Drug Delivery & Formulations

Atomic Force Microscopy in the Characterisation of Drug Nanoparticles

With nanoparticles becoming more widely used in drug delivery, atomic force microscopy (AFM) provides a convenient, easy-to-use method of nanoparticle sizing and characterisation.

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Pharmaceutical nanoparticles are being extensively used for drug delivery systems. They have many significant advantages over conventional methods such as applications in controlled and targeted delivery, providing efficient administration performance and enhanced bioavailability. Pharmaceutical nanoparticles come in different forms such as liposomes, microemulsions and colloidal crystals. While their use continues to increase, the need for characterisation at the nano scale is becoming more and more challenging. Conventional particle sizing methods may no longer be up to the task. This may be because of a limitation in particle size range, or be caused by a chemical or physical change in the particles, for example, agglomeration.

This article will discuss how atomic force microscopy (AFM) can be used to successfully overcome such problems, and provide a convenient, easy-to-use method of nanoparticle sizing and characterisation. It will introduce examples of how AFM may be used to characterise nanoparticles, and provide a background into the technology and its development over the past twenty years into a powerful metrology tool. It will also consider the challenges of sample preparation and provide insight into new ways of mounting nanoparticles for convenient study and measurement.

WHAT IS AFM?

AFM is the most common of the family of techniques known as scanning probe microscopes (SPM), and is now routinely used for metrology and surface characterisation of a multitude of materials. Invented in 1986 by Binnig, Quate and Gerber (1), AFM has developed beyond being a tool to produce high-resolution topographic information to one which is applied to many scientific disciplines, from semiconductors to the life sciences. While most measurements are made in air, systems are available enabling the user to study samples in different environments – from liquids to gases, from ambient to high vacuum.

Like all other SPM techniques, AFM uses a sharp probe (usually made from silicon or silicon nitride) moving over the surface of a sample in a raster scan. The tip is at the end of a cantilever which bends in response to the change of force between the tip and the sample. Most AFMs use an optical lever technique to detect flexure of the cantilever. A laser is focused onto the back of the cantilever and is reflected onto a four-quadrant photo detector. This enables topography to be measured by the up-down movement of the cantilever, while frictional or lateral forces may be measured by following the twisting of the cantilever (Figure 1).

Recently, a new force sensor, based on a crystal resonator, has shown promise for making AFM much simpler to operate (2,3); it provides the very high force sensitivity which is required for high resolution imaging. There are many different modes of AFM. Topographic modes use the force interaction between tip and sample. In contact mode, the tip is working in the repulsive region very close to the surface when the cantilever is pushed away from the surface. In the various non-contact modes, the cantilever is oscillated at its resonant frequency and is attracted to the surface. A change of amplitude or shift in frequency may be monitored to measure the force interactions.
Specific properties may also be measured using AFM. For example, the tip may be coated with an appropriate metal to enable properties such as magnetic or electrostatic field changes emanating from the sample surface to be measured. Again, vibrating techniques are used yielding simultaneous spatial topographic and materials properties. Nanoparticle characterisation requires a very sharp tip; a diameter of 20nm or less is ideal.

SAMPLE PREPARATION
To use the AFM, the particles need to be anchored to a flat surface in a way that does not change any of their properties. They should retain their morphology and ideally remain chemically active when studied at the nano scale.

Particles may be categorised in one of two groups: engineered and non-engineered. Engineered or artificially created particles break down to organic and inorganic categories; these in turn have additional subdivisions, for example powders, suspensions and embedded particles. There are many different approaches available in sample preparation. It is important to take into account the particle’s size, hydrophobicity and biocompatibility as well as the environment for the measurement.

There are three general rules to follow when using an AFM to image particles:
1. The particles must be rigidly adhered to a flat substrate
2. The particles must be uniformly dispersed on the substrate
3. The substrate roughness must be less than the size of the nanoparticles

Often an adhesive is required for affixing the nanoparticles to the substrate. There are a large number of choices of adhesive for small particle deposition. The most commonly used chemicals are poly-L-lysine, poly-D-lysine, PEI (polyethyleneimide) and APTES (aminopropyltriethoxy silane). These facilitate chemical bonding between particle and substrate. Functionalised surfaces may be used to either promote adsorption or allow covalent bonding. Sometimes, hydrophobic substrates are preferable for biological applications, for example, when imaging DNA and proteins. Glass, mica and silicon work very well for materials such as bio-cells, colloids, quantum dots and carbon nanotubes. Sometimes polymer membranes, filters or macromolecular gels may be used to immobilise larger particles.

IMAGING NANOPARTICLES WITH AFM
In a previous publication (5), the authors provided a comprehensive review of the numerous combinations of nanoparticles, substrates and adhesives that have been demonstrated to successfully work with AFM. In this review, focus has been kept to materials relevant to the pharmaceutical industry, from depositing simple particles for imaging in air to the stable immobilisation of bio-particles for imaging in liquid.

Imaging in Air
Many particles are produced or distributed as dry powders. The most commonly used substrates for ultra-fine powder deposition are glass slides, HOPG and mica. In order to increase the adhesive requires experimentation. There are several competing factors influencing this process. On a large scale, exposure time and dilution of the particle solution must be considered. On a smaller scale, the interfacial free energy and electrostatic energy associated with the nanoparticles tend to cause them to clump together or keep them far apart. On the other hand, hydrophilic-hydrophobic forces interacting between particles, substrate and the solution can cause agglomeration and coalescence. Care must be taken in the choice of additives and surfactants in the dispersion process as these may change particle properties during and after evaporation.

The choice of substrate is critical. The size of the particles must be greater than the topographical features of the substrate. The most commonly used substrates include glass cover slips, mica, HOPG (graphite), silicon oxide wafers and atomically flat gold. Atomically flat substrates are preferred for biological applications, for example, when imaging DNA and proteins. Glass, mica and silicon work very well for materials such as bio-cells, colloids, quantum dots and carbon nanotubes. Sometimes polymer membranes, filters or macromolecular gels may be used to immobilise larger particles.
properties of the substrate, poly-D-lysine is deposited on the substrate’s surface. Once a substrate is chemically treated and dry, it is immediately ready for powder deposition. Powder distribution is achieved by dusting a small amount of powder over the entire area of the substrate, and setting it aside for a few minutes. The substrate is then flipped over to remove any large agglomerates of particles (Figure 2, page 75).

Dry adsorption works very well for superfine powders with a particle size of less than 150nm, however, deposition rate and density of deposition are two of the challenges associated with this method.

If the granular size is larger than 500nm, a different method is used. A polished metal disc works well as a substrate with thermal wax as the anchoring medium. Wax is placed on the metal disk which is warmed up on a heating element until the wax softens (~60-70°C) to form a visible liquid interface on the surface. The disc is then removed from the heater, and as the surface starts to solidify, powder is sprinkled over the sample area. The sample is ready for AFM imaging when the thermal wax becomes solid and the metal disk is at room temperature – typically after 10 to 15 minutes. Experimentation is often required to obtain the optimal particle surface density. The depth of the embedding depends on particle weight and size, as well as the temperature of the thermal wax (Figure 3, page 75).

Droplet-evaporation or adsorption methods may be used for preparing AFM samples from liquid suspensions (Figure 4). A droplet of liquid is deposited on freshly cleaved mica or a poly-L-lysine covered slide. The droplet is then carefully washed after allowing the sample to sit for about 10 minutes. To dry the sample before scanning, it is left overnight in a dust-protected environment, or a furnace/heater is used to accelerate the drying process.

When studying bio-particles, the correct sample preparation techniques are extremely critical, because an immobilized specimen may degrade during sample preparation or imaging. Once again, requirements for substrate flatness, chemical compatibility and reagent purity are rigorous. Surface charges, surface energy and hydrophobicity play very important roles in selecting the optimal sample preparation method. These include absorption, replication and mechanical trapping (Figure 5).

Physisorption (physical adsorption) or non-covalent methods – such as aerosol-spray deposition, immersion and droplet-evaporation – are achieved by adsorbing biological cells onto highly negatively charged mica. Additional chemical treatment – such as functionalising by salinisation – can be used to facilitate stronger bonding on the surface of the biological specimen. Tight affinity to the substrate is mandatory for successful AFM imaging. The downside of the non-covalent absorption method is that it could cause undesired re-arrangements of bacterial cells. If displacement or distortions are critical, or if the molecular object has to be integrated into a complex molecular assembly, then covalent methodologies should be used.

Sometimes fixing with glutaraldehyde is necessary to minimise tip-particle interaction, and to prevent possible damage of the biological sample. Studies show that mechanical trapping of biological objects in a membrane filter appears to be the most reliable method to measure surface topography.

Embedded nanoparticles may be visualised using physical imaging techniques such as vibrating phase and frictional force microscopy, also known as lateral force microscopy (LFM) (6).

Sample preparation from suspension is perhaps the most common way to prepare samples for AFM imaging, such as the study of drug crystallites (Figure 6).

**Imaging in Liquid**

AFM is a proven tool to identify topographical features of particles submerged in liquid. Applications include the study of soft polymers and bio-particles (cells, membranes, viruses). The imaging solution is chosen such that neither particle nor adhesive will dissolve during the experiment.

An important advantage of AFM over other microscopy techniques is the ability to image biological samples in a native aqueous environment. AFM offers the possibility of in vivo study of the dynamics of biological changes in living cells, viruses and micro-molecular crystals. Imaging in liquid with an AFM requires a stable immobilisation of biological specimens. Absorption on a polycationic treated surface or on an agarose coating provide stable fixation for experiments in liquid. The absorption of specimens directly from the buffer solution can be controlled by the electrolyte concentration and pH of the buffer solution. Glutaraldehyde fixing may be necessary for some bio-AFM samples; the fixing agent is applied after absorption. In fact, fixation destroys molecular functionality and can affect true structure. Hydrophobic substrates and bio-incompatible agents are not recommended for solution-based measurements and should be avoided.

Sometimes, it is desirable to image particles embedded in a solid medium, bio-tissue or polymer thin film. In the case of relatively soft materials like organic
tissue or soft polymers, it is important to cross-section the specimen, making a very smooth, clean cut. A microtome is typically used to produce sub 0.5 micron slices that are suitable for AFM imaging (Figure 7). The microtome-prepared slices must be firmly attached to a substrate before imaging. Chemical etching of semi-thin sections of an epoxy-resin embedded specimen is a very good technique for visualising embedded particles with an AFM. Being able to scan with an AFM in this case depends on sample preparation before the particles are embedded in the substrate.

Thin films can be spin-coated on silicon or glass substrates and then examined with an AFM with no additional treatment. If a composite material is the subject of investigation and the components have dramatically different elastic, adhesive or frictional properties, then material contrast using vibrating phase modes may be used. Vibrating phase imaging can provide unique information about local materials distribution in composites and thin films (Figure 8).

**CONCLUSION**

Speeding up the process of obtaining nanoparticle size data is critical for many pharmaceutical applications. In general, using AFM for particle size characterisation is both cost- and time-effective. AFM resolution is greater or comparable with traditional techniques – but the main advantage of AFM for particle characterisation is its capabilities for unambiguous morphology determination and metrology measurement.

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**References**

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