force Microscopy (AFM)	- High-resolution surface topography - Non-destructive to the sample	- Limited to surface analysis - Sample preparation may require spin coating	Sub-nanometer scale	- Surface topography - Nanostructure details	[22,23]
Scanning Tunneling Microscopy (STM)	- Atomic-level imaging - High resolution	- Requires conductive samples - Sample preparation can be complex	Angstrom scale	- Atomic surface structure - Electronic properties	[23]
Inductively Coupled Plasma Mass Spectrometry (ICP-MS)	- Highly effective for detecting trace elements - High sensitivity	- Sample preparation can be complex - Requires aerosol dilution for high-matrix samples	Atomic scale	- Elemental composition - Trace element analysis	[24]
X-ray Photoelectron Spectroscopy (XPS/ESCA)	- Detailed elemental composition - Sensitive surface analysis	- Sample must be protected from contamination - Sample storage is crucial	Nanometer scale	- Surface elemental composition - Chemical states	[25]

2. Analytical Techniques for Nano Formulation Characterization

2.1. Particle Size Analysis by DLS

Dynamic light scattering (DLS) is a technique that measures the size, hydrodynamic diameter, and the state of aggregation of nanoparticles present in the solution. It also can be used to measure the concentrations of colloidal samples.

2.1.1. Working Principle

When the sample is illuminated by a laser source, particles scatter light in all directions (Figure 2). The light scattered from the laser passes through a colloidal solution and the intensity of the scattered light is modulated as a function of time. An important feature of DLS is the Brownian motion wherein smaller particles move quickly and larger particles

move slowly. The correlation between the speed and size of the particle is based on the Stokes–Einstein equation which applies to infinitely dilute solutions [26].

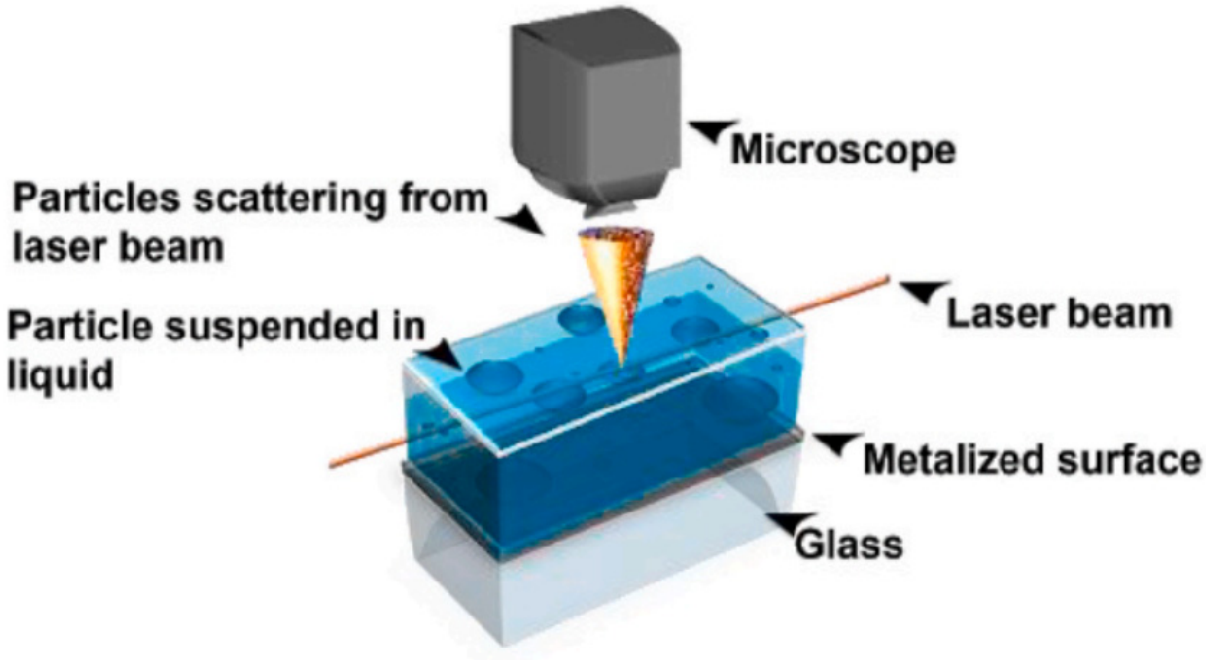

Figure 2. Dynamic light scattering. Reproduced with permission from reference [27]. 2021, Elsevier.

2.1.2. Sample Preparation

DLS can measure colloidal solutions like solid particles, emulsions, polymers, and proteins. Samples to be analyzed for DLS must be clear or slightly hazy. Highly concentrated samples require dilution as may cause multiple scattering and may give inaccurate readings. Protein samples should not be aggressively stirred for dispersing unless they are sturdy enough to be sonicated or vortexed

A. Dry sample dissolution

Powdered samples need to be suitably dispersed in appropriate solvents prior to measurement. Dry powders cannot be measured as such and appropriate surfactants must be used for wetting the powder. Particle size measurement by DLS should be carried out preferably in water with a trace amount of salt and not in pure deionized water. As the particles are surrounded by an electrical double layer, there is a possibility of electrostatic interaction between particles measured in deionized water. 10 mM of KNO_3 is an ideal salt for all concentrations of particles compared to NaCl [28]. The solvent used must disperse the particles well and not dissolve the particles. Ionic surfactants or dispersing agents may be used for complete dispersion. Pure solvents of high purity are to be used. The filtering of polar solvents is recommended due to the risk of dust when compared to non-polar solvents which do not dissolve or carry dust. Filtering is a good practice for aqueous diluents with salts. Impurities with a high mass can be removed via multiple filtrations.

B. Liquid sample preparation

Sample concentration affects DLS measurement. Concentrated liquid samples are ideally diluted with the same solvent that they are prepared in and with the same concentration of additives. Highly concentrated samples that are milky white or opaque in appearance can be diluted with a liquid of choice or can be centrifuged or redispersed. The dilution of the sample avoids the risk of interparticle interactions as well as multiple scattering. The measurement of the hydrodynamic radius is affected by concentrated samples, and highly diluted samples give insufficient signals (low signal-to-noise ratio).

The concentration of samples can be checked by performing a count rate check and the maximum count rate for the measurement should be 500–600 kcps.

The selection of the type of cuvette depends on the instrumental setup. A light scattering spectrometer has a typical cylindrical cuvette and square cuvette for special requirements. Nano lab 3D has three types of glass cuvettes and deposable glass cuvettes of 10 × 10 mm. Sufficiently homogenous solutions can be placed in the cuvette for measurement. Before analyzing the sample, the cuvette must be cleansed and should be free from dust. The cuvette can be cleansed with Hellmanex III (alkaline cleaning concentrate) from helma [29] or can be rinsed with milli Q. Bottom of the cuvette must be inspected for any settled larger particles as it indicates an unequal sample distribution or possible aggregation and is not suitable for DLS measurement. The walls of the cuvette should also be checked for bubbles. Colored solutions do not absorb laser light completely and are difficult to measure. The amount of solution to be added to the cuvette is important as a small sample volume may not allow the laser beam to pass through the sample, and larger volumes may influence the Brownian motion of the particles. Upon placing the cuvette in the sample holder, the sample in the sample holder is allowed to be equilibrated with that of the water bath. Further required parameters must be added and the data can be analyzed and interpreted. Standard DLS instruments require the sample to be diluted to avoid the risk of multiple scattering and inaccurate results.

Nano lab 3D has come up with a novel patented technology that does not require sample dilution and a technique that also suppresses the multiple scattering of light. The compact easy-to-use instrument offers a higher sensitivity and better sample characterization. Measurements up to the highest concentration are possible in Nano lab 3D [30].

Microfluidic measurement is an interesting concept with its microscale analysis system and the development of labs on a chip. The incorporation of miniaturized DLS instruments into microfluidic devices was investigated and was adapted for measuring very few fluid samples. Microfluidic devices come with the advantages of a small fluid volume, rapid sequential measurement, and precise measurement. Brown et al. developed a fiber optic probe in a homodyne DLS instrument as fiber optic connections simplified the splitting and mixing of signals [31,32]. In one study, five designs of miniaturized DLS instruments were designed with flow-through microfluidic devices, which caused less sample consumption and high-throughput analysis. The instrument accurately determined particles in the size range of 10–100 nm. The instrument was incorporated with stirring elements to blend together with stock solutions prior to measurement. It could also measure the critical micelle temperature (CMT) as some devices had a temperature-sensing ability. The instrument was tested by measuring solutions of latex polystyrene nanoparticles and block copolymers. Stirring elements were also stopped during measurement to prevent turbulence. High-throughput measurements were made on solutions of polystyrene-b-isoprene in a mixed solvent. The instrument allowed the systematic blending of solutions [33].

DLS primarily outputs an intensity distribution that represents the light-scattering intensity for each proportion of particle size. The distribution of particles in a sample might be deceptive when aggregates are present, as it is naturally skewed towards bigger particles that have a higher scattering intensity. In order to provide a more accurate depiction of the sample's composition, the intensity distribution can be transformed into volume and number distributions using Mie theory. This process necessitates making assumptions on the shape, uniformity, and optical characteristics of the particles. Although the conversion intends to offer a perspective based on mass or volume, it is important to recognize that these resulting distributions contain intrinsic inaccuracies caused by assumptions and the widening of peaks in DLS. Hence, when it comes to comparative studies and determining relative proportions in multimodal samples, volume and number distributions are more suitable than absolute quantification.

Since DLS measures the hydrodynamic diameter, the obtained diameter can be different from other methods. For instance, Hinterwirth et al. [34] found that the diameter

obtained from DLS is slightly larger than the diameter obtained by transmission electron microscopy (TEM) (Figure 3).

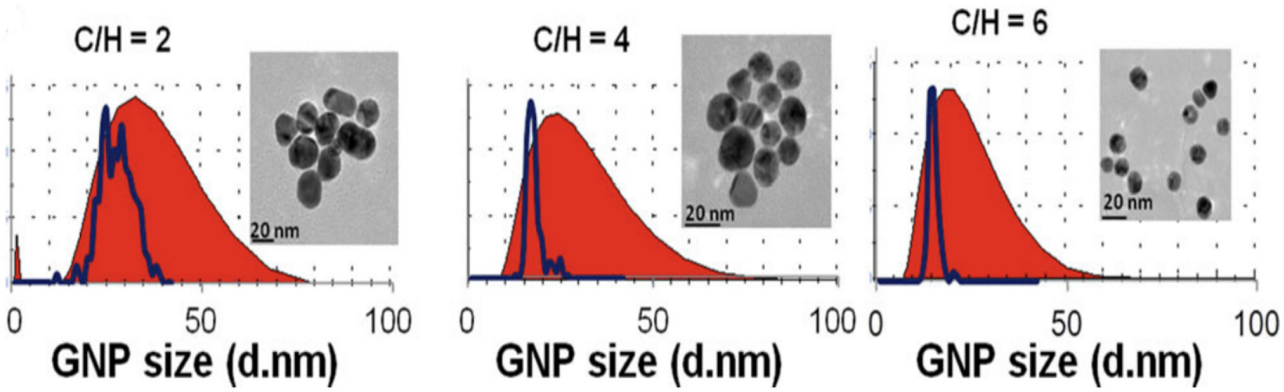

Figure 3. Comparison of hydrodynamic diameter and size distributions by DLS (red line) with TEM data (blue line). C/H is a ratio of citrate and $HauCl_4$ used for nanoparticle preparation. Reproduced with permission from reference [34]. 2013, Elsevier.

2.2. Scanning Electron Microscopy (SEM)

SEM is one of the main microscopic imaging techniques for the characterization of nanomaterials that delivers a high-quality resolution and superior material contrast in comparison to optical microscopy [35]. SEM is regarded as an effective analysis technique for the characterization of organic and solid inorganic samples from a nanometer to micrometer range [36].

2.2.1. Working Principle of SEM

The principle of SEM lies in the irradiation of the sample surface with a highly focused electron beam either from a thermionic emission gun (TE gun), field emission electron source in FE-SEMS, or Schottky emission gun [37]. Image formation in SEM is a consequence of the signal generated due to the interaction between an electron beam and atoms of the object. Topographical images generated of the sample can be visualized due to the two-dimensional scanning of an electron probe over the sample surface [38]. The instrument operates in a high-vacuum and dry environment to produce a beam of electrons that are needed for analysis, unlike optical microscopy which involves light and is easily transmitted in the air [39]. The specimen must be sufficiently conductive and coating the sample with heavy metal allows the spatial scattering of electric charges on the specimen surface. Biological samples require pretreatment and cannot be imaged in their native state, whereas air-dried samples or solid samples do not require special treatment and can be easily visualized [40]. Figure 4 represents the schematic representation of SEM.

2.2.2. Sample Preparation for SEM

A. Fixation

Fixation is the first and crucial step to stabilizing the microarchitecture of the specimen and making it resistant to further processing steps. Fixation is done to protect against any sample damage that may occur during the dehydration stage [42]. Primary fixation uses chemical fixatives like glutaraldehyde, formaldehyde, and tannic acid. Chemical fixatives act by denaturing and coagulating the biological specimen or by covalent binding to the macromolecules. Fixation stops the cellular processes and preserves the specimen. Formaldehyde is a cross-linking fixative used for electron microscopy [43]. This is followed by post-fixation carried out with osmium tetroxide [44]. Primary fixation is carried out for proteins and secondary fixation is carried out mainly for lipids which render them resistant to extraction by organic solvents. Physical fixation employs methods like cryo-freezing or heating.

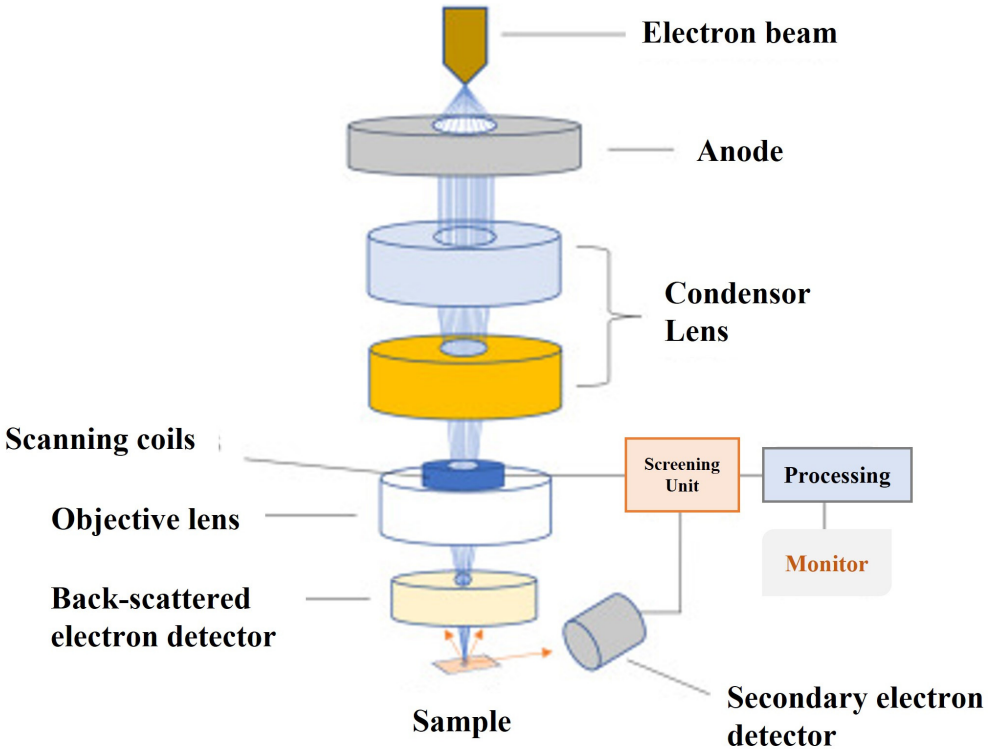

Figure 4. Schematic representation for SEM. Reproduced with permission from reference [41]. 2023, Elsevier.

B. Dehydration

The dehydration stage is a prerequisite in conventional SEM. Samples should be allowed to shrink gradually and prevent collapsing as they can also potentiate artifacts. A graded series of solvents like ethanol and acetone are used as dehydrating agents. Dehydration is distinct from drying as the former replaces water with organic solvents in the tissues.

C. Drying

The clarity of the micrograph can be compromised due to traces of water vapors which can obstruct the electron beam and affect the instrument's operation. Thus, drying is carried out before the introduction in the SEM chamber. Solvents like acetone or ethanol cause the micro-ripping of the sample surface and may hamper the integrity of the sample. To avoid this, solvents are replaced with hexamethyldisilane (HMDS) [45], PELDRI II [46], and freon 113. Air drying is a faster process; however, the structural integrity of the sample is compromised due to cell shrinkage. Critical point (CPD) is an established dehydrating method wherein, under certain temperature and pressure conditions, the liquid and gas become indistinguishable [47]. CPD provides distortion-free samples but is not a preferred technique; freeze-drying (FD)/(lyophilization) is a less complex process where a completely frozen sample is placed under a vacuum and sublimated from the solid to gas phase [48].

D. Mounting

Mounting on the stub is carried out to ensure electrical conductivity is maintained between the stub and the surface of the sample when it is mounted. Stubs are supports that are available in different diameters and make the imaging process of samples convenient. Mounts are usually made up of brass or aluminum. In this step, the sample is mounted on a sticky carbon disc to increase its conductivity. Double-coated carbon tape is placed on the sample stub, and then the sample is mounted on the tape. Double-coated carbon tapes are used commonly but they come with a risk of outgassing or creeping within

the SEM environment [49]. Thus, silver-containing adhesive glue is another alternative. Poly-L-lysine-coated coverslips are also used as attachments.

E. Sputter coating

Non-conductive materials or resin-embedded samples act as an "electron trap" [50] due to specimen charging, and the local accumulation of charge can result in hampered image quality, distortion, and imaging artifacts. Thus, sputter coating is a step carried out to localize the electrons on the surface and improve the signal-to-noise ratio as well as cause the emission of secondary electrons. Gold and platinum are ideal materials to coat the samples and give a high resolution due to their high metal conductivity. The alloy of titanium oxide and indium oxide can be used to create transparent conductive layers. On the other hand, metal samples do not require a coating as they are conductive and are sputter-coated just to increase their compatibility with SEM. An alternative to sputter coating is lowering the vacuum inside the specimen chamber which introduces a positive charge near the surface of the sample and neutralizes the electron. However, this solution comes at the expense of a poor resolution and signal-to-noise ratio.

2.2.3. Newer Advancements in SEM

To overcome the limitation of a low resolution due to drying and sputtering, a modification was made to a freeze-dryer wherein a magnetron sputter head was installed within a freeze-dryer. The technique was named "Cryosputtering" as a combination of freeze-drying and sputtering was introduced. It was developed with an aim to visualize cellular structures at a high resolution which become compromised when dried samples are exposed to air and are then coated. As the instrument coats the frozen dried samples in the chamber itself, the name Cryosputtering and the vacuum is maintained in between the two steps of drying and coating, and dried samples do not become exposed to humidity from the air before coating. The tungsten (W) magnetron sputter gives a finer coat to the sample surface in comparison with Au/Pd/Pt. A freeze dryer is connected with a pumping system, and thus dries the samples efficiently, and samples are not warmed to avoid the risk of recrystallization [51]. Environmental scanning electron microscopy (ESEM) is a modification to conventional SEM as biological specimens with a high water content and low conductivity cannot be observed [52]. ESEM operates with two major modifications and allows a visualization in the native state and without the additional step of metal coating. A multiple pressure-limiting aperture, which can adjust the chamber pressure by adjusting the input flow rate, along with a gaseous secondary electron detector (GSED), helps in the amplification of the original SEM signal as it generates secondary electrons that collide with water vapors and further generate positive ions which are attracted on the sample surface, and thus prevent charges and artifacts. Conventional field emission SEMs with high-vacuum systems require multiple processing steps to prevent sample damage, and these procedures prevent the direct observation of the biological specimens. Exposure to a high vacuum often leads to dehydration and loss of ultrastructure. It is reported that low-vacuum SEM or ESEM has a risk of low-quality image resolution. Thus, a novel technique of "Nanosuit" was introduced by researchers to visualize biological cells and tissues [53].

"Nanosuit" is a thin, vacuum-proof suit in which samples are encased keeping the multicellular organisms alive in high-vacuum conditions of EM. Nanosuit forms act as an electrically conductive barrier on the sample surface and the sample can be in its native state holding moisture. A native extracellular substance or ECS-mimicking substance is used to polymerize the plasma membrane. The modification of this technique was introduced when it was reported that tissues from living organisms were not protected by natural extracellular substances, and, thus, the new Nanosuit could also elucidate wet specimens in their intact cellular organization [54]. This Nanosuit technology was applied to the correlative light and electron microscopy (CLEM) analysis to examine paraffin sections. Zeiss FE-SEM from Gatan Inc. has come up with 3View® for the three-dimensional imaging of biological samples. It involves the serial block-face imaging technique which is a faster

and more convenient method to give high-resolution images with reproducibility. Serial block-face SEM has an in situ-placed ultramicrotome that will repeatedly cut layers of the sample and expose the resin-embedded cell and tissue sample for imaging. Block-face imaging can image multiple microns of the sample within minutes and gives a crisp quality image [55]. The focal charge compensation module for block-face imaging is a technique that was developed in collaboration with NCMIR by ZEISS that eliminates surface charging without interfering with the image quality and is used for most charge-prone samples. The system involves the use of a focal gas injection of nitrogen. The gas injection valve is placed precisely above the specimen surface and guides nitrogen onto a block-face surface. The needle is retractable and is mechanically coupled with the ultramicrotome and thus maintains high acquisition rates [56].

A new approach to sample preparation was attempted in a work by Ghomrasni et al. to obtain a well-dispersed population of particles from powdered forms or a suspension of nanoparticles for SEM measurements. The presence of clumps or agglomerates in the specimen have a negative impact on the measurement results and the possibility of significant errors is reduced by achieving the individualization of particles as they are more noticeable. The breaking up of agglomerates can be carried out by ultrasonication. In order to measure the powdered form of nanoparticles, an appropriate stable suspension is prepared by dispersing powder in a liquid medium, as the specimen for EM must be representative of the entire suspension or powder. A protocol for sample preparation for SEM was proposed in this article and was tested on four samples of particulate materials. The samples were obtained from various sources, out of which three samples were the (SiO_2, CeO_2, TiO_2) powders and the fourth sample was the food-grade TiO_2 (E171) sample. Sample preparation had three major steps: (i) extraction, (ii) redispersion, and (iii) spin coating.

Step 1 Extraction: TiO_2 particles were prepared by a hydrolysis process under microwave heating using a mixture of titanium butoxide and triethanolamine, and commercial TiO_2 nanoparticles were extracted and isolated from chewing gum (Freedent®).

Step 2: Powdered forms of samples were dispersed in ultrapure water, which was carried out in several steps. The steps differ according to the type of nanoparticles. Nanoparticles without a matrix are directly dispersed in the water and are further sonicated to improve the redispersion of particles. Ultrasonication is carried out for the deagglomeration of nanoparticles using a Vibracell 75,043 ultrasonifier bath and energy densities are calculated as it is the main parameter to determine the power delivered to suspensions to separate into particles. This is underlined by Retamal Mari et al. in their work [57]. The specific energy input is crucial as uncontrolled sonication may also induce the re-agglomeration of nano-systems [58]. The dispersions are sonicated in a cold-water bath which is maintained at a constant temperature as sonication may increase the suspension temperature and cause aggregation between nanoparticles; thus, the bath is constantly kept cold.

Step 3: The droplets of the particle colloidal suspension were spin-coated using the Lab Spin 6 SUSS Microtec. Coating involves two steps: (a) the spreading phase where the rotational speed of the coater is 1000 rpm and, during the first 60 s, the speed is 300 rpm/min (if the solvent is volatile) and 500 rpm/min with an aqueous solvent; and (b) the drying step with a speed of 8000 rpm/min. The colloidal suspension adheres to the center of the silicon wafer substrate which has an influence on the performance of the method. Most of the nanoparticles (metal or metal oxides) are negatively charged, and thus adhere easily to the charged surface of the silicon substrate. The attachment to the substrate can be promoted by changing the medium which will change the surface charge of particles or by the functionalization of the substrate surface with poly-L-lysine which is positively charged at the end. Thus, a bridge is formed between the negative end of the particle and the positive end of the substrate. The procedure for functionalization is the immersion of the silicon wafer in the poly-L-lysine suspension for 30 min with a fixed concentration of 0.1 g/L. For the assessment of the isolated particle using SEM for the measurement of the

size distribution, a Zeiss ULTRA-plus FEG microscope and an in-lens detector were used for imaging using semi-automatic software [59].

Lu et al. established a robust and systematic way to analyze the size of commercial ZnO nanoparticles with no clue of their surface functionality, as well as proposed a standard workflow for the preparation of SEM samples that can be applied to almost all types of commercially available ZnO nanoparticles in powdered form. The objective lies in preparing a well-dispersed nanoparticle suspension based on the chemical properties of the particles and the deposition of the developed particles on a substrate surface through the appropriate electrical properties. The commercially available ZnO particles possess different surface properties owing to their modification by a variety of functional groups. These modifications aid in achieving desired properties such as hydrophobicity, hydrophilicity, positive and/or negative surface charges, solvent compatibility, improved shelf-life, reduced aggregation, etc. Sufficient knowledge about basic chemical properties aids in the selection of appropriate solvents and dispersing agents. The schematic workflow as described in the study included selecting a solvent having sufficient wetting on the surface of particles, followed by the addition of a suitable dispersing agent for the measurement of the hydrodynamic radius by DLS. For the SEM analysis, the immersion method was used. The nanoparticles were attracted to the substrate surface mainly by electrostatic force and partly by gravitational force. The derivatized Si chips were used right after cooling down to room temperature. For particle deposition, the Si chips were fully immersed in the ZnO nanoparticle suspension for 2 h, with the derivatized side facing upward. Then, the chips were rinsed with isopropyl alcohol (IPA) and clean water, followed by clean air drying.

2.3. Cryogenic Scanning Electron Microscopy (Cryo-SEM)

Cryogenic temperature scanning electron microscopy (Cryo-SEM) is a highly specialized laboratory technique for imaging hydrated samples without causing the drying of artifacts [60,61]. Cryogenic microscopy allows the visualization of the intrinsic structure of specimens in their near-native state and maintains the ultrastructure by avoiding deleterious changes that can occur in the water removal steps and could cause cell shrinkage [62]. Cryogenic visualization ranges from macromolecular complexes to cells. The domain sizes of image resolution by cryo-SEM are wide-ranging from nanometers to several micrometers. Topographical imaging by SEM has certain challenges and cryo-SEM is an effective technique for imaging samples that would be difficult in a standard high-vacuum SEM.

2.3.1. Sample Preparation

Specimen preparation in Cryo-SEM is critical and samples must be prepared under a controlled environment by maintaining the appropriate concentration and temperature as specimen artifacts are formed at early stages. The temperature around the specimen and saturation of the gas phase must be controlled and must remain steady until the sample is completely thermally fixated. For example, nanostructured liquids change readily upon changes in conditions.

The basic instrument consists of a cryo-chamber and a cold-stage chamber. The cryo-chamber cleaves and coats the frozen samples and the cold chamber images them in the cold stage [63]. Preliminary fixation (thermal fixation by rapid cooling) is an important step before the samples are introduced into the cryo-chamber. Samples that contain moisture are fixed on a holder by using a conductive carbon-rich glue or cryogenic glue or clam based on the type of specimen. The sample must be as small as possible for optimum freezing and freezing is carried out to prevent damage due to ice crystals. The selection of the cryogen is important in plunge freezing [64]. Techniques like impact freezing, also known as the metal contact technique, are used, where the sample is pressed onto a polished metal plate or a jewel surface and is subjected to cooling by liquid nitrogen or by liquid helium. Fast cooling is a preferred technique as the specimen becomes vitrified and the nanostructure is preserved [65]. Physical fixation is a preferred method over chemical fixation for the fixation of labile specimens [66]. Lush of ethane and propane is best suited for sample

fixation. Fixation is followed by loading in the cryo-chamber. Frozen samples are loaded onto a specimen holder via an airlock system and are subjected to cutting with a gold knife.

2.3.2. New Technologies in Cryo-SEM

L. Issman and Y. Talmon, in a study, designed and built a methodology for cryogenic SEM preparation under controlled cryogenic conditions, and, further, the reliability of the system was also assessed by imaging the samples [67]. Controlled environment vitrification systems (CEVSs) is a concept introduced by Bellare et al. and has been applied for cryo-TEM vitrified specimens [68,69]. However, a new design of CEVS has been reported; the instrument had four modules: a temperature control module, an environmental chamber, a plunge module, and a cryogen box. The instrument was checked on samples and the results showed that the newer method was quicker with a reduced artifact risk.

Further, Gemini SEM 500 is equipped with cryo-ultramicrotomy attachments and diamond knives for array tomography. Cryo-chamber EM FC7 is a product of Leica microsystems that are equipped with ultramicrotomes for the cryo-sectioning of samples in minutes in the temperature range of −15 °C to −185 °C. Cleaved samples are sublimated using a heater which is known as "Sample Etching". The sublimation temperature of the ice depends on the vacuum present in the cryo-chamber. When the vapor pressure is higher than the vacuum present in the SEM chamber, sublimation occurs. The coating is carried out using cold sputtering, using gold-palladium (Au/Pd) or platinum in order to make it conductive for imaging in SEM. Leica EM ACE600 is a high-vacuum coating system.

"Vitrobot Mark IV system" is a fourth-generation automated vitrification system for the rapid, reproducible sample preparation for cryo-EM. Vitrification is a critical step that forms an amorphous solid by rapidly cooling water molecules and they have no time for crystallization. The amorphous solid does not alter or damage the structure of the sample. Vitrification is a common challenge in cryo-SEM and partial vitrification can destroy the ultrastructure of the biological sample. The developed system ensures that the critical fixation parameters are controlled, and thus ensures the high-quality cryo-fixation of the specimen prior to analysis. The system also has a coolant container with an anti-contamination device. The overall speed and quality of sample preparation are levelled up. ZEISS has come up with a correlative cryo-workflow that comes with high-resolution fluorescence imaging, sample protection, and high-contrast volume imaging. It has a multipurpose use in cryogenic conditions as well as at room temperatures. Gemini 500 SEM is an instrument that is equipped with a low kV argon ion gun that cleans the surface of cryo samples directly inside the cryo-chamber.

2.4. Transmission Electron Microscopy (TEM)

TEM is an electron microscopic technique and is similar to SEM imaging. The focused high-energy beam of electrons is passed through the sample. The process of sample preparation in TEM is critical and there is a need for thin samples in order to transmit electrons, which makes the process complex.

Sample Preparation

A. Primary fixation

Primary fixation stabilizes the microstructure of the cell and prevents damage. Fixation can be carried out by using chemicals like glutaraldehyde, thus known as chemical fixation. These substances are used to crosslink proteins. Cryofixation is a method for the rapid freezing of samples in liquid nitrogen or liquid helium. The rinsing of the sample is carried out using buffers such as sodium cacodylate to preserve and maintain the pH as fixation can increase the acidity of the sample.

B. Secondary fixation

Osmium tetroxide is used for the secondary fixation of the sample which induces stability and gives more contrast to the microstructure of the sample. The role of osmium tetroxide is to transform the protein into the gel.

Dehydration is a process carried out to replace the water content of the sample with organic solvents such as ethanol and acetone. Infiltration is carried out in order to bear the pressure of cutting and sectioning, wherein epoxy resin is used to penetrate the cells which occupy the cell spaces and makes the cells hard enough. Resins are first polymerized by keeping them overnight at 60 °C. Polishing is carried out to reduce the presence of any scratches that may hamper the image quality. The sample is cut into fine sections by using a glass or diamond knife using an ultramicrotome device. In TEM analysis, for the passage of electrons via the sample, the sample must be semi-transparent and, thus, cut into fine samples. The ultramicrotome device has a trough filled with distilled water in which the sections are collected. Ideally, the sections must be between 30 and 60 nm to obtain the best resolution. Staining is carried out after the dehydration step and after cutting. Heavy metals like uranium, tungsten, and lead are used to scatter the electron beam and to increase the contrast between structures in the sample. After sectioning, the sample is exposed to an aqueous solution of the metals.

TEM is a characterization technique that provides an excellent resolution and is a widely used technique in basic research; however, its scope is limited in industry. Conventional TEM preparation is a tedious, time-consuming process that requires the high skills of the operator and can give unreliable results. Focused ion beam (FIB) is a technique used to produce cross-sections of specimens. However, FIB also involves pre-FIB preparation [70]. Thus, an innovative and automated solution for TEM sample preparation was developed by researchers [71]. The instrument includes a micro cleaving system (SELA MC series) involved in precision cleaving, which is 20 min long. Micro cleaving gives a resultant specimen that can be considered as a pre-FIB input sample. The sample is transferred to a SELA TEM station which conducts the pre-FIB process, which is 30 min long. The finished pre-FIB specimen has two edges (a high-quality edge and a sawed edge). The thin specimen undergoes FIB milling for 1 to 1.5 h and produces a final TEM sample [72]. The entire process is automated and requires minimal operator intervention. Researchers have also developed a microfluidic device for enhanced reliability in TEM sample preparation [73].

Commonly used specimen preparation techniques for TEM are FIB, ion milling, ultramicrotomy, and mechanical polishing. Ultramicrotomy is usually used on biological samples and it works by slicing a thin film of the specimen using a glass or diamond knife that is placed on an epoxy resin. The formed flakes are floated on water and are then collected on a film grid. However, ultramicrotomy comes with the limitations of artificial defects due to the physical stress induced by diamond knives; moreover, samples that are prone to oxidation require a suitable inert liquid medium with the appropriate surface tension to float the sliced flakes. Secondly, the material needs to be sufficiently hard and plastic enough to readily fracture the sample during cutting, and then fragment during spreading. Wen et al. proposed a mechanical polishing method to prepare TEM samples where the bulk material is prepared from the powder–binder mixture and the prepared bulk is embedded in epoxy resin. The ratio of epoxy and powder mixture is critical to achieving a uniform powder distribution. The final samples are prepared by ion milling and polishing. Both these techniques are time-consuming and dependent on the skill of the operator [74].

Kim et al. proposed a novel and simple method to prepare samples for TEM to image powders in the range of 1–5 μm by using FIB. FIB is a method to directly produce samples for TEM by using Ga ions. The method uses a solder as an embedding agent and the lift-out technique is used to prepare specimens. Solder binder is a more effective technique under an electron beam than epoxy resin and it also prevents the oxidation of the powder. This sample preparation methodology was evaluated on Nd-Fe-B powder, prepared by spray drying and reduction/diffusion processes. The $Nd_2Fe_{14}B$ compound was prone to

oxidation and the particle size of the powder was in the 1–5 µm range. Gatan G-1 epoxy and Sn-based solder were used as embedding agents. The samples were prepared using a sample-embedding procedure and conventional solder for comparative analysis using G-1 epoxy wherein a mixture was made of epoxy and powder and was poured into a copper tube and the fraction was minimized using a rod and the pressure was applied to cure the mixture, followed by cutting. For the embedding procedure using solder, the powder was placed on a movable lower ram and a plate-shaped solder, preprepared, was placed on the powder. Further, to encapsulate the powder–solder mixture, an electrically conductive sample was poured. Heat (180 °C) and pressure (30 kN) were applied for 6 min to obtain the resultant solder-embedded specimen. Mounting was followed by polishing to avoid the oxidation of the powder using SiC paper. The polished samples were imaged by TEM [75].

Although TEM is a versatile technique, it damages the sample due to the heating by the electron beam. Thereby, results obtained by DLS may differ from those obtained by TEM.

2.5. Atomic Force Microscopy (AFM)

AFM, a subset of scanning probe microscopy (SPM), is a surface characterization technique through which a large range of materials can be imaged (Figure 5). SPM involves techniques that measure surface properties. When compared to SEM, the sample does not have to be conductive which makes the technique more user-friendly, and vacuum conditions are not required. AFM delivers a unique 3D topographical image of the specimen with a high-quality resolution (Figure 6). The high sensitivity of AFM is due to a force sensor between the probe and the target surface [76]. For AFM imaging, particles must be adhered to or dispersed to the substrate by using an adhesive.

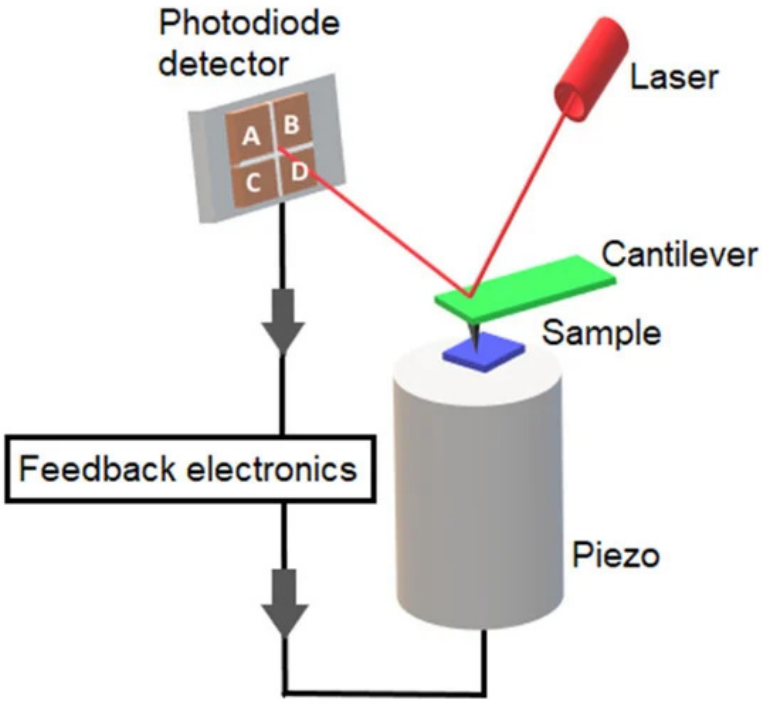

Figure 5. Schematic representation of main components of AFM. Reproduced with permission from reference [77]. 2023, MDPI.

Critical Components in AFM Sample Preparation

A. Adhesives

Adhesives are important to adhere the nanoparticles onto the substrate to resist the lateral forces exerted by the scanning tip. The affinity between the nanoparticles and

substrate must be greater than the affinity between the tip and particles. Poly-l-lysine, poly-D-lysine, and triethyoxysilanes are the most used adhesives for facilitating chemical bonds between the sample and substrate. In air-AFM, hydrophobic substrates are preferred to avoid any water monolayer formation. Hydrophilic agents can be used if imaging must be carried out in aqueous solutions.

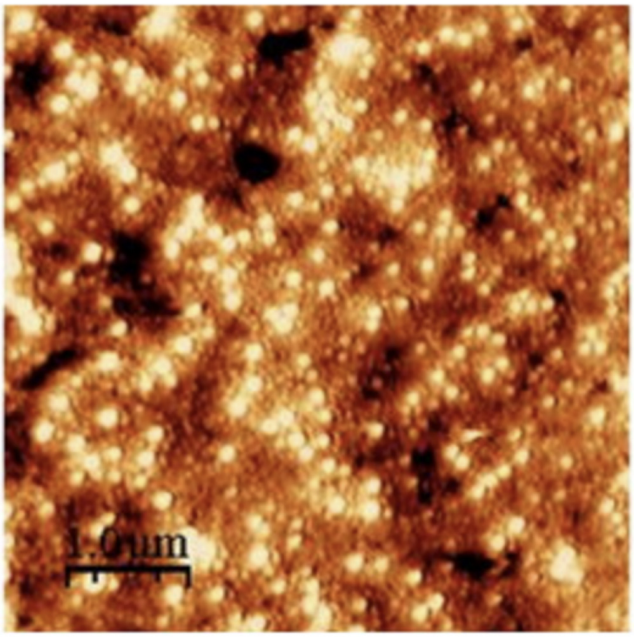

Figure 6. Representative AFM image of polyurethane nanoparticles on mica (contact mode). Reproduced with permission from reference [81]. 2008, Elsevier.

B. Substrate

Substrate selection is an important step in the imaging of samples. Glass, silicon, mica, and highly ordered pyrolytic graphite (HOPG) are excellent substrates in common practice. In AFM analysis, the substrate must be as flat or smooth and as small as the particle size of the sample. Glass as a substrate is used for imaging cells. Gold is chemically inert and is stable against free radicals and the coverslips are flat enough to carry out the imaging of cells. Glass is washed with concentrated acidic solutions to remove any contaminants or coated organic contaminants that may have adhered on its surface. Mica is a commonly used substrate for imaging biological samples like DNA, DNA–protein interactions, proteins, and lipids. Mica surfaces can also be modified with silanes to facilitate the covalent bonding to the biomolecules [78]. Mica can also be coated with carbon to yield a hydrophobic surface. The surface of the glass is too rough and silicon wafers are an alternative to them. However, silicon wafers as a substrate are much more expensive and difficult to handle. Template-stripped gold (TSG) is a method developed by Wagner for reducing the roughness of the glass surface [79]. In the case of bulky samples, substrates such as metal discs are adhered to using a carbon tap or thermal wax.

AFM characterizes individual nanoparticles as it has the required resolution but requires uniform arrays of complex nanostructures. However, a major challenge in the accurate analysis using AFM is the presence of particle agglomerates. Hoo et al. developed a method to prevent the agglomeration of nanoparticles and form a deposition on atomically flat substrates that were concrete and highly uniform. The article demonstrated spin coating for optimized size measurements by AFM and described factors that affect the spin-coating parameters. Spin coating prior to deposition was performed using an aqueous suspension of silica, polystyrene nanoparticles. Silicon wafers were used as substrates and modified by heating at 300 °C, which grows as a layer of oxide and increases the surface roughness. The procedure for spin coating comprised mounting substrates onto an AFM disk and coating

at a speed of 1000 to 5000 rpm with an increment of 500 rpm every 30 s. After this step, the solutions were deposited, and substrates were analyzed using an AFM microscope in tapping mode. The deposition of nanoparticles using a fluid cell was also attempted for comparison with spin coating and evaluated the effectiveness of accurate deposition for a better AFM analysis. It was observed that the fluid cell gives a more uniform and high-density deposition [80].

Figure 6 depicts the typical AFM image of the polyurethane nanoparticle on mica.

2.6. Scanning Tunneling Microscopy (STM)

2.6.1. Working Principle

This is a technique based on the quantum mechanical phenomenon, that resolves single atoms and produces real-space images of atoms [82]. In STM, a sharp metallic tip is scanned across the surface of the specimen (Figure 7). A finite probability of electrons can flow through this gap; the wavelike property of electrons permits them to tunnel in the space between the tip and the sample. Electrons on the surface are as tightly bound to the nucleus as the electrons present at the bulk. A small sharp tip is brought a few angstroms closer to the sample surface and is put under voltage; a tunneling current flows, which is proportional to the distance between them. STM can measure only electrically conductive samples and gives an atom-resolution image with their electronic structure. STM works in two modes—either with a constant height or with a constant current. When compared to AFM, which works for all samples and measures the deflection in the cantilever due to van der Waals forces in the tip and sample, STM has a better resolution due to the tunneling current, and individual atoms can be imaged. STM techniques work by scanning the surface at a very close distance. Highly-oriented pyrolytic graphite (HPOG) is a standard used for the STM technique [83].

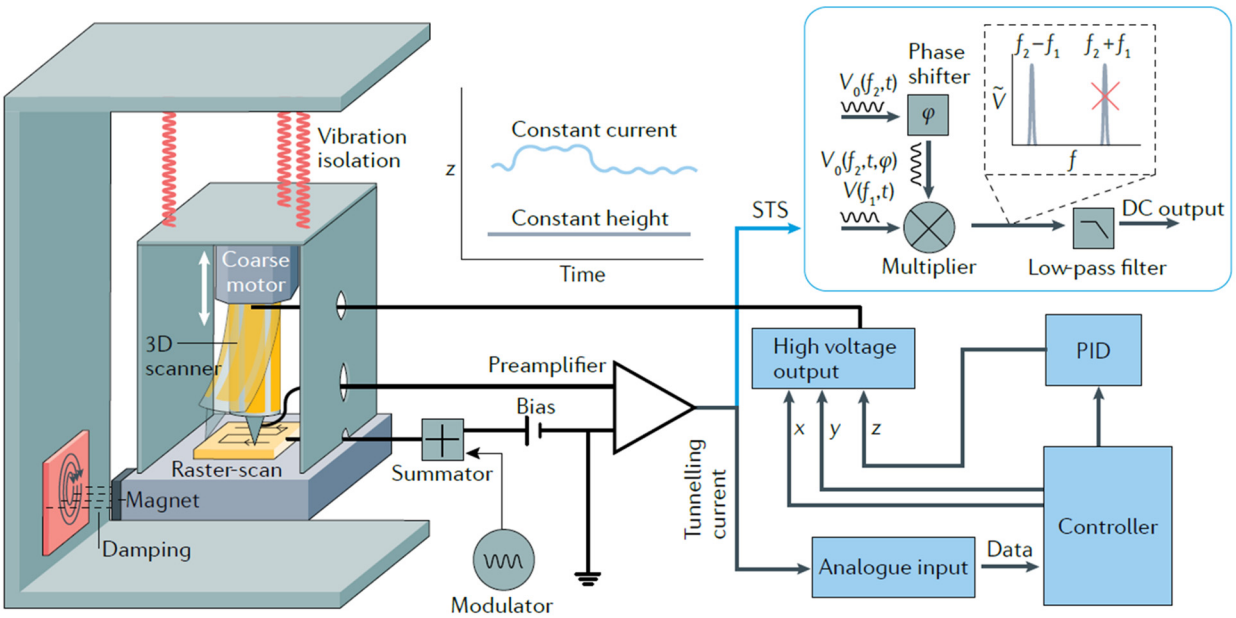

Figure 7. Scanning tunneling microscopy (STM) setup. Reproduced with permission from reference [84]. 2021, Nature.

2.6.2. Sample Preparation/Sample Installation

The sample holder should be checked for any source of contamination (dust or fingerprints) on the metal part of the holder. The metal part must be cleaned using ethanol by a cotton swab. The sample holder should then be placed on the sample holder guide bar of the STM head without touching the tip cleaving off the sample. Automated tip

conditioning for STM is a powerful tool to analyze the electronic structure of the single molecules, and the STM tips are critical for obtaining high-quality spectra [85].

XSTM is a cross-sectional STM microscopic technique, used to study the heterostructure of semiconductors III–V on an atomic level. A novel, effective in situ cleavage technique was developed for the sample preparation of cross-sectional STM (XSTM). This technique is easily adaptable to any STM system and aims to minimize the tough area that surrounds the scratch and, thereby, yield an atomically large surface. The technique was carried out on III–V semiconductor GaAs (Gallim Arsenide) sample surfaces under ultrahigh-vacuum conditions, and a 90° bent diamond tip was used to scratch the sample surface. The scratch length and depth were minimized to a few microns. After creating a scratch, the sample was transferred, and then a fracture was initiated which revealed a freshly cleaved cross-sectional surface. The SEM images were obtained to compare the in situ cleavage technique with the conventional method [86].

2.7. X-ray Diffraction (XRD)

XRD is a sensitive and non-destructive analytical technique for the microscopic elemental detection in samples and the phase identification of crystalline materials. It measures the intensity at which the crystalline matter will diffract the X-rays and the constructive interference between the X-rays and the crystalline sample when Bragg's law is satisfied [87]. The peak intensity is determined by the atom distribution in the lattice. The diffracted X-ray is detected and counted. XRD measures liquids, solid samples that have a polished surface, and thin films. Sample preparation should not alter the elemental distribution of the sample. Major components of a diffractometer are an X-ray tube, a sample holder, and an X-ray detector.

Sample Preparation Methodology for XRD

A. Grinding

Samples are cleaned, air-dried, and broken down from aggregates to a fine powder prior to the XRD analysis. The size of the sample influences the intensity and degree of randomness of the crystallites. Thus, grinding pure coarse powder is important. Coarse powder gives rise to inaccurate and imprecise intensities. Grinding can be carried out manually in an agate mortar but comes with a risk of loss due to dusting. Wet grinding is carried out with a liquid-like methanol or ethanol to reduce the loss of the sample. Mechanical grinding can be carried out by a ball mill or by using shatter boxes. Powder less than 10 μm is preferred for analysis as grinding to this size gives a very high number of crystallites in the analytical volume. RETSCHs XRD Mill McCrone is designed for the homogenization of samples prior to the XRD analysis [87]. It carries out the size reduction based on friction. It produces an optimum grain distribution and preserves the sample structure due to the gentle homogenization of samples and works on low energy input [88]. It comes with a danger of contamination due to grinding elements like ZrO_2, corundum, and quartz. The planetary ball mill and mixer mill generate a higher energy input compared to XRD Mill McCrone.

A novel method of XRD sample handling was developed to reduce the efforts for sample preparation and the characterization of coarse-grained samples. They aimed to induce random motions in the sample via sonic or ultrasonic vibration, as the rotating or rocking of samples increases the exposure of crystallites to the X-ray beam. The new method improved the quality of the data for the coarse powder analysis [89]. The powder mount made for the XRD analysis can be loaded by front loading, back loading, or slide loading. Front loading has standard holders and powder is pressed with a glass slide and the surface is roughened by a razor blade. Back loading is carried out by using Philips/Panalytical equipment. The sample is then placed in a sample holder by creating a proper flat surface. A glass slide can be used to uniformly smear the surface. Shadowing and errors arise due to the improper surface of specimen; thus, smooth and flat surfaces are necessary. Unit cell

determination can be carried out by placing a small amount of the standard. Samples are then analyzed using an X-ray diffractometer by measuring the intensity of diffracted rays.

The analysis of mineralogical samples requires random powder mounts for the differentiation of subgroups by reflections, and achieving random orientations is a limitation of XRD analysis. Zhang et al. describe a simple sample-mounting method, "Razor tamped surface" (RTS), for XRD. This mechanical technique prepares random power mounts without the need for pre-processing the dry sample powder. The procedure for RTS is as follows:

1. The standard sample holder is selected to perform the RTS method and a glass piece with dimensions greater than an empty window in the Al-holder is selected. It is covered with a double-sided sticky tape.
2. Transfer the powder onto the holder, which is premixed to avoid pre-existing orientations; the powder should be 3–5 mm deep into the cavity.
3. A sharp razor edge is used to gently chop the powder in random directions which causes the initial compaction and uniform packing in the cavity. The movement of the blade is kept vertical to avoid a shearing motion on the surface of the holder.
4. The powder is tamped in a horizontal motion path from the center to either direction by pushing the blade up and down at a 30–45 angle to vertical. This helps to remove any excess powder from the cavity.
5. Air is blown across the surface of the holder to remove a loose surplus. The loose surplus should not be removed with a razor blade.
6. The steps must be repeated multiple times until a flat and smooth powder mount is obtained [89].

2.8. Inductively Coupled Mass Spectroscopy (ICP-MS)

ICP-MS is an analytical technique that is a combination of an ion-generating plasma source with mass spectroscopy and is used for trace elemental detection in biological fluids and to determine isotopic concentrations in samples [90]. ICP-MS is capable of multi-element detection present in low concentrations as minute as nanograms per liter. This technique has a higher speed, sensitivity, and precision when compared with atomic absorption spectrometry. ICP-MS uses an inductively coupled plasma to ionize the sample and the generated polyatomic ions are detected by a mass spectrometer. Isotopic labelling can also be carried out via ICP-MS. ICP-MS primarily detects liquids or gases. Gases can be analyzed directly by plasma, whereas liquids must be aerosolized using a nebulizer and by using an ablation device for solids. Sample tubes are to be filled with nitric acid and heated at 60 °C. Samples are rinsed with distilled water and stored with the same. Before use, the water is discarded and the samples are dried. Samples should be delivered in 12–15 mL tubes. As ICP-MS detects minute concentrations, the collection and storage of samples are critical as contamination can occur. When samples are stored, glassware should be avoided to minimize the risk of leaching impurities, and, if plastic containers are used, teflon is preferred.

2.8.1. Sample Preparation for Liquids

Samples are diluted or thermally digested using concentrated trace-metal-grade HNO_3. The sample is to be heated for 2 h at 90 °C. If particles remain, trace-metal-grade hydrogen peroxide can be added and can be further heated for a few hours. Liquid samples are then diluted in an aqueous matrix to obtain a final acid strength of 2–5%. The addition to an aqueous matrix stabilizes the elements as ionic solutions. If the amount of total dissolved solids (TDS) is less than 0.2%, then liquids can be directly analyzed without any dissolution by using a standard ICP-MS kit. Organic samples can be used and additions like oxygen must be carried out on the plasma to prevent the deposition of carbon or any interference in signals. Nitric acid digestion is usually sufficient to digest most of the samples. If samples have elements like gold or platinum, aqua regia is used.

2.8.2. Sample Preparation for Solids

Samples must be homogenously dried. Solids are ground to a fine powder and passed through a sieve. The first sample is discarded to avoid contamination from the mortar and pestle. Strong acids ranging from hydrochloric acid, hydrofluoric acid, and chloric acid can be used for digestion if the sample has a higher content of organic matter or a high silicon dioxide content. Hydrogen peroxide is efficient in breaking down organic matter into CO_2 and NO. Laser ablation (LA), electrothermal vaporization (ETV), and spark ablations are methods used to convert samples into aerosols. LA is a microanalytical technique that identifies small spots on solid samples by using high-irradiance UV lasers. The ETV method is a bulk method and interacts with the complete sample. Spark ablation identifies spots on a conductive sample and can be considered a semi-bulk interactive technique. Digestion is usually performed in a hot water bath or high-pressure microwave.

2.8.3. Sample Introduction

The instrument has a sampling interface, a pump leading to a nebulizer, a spray chamber, a plasma torch, and a detector. Solid pneumatic nebulizers are commonly used to generate aerosols. Internal standards like germanium are added to neutralize the matrix effects. The sample, after becoming nebulized, enters the spray chamber that filters out larger aerosol droplets. Smaller droplets are moved to a plasma torch where they are ionized. Plasma is inefficient in dissociating larger droplets; thus, filtering out large droplets is important. The ionized ions are separated in the mass detector.

ICP-MS, a commercial technique used for trace elemental detection, has limited acceptance owing to the barrier faced due to the high matrix levels encountered in samples. As ICP-MS uses an interface (plate or cones) for the separation and detection of ions into a high-vacuum region, samples with a high TSC (total solid content) are deposited in the interface's orifice and have an impact on the overall signal and lead to instability. A high-matrix elemental concentration affects sample transportation and causes matrix suppression and hampers sensitivity. Thus, a newer approach to sample handling was reported—"Aerosol dilution"—which eliminated manual sample handling and allowed the sample analysis with high levels of the matrix of up to 25% salt, that surpasses a higher than accepted maximum level. The standard ICP-MS sample introduction hardware is retained but an additional argon gas stream is introduced between the spray chamber and torch that dilutes the aerosol sample. Thus, the amount of the sample aerosol reaching the plasma was reduced by dilution, further reducing plasma loading and resulting in more energy remaining for the decomposition of the matrix and the ionization of the analyte. The method was compared using matrix samples with variable concentrations ranging from 0% to 25% NaCl and their relative intensities for internal standards. The accurate spike recovery of elements demonstrated that aerosol dilution was effective in minimizing the impact of ionization [91].

Jantzi et al. reported a novel sample preparation strategy, "filter pellet", for a quantitative analysis of filter-bound sediments. Sediment fingerprinting is a common application of LA-ICP-MS for the source determination of sediments to identify soil erosion. The filter pellet is a green chemistry approach for the same. The technique requires around 50 mg of sediment to homogenize and pelletize the specimens. The sediments bound to the filters are processed into pellets using LA-ICP-MS. The conventional technique requires 0.1 g of sediment on a cellulose nitrate membrane filter. The performance of pellets by this technique was similar to that of the conventional technique and the quantitative data were generated without the need for acid digestion [92].

2.9. X-ray Photon Electron Spectroscopy (XPS) or Electron Spectroscopy for Chemical Analysis (ESCA)

ESCA is a quantitative surface-sensitive technique based on the photoelectric effect to identify the elemental composition within the sample or on the surface of the specimen. XPS is an important technique that gives qualitative information about the nature, composition,

presence of contamination, and elemental state of the nanoparticle. The uniformity of the elemental composition can also be determined before and after sample preparation, which involves sample etching, finishing, or the coating of surfaces. Figure 8 represents the main components of XPS.

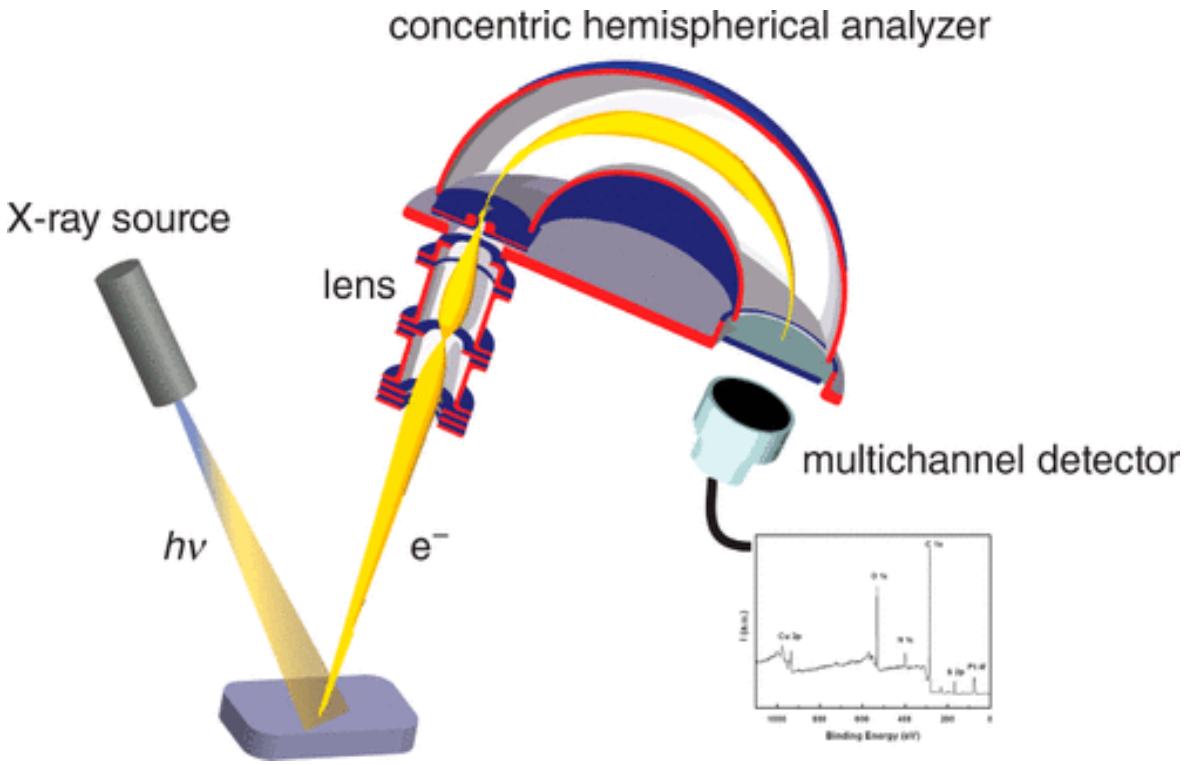

Figure 8. Schematic representation for X-ray photon electron spectroscopy. Reproduced with permission from reference [93]. 2013, The American Chemical Society and Division of Chemical Education, Inc.

2.9.1. Working Principle

The surface of the specimen is irradiated with soft X-rays. Photons interact with the atoms in the surface region and cause the emission of electrons. Emitted electrons have kinetic energy given by an equation: KE = hv − BE − Q [94]. Every element has a unique binding energy. A spectrum is plotted of electrons detected per energy interval vs. kinetic energy.

2.9.2. Sample Preparation for XPS

The sample must be protected from contamination as this can affect the sensitivity. Sample storage is important and samples must be stored in appropriate glassware or fluoroware, or properly wrapped using aluminum film. Plastic samples should be avoided as they may contain PDMS (polydimethylsiloxane). The fingerprint region should not be contaminated with gloves or with tweezers, and, preferably, nitrile gloves should be used. The tools must be cleaned with solvents like methanol. The size reduction of the sample is carried out by using serrated-blade scissors or a diamond saw that is lubricated with deionized water. If the sample is porous or polymer-based, it may be incorporated with volatile components. Thus, it is recommended that volatile components must be removed by pumping in a separate vacuum system. If, in some cases, the volatile layer is of interest, then the sample can be cooled to lower temperatures. If the sample has any organic impurities, the appropriate organic solvents can be used to eliminate the same. The etching of the sample surface is carried out in order to sputter clean the surface, whereas instruments are used. Ion-sputtering techniques or erosion techniques that use oxygen

plasma on organic materials are used to remove any surface contaminants. This is used when the surface has adventitious carbon or any other contaminant. Sputtering can be carried out with Ar sources to obtain information on the composition as a function of exposure time. Ar etching is carried out particularly for pure metal surfaces to produce clean surfaces. Sputtering is also carried out to obtain an XPS depth profile. Other sources other than Ar include C_{60}, which also provides depth profiles, and they remove adventitious carbons [95].

3. Conclusions

Ultimately, the ongoing development of advanced techniques for analyzing nanomaterials is leading to an improved accuracy and broader functionalities. SEM is still crucial for achieving high-resolution images. Recent developments, such as environmental SEM (ESEM) and the "Nanosuit" technology, have made it possible to image living material without the need for drying. Cryo-SEM is a technique that allows for the high-resolution imaging of objects in their hydrated state, without any distortions or artifacts. This method utilizes advanced technology including controlled environment vitrification equipment and cryo-ultramicrotomy. DLS is a technique used to determine the size and aggregation of nanoparticles in a solution. Recent advancements, such as Nano lab 3D for measuring high concentrations and microfluidic devices for quick analysis, have enhanced the accuracy and efficiency of DLS. XRD is a method that allows for the identification of phases and the detection of elements. Recent breakthroughs in this field include the use of automated micro cleaving devices and FIB techniques, which enable more accurate sample preparation. TEM provides high-resolution pictures of thin materials, utilizing automated sample preparation and microfluidic devices to improve accuracy. AFM is used to capture high-resolution pictures of the surface topography. These images are enhanced using spin coating and microfluidic devices, which ensure a uniform deposition of nanoparticles. STM allows for the imaging of surfaces at the atomic level. It now includes automated tip conditioning to improve the quality of spectra. ICP-MS is highly effective in detecting trace elements. It utilizes techniques such as aerosol dilution for samples with a high matrix content and the filter pellet approach for analyzing sediments. XPS/ESCA is a technique used to examine the elemental composition of a surface. Recent improvements in sputtering, etching, and sample storage procedures have increased the precision and dependability of this analysis. Together, these developments enhance the precision of methodologies used to analyze nanoparticles, pushing the research forward and expanding the range of possible uses for nanomaterials.

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