Experimental Nanobiotechnology

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Lecture 17: In vitro Methods to Study Antibacterial Properties of Nanomaterials

Hello everyone, today we are going to learn in vitro methods to study the antibacterial properties of nanomaterials. In today's lecture, we are going to learn various techniques to assess the antibacterial properties of nanomaterials and the antibacterial mechanism of nanomaterials. And at the end of the lecture, we will also have a practical demonstration to assess the antibacterial properties of nanomaterials. Before I talk about the various methods for assessing the antibacterial properties of nanomaterials, let us briefly learn why there is a need for developing nanomaterials with antibacterial properties. Bacterial infections are a major problem as they can lead to serious illness and even death.

Bacterial infections can be controlled by the use of antibiotics, but extensive antibiotic usage leads to multidrug-resistant bacterial strains. To overcome that, we need new materials with antimicrobial properties. In search of novel materials, researchers found that nanomaterials exhibit significant antibacterial activity. And they are less likely to promote bacterial resistance. These are the advantages of nanomaterials. That is why nanomaterials with antimicrobial properties are gaining much importance in the biomedical field.

Let us learn about various methods to assess the antibacterial properties of nanomaterials. The first method is the turbidity assay. Using this assay, we can identify the MIC and MBC. MIC means minimum inhibitory concentration. That is the lowest concentration of an antimicrobial agent that will inhibit the growth of a microorganism. And MBC means minimum bactericidal concentration.

That is the lowest concentration of an antimicrobial agent that will prevent the growth of a microorganism. That means the MIC concentration: if you use this MIC concentration, it will inhibit the growth of bacteria. If you use this MBC concentration, it will kill the bacteria. To detect the MIC and MBC, we have to inoculate 300 microliters of 0.5 OD

bacteria into 3 ml of bacterial growth medium. Then we have to add various concentrations of nanomaterials and incubate overnight in the incubator.

For example, if I make new nanomaterials and you want to study their antibacterial properties, we don't know at what concentration they will inhibit or kill the bacteria. For example, in this case, we can add concentrations like 2 micrograms, 4 micrograms, 6 micrograms, 8 micrograms, 10 micrograms, and 12 micrograms. So, once you add various concentrations of your nanomaterials, you can incubate them overnight. After overnight incubation, you can observe the turbidity.

Turbidity means the bacteria have grown. In the test tube, that is the control, which means it is untreated, where you did not add any nanomaterial. There is no nanomaterial. So here, the bacterial growth is complete, and you observe full turbidity.

Whereas you added the nanoparticle at the low concentration like 2 microgram or 4 microgram, still you are able to see the turbidity. That means the bacteria has grown in these test tubes. You can observe that in these three test tubes, there is no bacterial growth. From this we have to identify which is MIC and which is MBC. For that what we have to do is we have to re-inoculate from this test tube to a new test tube with 3 ml of bacterial growth medium.

We have to inoculate 300 microliter from this test tube and after incubating for overnight you can see that there is a growth in the 8 and 10 microgram concentration and there is no growth in the 12 microgram concentration nanoparticle. It shows that this 8 and 10 microgram is the MIC. That means it is able to inhibit the growth of bacteria. That is why when we re-inoculate into the fresh test tube, it is able to regrow again. Whereas in case of this 12 microgram, there is no growth.

That means this is the bactericidal concentration that is the killing concentration. And you can also do the spread plate method by taking 100 microliter from each test tube and incubate it overnight to observe the bacterial colonies. You can take 100 microliter from each test tube and you can do the spread plate technique and after overnight incubation you can see the colonies growth and you can also count the number of colonies. You can see here in the control there are a lot of colonies and in case of MIC also you can see some few colonies are there.

But in the case of MBC, there are no bacterial colonies. It confirms which is the MIC concentration and which is the MBC concentration. From this data, you can understand

this is a control. That means we do not add any nanomaterials. Only bacteria are added. So it is the complete growth of bacteria. You can observe the complete growth of bacteria. That is the turbidity.

Complete turbidity is there, and in the case of different concentrations of nanomaterial, we can see here there is some growth. But in these two concentrations, there is no growth. And by using this kind of re-inoculation as well as the spread plate method, we can identify which is MIC and which is MBC. And here we used bacteria that is GFP E. coli. GFP means green fluorescent protein-expressing recombinant E. coli bacteria.

And it was treated with different concentrations of AgZnO nanocomposite. We can also identify the MIC and MBC by optical density measurement, that is the OD. You can also see that when you measure the OD at 600 nanometers, you can see that the bacterial growth is going down with respect to the concentration. And whenever we perform antibacterial studies, we have to use at least one gram-positive bacteria. In this case, we used Staphylococcus aureus and at least one gram-negative bacteria, which in this case is E. coli.

So we are always we have to use one gram positive and one gram negative bacteria to understand whether our nanomaterials is having broad spectrum of antibacterial activity against both gram positive as well as gram negative bacteria. Also this MIC and MBC also varies with respect to the bacteria. You can also see that in S.aureus, this MIC concentration is different and in the E. coli, the MIC concentration and the MBC concentration is different. So, we have to use the right concentration to restrict the complete bacterial growth and here you can see that N equal to 3.

That means this experiment was done 3 times and the data represented here, these are the average of that Or it can be a triplicate samples and this error bar represents the standard deviation of the data. So you can observe here for S.aureus the same nanocomposite it is giving 60 microgram as MIC. For E. coli it is giving a 550. So depends on the nanomaterial and depends on the bacteria this MIC and MBC will vary. And we can also do the time dependent study to understand at what time point the nanomaterial is killing the bacteria.

And in this case, we used a silver nanoparticle and the silver nanoparticle is made using sodium borohydride as a reducing agent. So to confirm that the bacterial death is due to silver nanoparticle and not due to the reducing agent, we have to use sodium borohydride as another control. One is the negative control where you are not adding any nanomaterial. In this case, you can see that bacterial growth is proper and it is growing more than 12

hours. And similarly, when you are adding the reducing agent, that is sodium borohydrate, there also there is no bacterial death.

It is equal to the control. It is growing nicely. When you use different concentrations of silver nanoparticles, you can see that, with respect to the concentration and time, the bacterial growth decreases. If you use a low concentration, bacterial growth is still present but is less compared to the control. Whereas in this case, you can see here that the bacterial growth decreases when you use the optimum concentration. There is no bacterial growth, so we can perform both time-dependent and concentration-dependent studies.

For understanding the antibacterial properties of nanomaterials, you can see here in the control (untreated) that there is no effect. When you use only the reducing agent, sodium borohydride, there is no effect. In nanoparticle-treated cells, as I mentioned earlier, bacterial growth decreases with respect to concentration. We can also perform the colony counting method, as I mentioned earlier, with respect to different time points. You can take 100 microliters of bacterial culture and perform the spread plate method, then count the number of colonies and plot it.

We can see that, with respect to time, the number of bacterial colonies decreases, as well as with respect to concentration. You can see that at the 3-hour time point, there is some bacterial growth, but at 6 hours and 12 hours, there is no bacterial growth. So, by using this colony counting method, we can also understand the antibacterial properties of nanomaterials. As I mentioned, in this study, we used a bacterium that is a green fluorescent protein-expressing E. coli. This is a type of recombinant bacterium, and it emits green fluorescence under UV light.

And this is a control. The control is untreated. That means no nanoparticles were added to these bacteria. You can see here the intact bacteria. This is an E. coli bacterium. This is a rod-shaped bacterium.

You can see the intact rod shape under the microscope. Whereas with the cellulose nanoparticle treatment, you can see that the bacteria are damaged. They are undergoing cell death. The advantage of this green fluorescent protein-expressing E. coli is that we do not have to add any additional fluorescent dye or stain to see the bacteria under the microscope. It has its own GFP, and under UV light, we can easily observe the GFP expression.

So that is the advantage of this GFP E. coli. Here, we can also do time-dependent and concentration-dependent studies. We can see here that this control is untreated. Over time, the number of bacteria increases. You can see at 0 hours, the number of bacteria is lower.

Whereas at 12 hour, you can see the number of bacteria is more. And if you are using a different concentration, you can see here the number of bacteria when compared to control, the bacterial number is going down. So by using this time dependent and concentration dependent study, we can confirm the antibacterial activity of your nanomaterial. An additional advantage of using this GFP E.coli is we can also do the fluorescent analysis for assessing the antibacterial activity.

For example, in this case, when we are treating with the Ag ZnO nanocomposite, we can see here this untreated E.coli. In untreated E.coli, this is giving a very high fluorescence and with respect to concentration of nano composites you can see that the fluorescent intensity is going down and you can see that at high concentration is completely gone down fluorescence is completely gone down and here we used only nano composites in the bacterial growth medium as a negative control without any bacteria so there is a negative control and with respect to the concentration you can see that the fluorescence is going down

By using this fluorescence analysis also we can assess the antibacterial activity of nanomaterials. The next method is the disk diffusion method. So this is a rapid and easy semi-continuity method for assessing the antibacterial activity of diffusible antimicrobial agents from different drug delivery platforms. In this we have to add 100 microliter of overnight grown culture and you can use a sterile swab and you can Spread it on the complete agar plate like this.

Either you can use the sterile swab or you can use this kind of spreader and spread the bacteria completely by rotating the plate with bacterial culture. Then you have to place the disc of different sample. For example, you made some nanofiber membrane and you want to understand their antibacterial property. you can cut the nanofiber membrane into small small circular disc and that can be kept it and in addition to that you can keep like a negative control that is only filter paper

and that is another one is positive control that is a antibiotic disc that means completely it will kill the bacteria so we can use one positive control and one negative control and also we can use the sample so the positive control is antibiotic disc so antibody disc where we can see the clear zone of inhibition and negative control is only filter paper or only

nanofiber without any antibacterial agents and third one is your test sample nanofiber loaded with some antimicrobial agents and after incubating the plate you can observe the plate and measure the zone of inhibition for test sample

you can see here so this is your circular disc and followed by that there is a no growth in this region so that is a clear inhibition of growth so that is called zone of inhibition for example this is your sample and in the surrounding there is no bacterial growth and whereas here there is a bacterial growth that means so this is called zone of inhibition this is called zone of inhibition in this zone it is not allowing the bacteria to grow and by using this vernier caliper we can also measure the the centimeter of how much or millimeter how much it is inhibiting the growth of bacteria and we can plot it so in this case We used silk fiber. This SSD is the Silver sulfadiazine and SS is the silk sericin and SF is the silk fibroin.

So this is a different nanofiber membrane. One is loaded with the antimicrobial agent, and the other one is a simple nanofiber. And you can see that when you use the negative control, which is only filter paper, there is no zone of inhibition. And when you use only the scaffold, which is nanofiber without any antibacterial agent, there also we do not observe any zone of inhibition. And when you use the drug at different concentrations, you can see a clear zone of inhibition. Here, you can clearly observe there is no growth. So that is called the zone of inhibition. It can be measured and also plotted.

To understand the antibacterial efficiency of the particular nanomaterial. We can also use various microscopic analyses to understand the morphology of bacteria undergoing cell death. For example, we can use the atomic force microscope. This is the untreated E. coli. You can see that it is a rod-shaped bacteria, as I mentioned earlier. And this is the untreated Staphylococcus aureus. You will see a bunch of grapes-like structure. The bacteria will look like this. When you treat this E. coli and S. aureus, you can see that the bacteria's cell wall is damaged.

This can be studied using atomic force microscopy or scanning electron microscopy (SEM) analysis. We can see that the bacteria's cell wall is damaged by the nanocomposite. As I mentioned in the electron microscopy lecture, if you want to confirm that this damage is caused by the nanocomposite, you can select this area and perform elemental analysis. When you perform the elemental analysis, if you detect peaks for silver and zinc, it means the silver and zinc are attaching to the bacterial cell wall and destroying it. We can also study the bacteria under a transmission electron microscope.

So this is a single bacterial cell that is an E. coli bacterium. And you can see here the bacterial cell wall is damaged by the nanoparticle. So we can confirm it when you enlarge this and see that there are black-colored dots. These black-colored dots are silver nanoparticles. So this confirms the presence of silver nanoparticles on the cell membrane of bacteria.

We got the overall idea of how to assess the antimicrobial properties of nanomaterials. Let us see what the mode of action of nanomaterials is. The first one is due to the electrostatic interaction. If you are using nanoparticles with positive zeta potentials, that means your nanoparticles are positively charged, and most of you know bacteria and cells are negatively charged. So through electrostatic interaction, these positively charged nanoparticles can easily attach to the negatively charged bacteria or cells, and they can attach to the cell wall of bacteria and damage it.

The other mechanism is that they can also produce ROS, which are reactive oxygen species, and they can damage the bacterial cell wall. And as I told you, DNA is also negatively charged. So these positively charged nanoparticles can easily attach to the negatively charged DNA and inhibit its replication, leading to cell death. Based on various antibacterial studies and literature, numerous antibacterial mechanisms have been postulated. The first one is electrostatic binding. As I mentioned earlier, positively charged nanoparticles can easily bind to negatively charged bacteria through electrostatic interaction.

These metal nanoparticles induce surface oxidation and the generation of reactive oxygen species, enhancing oxidative stress, which leads to membrane disruption and kills the bacteria by damaging the cell wall. Once the cell wall is damaged, it leads to the oozing out of damaged cellular material. These nanoparticles can also kill bacteria by inhibiting DNA replication, transcription, and translation processes. The positively charged metal nanoparticles bind to the negatively charged DNA and inhibit DNA replication. They also bind to the ribosome and inhibit protein synthesis, which leads to bacterial cell death.

I hope you got the overall idea about the antibacterial mechanism of metal nanoparticles. Let us see what the various crucial factors affecting the antibacterial mechanisms of metal nanoparticles are. The first one is nanoparticle size. As you know, smaller nanoparticles have a greater surface-to-volume ratio, which allows them to attach more easily to bacteria. The smaller the nanoparticle size, the stronger the antibacterial potential. Compared to standard antibiotics, small-sized nanoparticles have higher antibacterial activity.

The next one is nanoparticle shape. The shape of the nanoparticles can affect how they interact with bacteria. For example, spherical-shaped nanoparticles have higher antibacterial activity compared to other shapes. The third one is nanoparticle surface charge. As I mentioned earlier, positively charged nanoparticles are more likely to interact with negatively charged bacterial surfaces.

The next one is the capping agent. So, that depends on the type of capping agent used to stabilize the nanoparticles. So, that will also affect the antibacterial properties. For example, if you are using PEI, it will give a positive charge to the nanoparticle. So, it can easily attach to the bacteria.

And if you are coating the nanoparticle with PEG, it will have a negative charge, so based on your capping agent or coating agent, the antibacterial properties will also vary. And the next one is environmental factors: the pH and ionic strength of the medium also affect the antibacterial activity of the nanoparticles. And suppose if the pH and ionic strength are not optimal, that leads to the aggregation of nanoparticles. Once the nanoparticles are aggregated, the interaction with the bacteria will be less, and that leads to reduced antibacterial activity.

So, to achieve high antibacterial activity, we have to optimize the parameters. I hope you got the overall idea of how to assess the antibacterial properties of nanomaterials. Let us go to the lab and learn this technique in more detail. In this experiment, we will learn how to assess the antibacterial properties of nanomaterials. To proceed with the experiment, we require nanomaterials.

Today, we will first synthesize silver nanoparticles. For this, we will use AgNO3 as a precursor, sodium borohydride as a reducing agent, and SDS, that is sodium dodecyl sulfate, as a capping agent. Additionally, we will need microtips, micropipettes, ultra-pure water, and two microcentrifuge tubes. One for the control and another for synthesizing silver nanoparticles. First, we will add 1 mL of ultrapure water to both tubes.

Next, we will add 3.34 microliters of 0.1 molar AgNO3 solution to the microcentrifuge tube. We will then add 2.66 microliters of freshly prepared 2 mg per mL sodium borohydride as a reducing agent to the microcentrifuge tube. While in the control tube, we will add the same amount of ultrapure water. Finally, we will add 90 microliters of 0.1 molar SDS as a capping agent to both tubes. Take both tubes and shake them vigorously.

Here, you can observe that the tube where we added the reducing agent turns pale yellow. This confirms the synthesis of silver nanoparticles, while the control tube remains transparent where we added only ultrapure water instead of the reducing agent. Now, we have synthesized silver nanoparticles. We will assess the antibacterial properties of silver nanoparticles using the disc diffusion method. The first step is to sterilize all the materials needed for the experiment under a laminar flow hood.

We will begin by dipping a triangular glass spreader into ethanol, flaming it to sterilize,then shaking it to cool down. Place the glass spreader in a stand to allow it to cool down further. Next, we will add 5 microliter of the following samples to separate sterile disc such as the positive control that is antibiotics, negative control that is the LB broth and test sample that is the silver nanoparticles and allow the disc to dry under sterile conditions.

Now we will add 100 microliter of overnight grown bacterial culture to a LB agar plate. Using the sterile triangular glass spreader, we will spread the bacterial culture evenly on the LB agar plate. After that, we will place the earlier prepared three discs, positive control, negative control and silver nanoparticles on the plate. Now we are going to place the three discs on the plate with respect to its naming on the plate. Once it is done place the negative control plate as well as the test plate in the incubator.

After overnight incubation period Here we can observe zone of inhibition for our nanomaterial treated disc as well as the positive control disc which is having antibiotics. So today we learnt the synthesis of silver nanoparticles and their evaluation for antibacterial properties using the disc diffusion method. Here we observe the antibacterial properties of silver nanoparticles under normal light and UV transillumination. The silver nanoparticles particles exhibit a zone of inhibition against E. coli bacteria which is compared to the positive control that is the antibiotics and the negative control so that is the only simple broth. So here you are not able to see any zone of inhibition but in the test sample where the silver nanoparticles is there you can see the clear zone of inhibition and in the positive control also we are able to see the clear zone of inhibition.

Whereas, in the case of the negative control where we added only the broth, there is no zone of inhibition. As a summary, in today's lecture, we learned various techniques to assess the antibacterial properties of nanomaterials. We also learned the antibacterial mechanism, and through practical demonstration, we learned how to assess the antibacterial properties of nanomaterials. Thank you for your kind attention. I will see you in another interesting lecture.