Experimental Nanobiotechnology

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Lecture 10: Electron Microscopy

Hello everyone, today we are going to learn about electron microscopy. Electron microscopy is one of the important techniques in nanomaterial characterization. In today's lecture, we will learn about the theory and principles of scanning electron microscopy and transmission electron microscopy. Through practical demonstration, we will also learn this technique in more detail. Let us see what scanning electron microscopy is.

Scanning electron microscopy is an imaging technique that uses a focused beam of electrons to provide high-resolution images of the surface morphology of materials. SEM is widely utilized in fields like material science, biological science, and nanotechnology for material characterization. These are the various parts of a scanning electron microscope. The first one is the electron gun. That is the source of electrons.

Next, we have the condenser lens, which focuses the electron beam. We have the objective lens, which further narrows the beam to focus on the sample. We have the scanning coil, which moves the beam across the sample. And we have the sample chamber, where the sample has to be placed under vacuum conditions. Once the signal is generated from the sample, it will be detected by the detectors.

So we have three detectors. One is a backscattered electron detector, an X-ray detector, and a secondary electron detector. The signals will be amplified, and the image will be generated. So let us see the working principle of a scanning electron microscope. The scanning electron microscope, that is SEM, utilizes a focused beam of high-energy electrons and has a magnification of 20 to 1 million times.

The principle is that electrons interact with atoms on the sample surface. This generates various signals such as secondary electrons, backscattered electrons, and X-rays. The detectors collect these signals generated from the sample and provide information about the sample. For example, it provides information like surface topology, composition, and

physical properties of your sample. How do we get an image from the scanning electron microscope?

Here, the first step is the electron beam is generated from the electron gun, and the beam is focused on the sample by electromagnetic lenses. The beam scans the sample, and the signals, that is, electrons emitted from the sample, will be detected by the detector. And the image will be constructed on the screen. So it will scan the complete sample, and finally, you will get the image that will be generated on your computer using the software. So let us see what the various types of reflected electrons are and what kind of information we can get from these reflected electrons.

The first one is secondary electrons. From the secondary electrons, we can get information like surface topography and fine surface details. And from the backscattered electrons, we get the atomic number contrast and material composition of the sample. The third one is X-rays. By using that, we get the elemental composition and elemental mapping.

So let us see this in detail one by one. So let us see what EDX is. EDX is Energy Dispersive X-ray Spectroscopy. EDX is a technique used for the elemental analysis of a sample. So what is the principle?

The principle is that X-rays are emitted due to the ionization of the sample upon interaction with the electron beam. And these X-rays are unique to each element. This will help us identify and quantify each element present in the particular selected area of the sample. For example, in this picture, If this area is selected, we can understand what different elements are present in this particular selected area.

In this case we can see that carbon which is 70 percentage weight and oxygen which is around 29 percentage by weight. By this technique we can understand the elemental composition of the particular sample in the selected area. and next one is elemental mapping this elemental mapping is a technique used to visualize the spatial distribution of elements within the sample in this case we can select the particular area in the sample or we can the complete sample can also be analyzed for the spatial distribution of various elements within the sample in this eds data we can understand the elements are represented in the color coded maps

For example here, the red color denotes the carbon and this yellow color denotes the nitrogen and the green color denotes the oxygen. So, this will give you an overall idea about the what are the various elements present in the particular sample. Let us see the

difference between the SEM and FESEM. The main difference is the electron source. In the scanning electron microscope, we will be using the thermionic electron gun which is the hot emission.

In the FESEM, we will be using the field emission gun This is a cold emission. And in the FESEM, we get the higher resolution, but it requires ultra high vacuum. Whenever we are using these biological samples or non-conductive samples, we get poor resolution and sometimes the sample may get damaged. So how to overcome that?

We can overcome that by sputtering of conductive materials. How we can do that? We can do the sputtering of conductive materials like gold, which will enhance the image quality. How it is increasing the image quality? It will increase the image quality by increasing the conductivity.

So once the conductivity is increased, the secondary electron signals are emitted more efficiently, which results in sharper and higher resolution images with improved contrast. And this fine grain structure of coatings provides a smooth and uniform layer which will enhance the fine details of the sample surface. In case if you are using a non-conductive samples, these coatings create a conductive path for excess electrons to dissipate. So that will reduce the sample surface charging and it will improve the image stability.

So let us see the difference between the uncoated and gold coated sample. So this is a non-conductive sample. When you are using the uncoated sample, you can see that the resolution is poor and the sample get damaged. And when you are using the uncoated sample and if you are using the low voltage or low vacuum, the resolution is still poor. The same sample when you are coating with the gold, you can see that you get a better resolution and you get a better quality images.

So that is the advantage of this gold sputtering on the samples. Let us see some of the important factors for conductive sputtering. The first one is the coating material. We can use gold, platinum, silver, or chromium, depending on your sample. Gold is the popular choice because of its chemical inertness and high conductivity.

The thickness of the coating also plays a very important role. Usually, it should be between 2 to 20 nanometers, and it depends on the sample as well as your desired resolution. The grain size of the gold coating should be in the range of 8 to 12 nanometers. If you use a lower sputtering current, it can produce smaller grain sizes and denser layers, and high

vacuum results in finer grains. So these are the parameters we have to follow when doing conductive sputtering to get a better image.

Let us see the relation between voltage and image quality. Whenever you use a higher voltage, you get better resolution. But there is a chance your sample may get damaged if you are using some biological or polymeric samples. At lower voltage, it reduces sample charging and also reduces damage. But again, the resolution will be very low.

We have to use the optimal voltage settings to improve the image quality. Let us see some of the challenges or problems in the scanning electron microscope technique and how to overcome them with some troubleshooting tips. The first problem is charging of the sample. Charging means the accumulation of electric charge on the surface of non-conductive samples when the sample is exposed to a high-energy electron beam. These non-conductive materials cannot dissipate the electrons efficiently.

So, that leads to charge buildup. This leads to image artifacts or bright spots on your image. So, how to overcome this? By applying a thin conductive coating, you can use gold or palladium, or you can use the low-vacuum SEM mode, or you can reduce the beam voltage. By doing that, we can overcome the charging of the sample.

From this picture, we can clearly understand that when excessive charging is used, the sample gets damaged. So, excessive charging on the polymeric nanoparticles causes damage and burning of the sample. The next important challenge is moisture in the sample. If there is moisture in the sample, it will evaporate under the vacuum conditions of SEM, leading to physical changes such as shrinkage or deformation. The interaction of water molecules with the electron beam can disrupt the path of electrons, leading to unstable focus and poor image quality.

And this moisture can also result in additional charge accumulation due to interaction with the electrons, especially in the case of non-conductive samples. And poor vacuum conditions arising due to water evaporation can scatter electrons, reducing the focus of the electron beam. How to overcome this? We can dry the sample thoroughly using an IR lamp or in a hot air oven, or you can store the samples in a desiccator. From this picture, we can clearly understand that the moisture in the sample causes a change in the surface morphology of the sample while imaging. Let us learn how to prepare a sample for SEM analysis.

The first step is to clean the stub. We clean the surface of the stub using isopropanol or ethanol. So, this is a stub. We have to clean it with isopropanol or ethanol and apply conductive tape over the stub. If it is a liquid sample, cut a glass slide into one-centimeter pieces, stick the glass slide using conductive tape on the stub.

You can add the nanoparticle solution on top of that. You can add a few drops of the sample on the glass slide, then irradiate the sample under an infrared lamp for drying. Once the sample is dried, keep the dried sample in a desiccator to avoid moisture. Then coat the sample with gold or other conductive materials to get a better image. And these are the gold coating parameters; we have to maintain these parameters to get better-quality images.

The next one is a solid sample. If you are using a solid or powder sample, the dried powder sample can be placed on the conductive tape using a spatula. It has to be evenly spread over the conductive tape. Then, remove the excess powder by blowing air over the sample. After that, coat the sample with gold or other conductive material. When performing this second step, avoid pressing the sample with the spatula to prevent disturbing its original morphology. The third step is, if you are using a solid whole sample, you can stick it on the stub using conductive tape and coat the sample with gold or other conductive material. Once the gold-coated sample is ready, place the prepared stub in the stub holder. Insert the stub holder into the SEM machine and start imaging.

You will get this kind of final image. This is a mammalian cell. You will get this kind of image in the scanning electron microscope. I hope you understood the basics and theory of the scanning electron microscope. The next technique is transmission electron microscopy.

Transmission electron microscopy is a powerful technique that uses a beam of high-energy electrons transmitted through an ultra-thin sample to produce detailed images. Here, the electrons are transmitted through the sample. That is why it is called a transmission electron microscope. TEM provides near-atomic resolution and is very useful for understanding structures at the nanoscale. It also provides information about the size, shape, morphology, crystallographic structure, and composition of the material. Now, let us see the various parts of TEM.

The first one is the electron gun, which is the source of electrons. In the TEM, we will be using a thermionic or field emission gun. The next one is the condenser lens, which will focus the electron beam onto the sample. Then we have the specimen holder, which holds the thin sample in place under vacuum conditions. Next, we have the objective lens, which forms the initial image by further focusing the electron beam.

Then we have the projection lenses, which magnify the transmitted image before it reaches the detector, and we have the imaging detector to capture the image. We also have the spectroscopy detector for elemental analysis. Let us see the working principle of the TEM. Here, the electron gun generates high-energy electrons, and the electron beam passes through the thin sample. Then, the electrons are either transmitted through, scattered, or absorbed by the sample.

The magnetic lenses focus the transmitted electrons onto a phosphor screen or a CCD camera to generate the image. Thicker or denser areas scatter more electrons and appear darker. From this picture, you can clearly understand: this is the core-shell nanofiber; this is the core, and this is the shell. The core is loaded with the anti-cancer drug. That is why the core appears darker when compared to the shell. Let us understand the diffraction patterns.

That is, SAED is a selected area electron diffraction. SAED is a crystallographic technique used to analyze the crystal structure of a selected area of the sample. These patterns provide information about the atomic arrangement and orientation of the material. These include spot patterns. If your sample is a single crystal, you get spot patterns.

If your sample is polycrystalline, you get ring patterns. If the sample is amorphous, you get diffuse halos. So, let us see the diffraction pattern with some examples. The first one is crystalline materials. The diffraction pattern consists of distinct spots.

These spots correspond to specific crystallographic planes in the sample. The arrangement and symmetry of the spots reveal the crystal symmetry and orientation of the sample. For example, in the case of single-crystal gold, you get this kind of distinct spots. Let us see the next example: polycrystalline material. When the material is made up of many small crystallites, it produces a concentric ring pattern.

The sharper and more distinct the rings, the larger the grains. The diffuse rings indicate smaller grains or amorphous material. The spacing between the rings provides information about the distance between the atomic planes, which can be used to determine the lattice parameters, such as edge length and edge angles. If you have a single crystal, you will see this kind of dotted pattern. If you have a polycrystalline material, you will see this kind of ring pattern.

This is an example of polycrystalline aluminum. If you zoom in on this image, you will see this kind of dotted pattern. The next example is amorphous material. For amorphous or

non-crystalline materials, a broad, diffused halo in the diffraction pattern will be observed. The halo depicts the lack of long-range order in the material's structure.

From this picture, you can see that this is amorphous quartz glass. You can see the diffused ring pattern. Now, let us discuss some common problems encountered while performing TEM and how to overcome them. The first one is sample drift. During imaging, the sample may shift due to thermal or mechanical instability, resulting in a blurred image.

The second reason may be contamination. Hydrocarbon deposition from the vacuum system can contaminate the sample. The third one is radiation damage. The high energy electron beam can damage the biological or some of the sensitive samples. The fourth one is the charging effects.

If you are using a non-conductive samples, accumulate more amount of charges which leads to artifacts and poor image quality. The fifth one is the vacuum leaks. If the TEM operates under high vacuum, there is a chance the vacuum can leak and lead to poor image quality. So how to overcome these problems? If there is a contamination, clean the sample holder and vacuum system regularly.

use the plasma cleaning to remove the hydrocarbons if there is a damage due to radiation lower the beam intensity or if you're using a very sensitive sample temperature sample you can go for cryo tem for the sensitive samples and to overcome the charging effects we have to coat the sample with the thin conductive layer to overcome the vacuum leaks we have to regularly inspect the o-rings and vacuum seals in the grid holder and to overcome the sample drift We have to allow time for thermal stabilization or we can use the software and correct the drift in the software.

If it is a low-contrast sample, such as some biological samples or polymeric nanomaterial samples, to overcome the low contrast, we have to stain the sample with heavy metal salts. For example, we have to use uranyl acetate or phosphotungstic acid to enhance the contrast. Here, you can see this is the negative staining of a dendrimer nanocarrier. So, negative staining means you will stain the background, and you can see the nanoparticle. So, let us see the difference between TEM and HRTEM.

So, the main difference is the resolution. In HRTEM, which is the high-resolution transmission electron microscope, you will get sub-angstrom resolution. And you get ultra-high magnification up to several million times, but you need ultra-high vacuum for this atomic resolution. And this HRTEM plays a very important role in nanomaterial

characterization, especially if you are using small-size carbon dots or nanoclusters; the HRTEM will be very useful.

Let us see how to prepare the sample for TEM analysis. Once you prepare the nanoparticle, dilute it in the appropriate solvent, then drop-cast it onto a TEM grid. The TEM grid is placed on a filter paper, and you can add a drop of your nanoparticle solution. Then, you have to dry it overnight in a desiccator. The excess nanoparticle solution can be absorbed by the filter paper.

This is called drop casting method and this is a TEM sample grid. Here the TEM grid will have a layers The first layer is the carbon, the next one is formvar and the bottom is the your grid. The thin carbon layer on the top of theformvar layer of copper grid, it will dissipate the excess electrons and stabilize the sample. And this carbon has a low electron scatter, so it does not interfere with the material image and which will help in the getting the better image.

Let us see how to prepare biological sample for the tem analysis. The first step is fixation. The biological sample should be fixed using glutaraldehyde or paraformaldehyde. Then, followed by that, we have to dehydrate it using ethanol or acetone solution. Then, you have to embed the sample using the suitable resin.

Followed by that, we have to do the sectioning. That means, you have to make ultra-thin sections using the ultra-microtome. Followed by that, you have to do the staining. So, as I told earlier, biological samples or some of the polymeric samples, we have to stain with the heavy metal strains such as uranyl acetate or lead citrate to improve the contrast. So, this is called negative staining.

That means it stains the background so that you can get better quality images. I hope you understood SEM and TEM. Before we go to the practical demonstration, let us briefly understand the difference between SEM and TEM. The operating voltage in SEM is 0.5 to 30 kV, and in TEM, it is 80 to 300 kV. Here in SEM, we get a resolution of 1 to 10 nanometers.

In TEM, we can get 0.5 nanometer to sub-angstrom resolution. The application in SEM is useful for surface morphology and material composition detection. In the case of TEM, we can get structural and crystallographic analysis information and even atomic-scale imaging. I hope you understood the theory and principle of SEM and TEM. Let us go to the lab and learn this technique in more detail.

We are going to learn how to perform scanning ultramicroscopic analysis. BSA nanoparticles, stubs, glass slides for drop-casting the material, a glass cutter to cut the slides, pipette, tips, carbon tape, forceps, a beaker for discarding waste, tissue paper, and ethanol for cleaning the glass slides. First, we are going to serially dilute the stock solution of BSA nanoparticles to get a proper image. We will prepare 1:10, 1:100, and 1:1000 dilutions of the stock solution. So, we will add 1 ml of the stock solution to 9 ml of ultrapure water so that the final dilution will be 1:10.

Mix it properly. Similarly, using a fresh tip, we will add 1 ml of the 1:10 dilution to 9 ml of ultrapure water so that the final dilution of this solution will be 1:100. Similarly, we will take 1 ml of the 1:100 dilution and add it to 9 ml of ultrapure water to get a final dilution of 1:1000. We are going to follow the drop-casting method. For that, we need a glass slide, and we have to cut it into 1 cm by 1 cm pieces.

We can use a glass cutter to cut the slides. Carefully cut the slides into 1 cm by 1 cm pieces. Now, we will drop-cast the sample onto the cut glass slide using a micropipette. We need an IR lamp to dry the sample. Carefully add the sample, ensuring that it does not flow out of the slide.

Now, we will dry the sample for about 10 to 20 minutes. We can see that the sample has completely dried. Now, the next step is to place the glass slide on the stub. Stick a piece of carbon tape on the stub. It is a double-sided conductive tape.

Remove the white cover using forceps. Hold the glass slide carefully at a corner and stick it on the stub. Tap it in a corner to secure it properly on the stub. This is a sputter coater, which we will use to coat our sample with a thin layer of gold to make it conductive. Select proper sputtering settings.

Now, place the stub in the machine and start the process. We are using 20 mA of current and 45 seconds of sputtering time. You can see that the sample has been coated with a thin layer of gold. This is a scanning electron microscope, which we are going to use to analyze the surface of the sample. The first step is to vent the chamber.

Then, place the stub in the sample holder and close the chamber. After that, we will pump the chamber so that a vacuum is formed inside. Once the vacuum has formed, we will move the sample holder and adjust the height of the stage accordingly. Use the screen on the bottom left to navigate around the sample. We need to bring it to an appropriate working

distance. After that, we will first adjust the voltage and magnification and then turn on the beam. Select the appropriate detector.

You can see that the image is starting to appear on the screen. We will increase the magnification and then focus on a part of the sample. The focus and contrast are adjusted until we get a clear image of the sample surface. We can see that the sample surface is being scanned and sharp images are forming on the screen. You can save this image in the folder with a proper sample name and date.

This image depicts the surface morphology of the synthesized BSA nanoparticles, obtained using a scanning electron microscope. In a 1:10 dilution of the stock solution, you can see densely packed nanoparticles. Upon further dilution and magnification, individual nanoparticles can be observed, with sizes falling within the nanoscale range. For TEM analysis, we have a 1:100 dilution of BSA nanoparticles, and we need TEM grids. This is the box in which the TEM grids are kept.

We require special forceps with a very fine tip to hold the grid. We should be very careful while handling this, and we'll need filter paper for sample preparation. First, we'll take out a new TEM grid. You can see that it has two sides: one shiny side and another carbon-coated, non-shiny side. We have to place it with the carbon-coated, non-shiny side facing up.

Then, put a drop of the sample solution on the TEM grid. The excess sample solution will be absorbed by the filter paper. Use an IR lamp to dry the sample. Alternatively, we can also dry the sample overnight in a desiccator. Once it is dried, we will put the TEM grid in the TEM sample holder. This is a sample holder for TEM. First, open the latch, and then carefully place the TEM grid on the sample holder.

Close the latch to secure the TEM grid and tap it a few times to ensure that it is properly fitted. You can see the TEM sample holder with the grid at the tip. This is a transmission electron microscope, which we are going to use today for the analysis of our sample. The sample holder will go inside through this port. We will insert the sample holder into the sample port and then pump the chamber to form an ultra-high vacuum.

After the ultra-high vacuum is achieved, the sample holder is inserted further into the machine and rotated into the closed position. This is the setup to control the machine. The voltage is set, and the beam is then turned on. The magnification and focus are adjusted to

get a clear image of the sample. You can see the TEM image of the BSA nanoparticles on the screen.

Final adjustments need to be made to get a clear image. Now we move on to the SAED pattern. The beam stop is inserted over the sample, and the beam is focused on the center area. Then the diffraction pattern is recorded. The diffused rings denote the amorphous nature of our sample. As a summary, in today's lecture, we learnt about the principle and theory of SEM and TEM, and also, by practical demonstration, we understood these techniques in detail. Thank you for your kind attention.

I will see you in another interesting lecture.