Experimental Nanobiotechnology

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Lecture 15: In vitro 3D Cell Culture

Hello everyone, today we are going to learn about in vitro 3D cell culture. This in vitro 3D cell culture is one of the important tools or techniques for understanding the toxicity of nanomaterials. In today's lecture, we will be learning about what is 3D cell culture? What is the difference between 2D and 3D cell culture? And what is the need for 3D cell culture?

We will also learn about the applications and types of 3D cell culture. In today's lecture, we will learn about the alginate bead-based 3D cell culture model. Through practical demonstration, we will also learn how to prepare the alginate bead-based 3D cell culture model. Let us see what is 3D cell culture. The 3D cell culture model mimics living body environments outside the body.

It is composed of an extracellular matrix scaffold. The 3D matrix supplies nutrients, oxygen, and drugs through diffusion gradient and permeation. This 3D cell culture allows cells to grow and interact in all three dimensions. We can prepare a 3D porous scaffold. Scaffold means support.

Which can support the growth of cells and the movement of cells. Let us see the difference between 2D and 3D cell culture. In 2D cell culture, we usually grow the cells in the lab on plastic plates. So, cells attach to the plastic plates. And grow as a monolayer.

But the drawback of this 2D cell culture is less cell-to-cell contact and unrestricted accessibility to nutrients, oxygen, and drugs. For example, if you're adding an anti-cancer drug and you want to check whether this anti-cancer drug is efficient in killing the cancer cells. When you add the anti-cancer drug, you'll get a uniform distribution of your anti-cancer agents. So that is why this 2D cell culture may not be suitable for translational research. Because inside our body, we have all the organs in 3D.

If you are using the three-dimensional cell culture, it will closely mimic your in vivo condition, that is, the inside-the-living-system condition. We can use the 3D cell culture as an alternate or equivalent for understanding what is the toxicity or what is the therapeutic efficiency of the anti-cancer drug using a three-dimensional cell culture model. When you are using this three-dimensional cell culture model, we can have enhanced cell-to-cell contact, and cell-ECM interaction will also be more.

And here we will have a restricted access to nutrients and oxygen. That is why we will have a variability in drug interaction. For example, in 2D, all the cells got the uniform distribution of chemotherapeutic agents or anti-cancer drugs. But in case of 3D, whenever you add an anti-cancer drug, So some cells on the top will get the anticancer drug and some drug can penetrate and go inside and kill the cells.

We'll have a differential drug exposure in layers. So that's why this will closely mimic like your in your condition. That's why this 3D cell culture is one of the best model for understanding the toxicity of nanomaterials or if you want to study the any test compound, this 3D cell culture can be one of the alternate tool

for understanding the toxicity of nanomaterials or any cytotoxic drugs. As I told in the previous slide when we compare the 2D cell culture and 3D cell culture the 2D cell culture will get flat and stretched cell shape whereas in 3D cell culture we get the ellipsoid or polarized one and 2D cell culture is easy to culture in short time and 3D cell culture need a long time to grow the cells in the three-dimensional scaffold. And as I told earlier, we will also have that all the cells directly exposed to the drug and cells respond sensitively.

Whereas in three-dimensional scaffold, cells tends to develop resistance against drug treatment. That means, as I told earlier, We have a three-dimensional scaffold. Some of the cells on the top will get the anti-cancer drug and only few drugs can enter into the scaffold and it can kill the cells. And when compared to the 2D, the 3D cell culture have the better bioequivalent.

That means it biomimic your original tissue. Let us see some of the demerits of 2D cell culture. Usually the mammalian cells are cultured on petri dishes where the cells will grow as a monolayer. But this monolayer does not mimic the cells residing in the complex microenvironment of a tissue. Pharmacokinetics of drug in 3D culture models varies significantly from 2D models, which affects the drug efficacy and toxicity analysis.

For example, if you are using 5 microgram of anti-cancer drug, The drug efficacy will be different for 2D and 3D cell culture model. Similarly, cell to cell and cell-ECM interactions are very important for drug discovery in pharmaceutical field, but it cannot be achieved by 2D cell culture. These are the some of the demerits of 2D cell culture. Let us see some of the applications of 3D cell culture.

The first one is translational. This 3D cell culture have more translational value. For example, if you are using a 3D cell culture model using human cells, which can overcome the drawbacks of most models and it will also reduce the number of animal model studies. And we can also go for precision medicine.

For example, if you are having a patient with a tumor, we can have the tumor cells. The tumor cells can grow in the lab environment and we can develop the three-dimensional tumoroid. So once you develop this three-dimensional tumoroid, we can add the anti-cancer drug and we can check it which anti-cancer drug is efficient. For example, if you're having two types of anti-cancer drug, we can add it on the tumoroids and we can understand

which anti-cancer drug is very efficient in killing this tumor cells. Then we can give the particular medicine to the patient to reduce the side effects due to the over dosage of various types of anti-cancer drugs. And this 3D cell culture can also play a very very important role in new drug development. It will increase the success rate of drug development of new drug candidates.

Whenever any new drug is discovered, we can evaluate using this three-dimensional cell culture model system for understanding the toxicity as well as the therapeutic efficiency of these new drug candidates. Before I talk about the various types of 3D cell culture, let me briefly explain about what is the difference between spheroids and organoids. The spheroids are simple and round.

This is a kind of cluster of cells. These are called as spheroids. If the spheroids are made up of tumor cells, then it is called as tumoroids. And when compared to these organoids, these organoids have this complex structure. And in the spheroids, we'll be using the single cell type, usually from the cell lines.

And in the organoids, we'll be using the stem cell derived mixture of different cell types. And that it mimics exactly the diversity of organs. So that's why it is called as organoids.

So it mimics like your original function of the organs. So that's why these are called as organoids.

And these spheroids can be cultured. For a certain amount of time, but these organoids can be cultured for several months. The application of spheroids can be used for disease modeling, drug screening, and cancer research. In the case of organoids, in addition to this, we can also use them for personalized medicine. There are two types of 3D cell culture.

One is scaffold-free, and the other one is scaffold-based. Let us learn about scaffold-free 3D cell culture. Under scaffold-free 3D cell culture, the first one is forced floating. Here, we'll be using a non-adherent surface. Due to the non-adherent surface, the cells will try to form a clump and aggregate, forming spheroids.

The next one is the hanging drop. Here, we'll have a suspension of cells, which will be in the form of a hanging drop. So the cells will try to form an aggregate, and this way, we can obtain the spheroid using the hanging drop method. The third one is magnetic levitation.

Here, the cells are labeled with magnetic nanoparticles, and when you use this external magnetic field, the cells will form a clump and create spheroids with the help of the external magnetic field. The fourth one is a cell sheet, where we will have the layering of a 2D cell monolayer. We will be adding the layer-by-layer cells, and we can have scaffold-free 3D cell culture.

The fourth one is dynamic culturing, where you will use agitation, and when you apply agitation, these cells tend to join together and form a clump, creating spheroids. The next one is scaffold-based 3D cell culture, in this scaffold-based 3D cell culture, we can use a manual assembly technique, such as

chemical, physical, or mechanical modification to create a porous scaffold or hydrogel structure, where the cells can attach and grow. This is scaffold-based 3D cell culture, and it is a manual assembly technique. The next one is We can use the CAD model, which is the computer-aided design model, to create a precise architecture of the scaffold or hydrogel.

And we can use the 3D bioprinter. We can use the 3D bioprinter and we can make the particular three-dimensional scaffold with the cells. And the third one is the hybrid

model. We can say this is a microfluidic device-assisted. And we can also say the other way, this is a lab-on-a-chip or organ-on-a-chip.

Here we will have small microfluidic channels which mimic like your original organ or tissue. So it will mimic like your native tissue micro architecture and these micro channels can act like a kind of blood vessels and we can add the, for example, if you want to test whether the particular drug is toxic to the liver or spleen, we can have the liver cells and we can add the anti-cancer drug And this can go through the microfluidic channel and it can, if it is killing the liver cells, then we can say this drug is toxic to the liver.

So this is a scaffold-based 3D cell culture. Let us see how to prepare alginate bead-based 3D cell culture model. For that, we have to use the sodium alginate. This sodium alginate is a naturally occurring gelling substance which is produced from the cell walls of brown algae. And this sodium alginate can be cross-linked with calcium.

When it cross-links, a substantial amount of water is absorbed and bound into the egg box structure. Thus, generating a stable hydrogel, this is called the egg box model. Let us see some of the advantages of the alginate bead-based 3D cell culture model. Here, the sodium alginate has a very good biocompatible nature as well as biodegradability, and it is low-cost as it is obtained from natural sources, easy to gelate, inert in nature,

non-toxic, and it aids in regeneration. These are some of the effective parameters that affect the alginate bead-based 3D cell culture model which I will discuss in more detail in the subsequent slides. The first parameter is bead size and shape. The surface tension of the alginate solution influences the droplet size.

Hence, it impacts the overall bead size. The surface tension force in the alginate solution droplet is crucial for the droplet to restore its spherical form. The next one is the concentration of calcium chloride in the gelation bath. The bead shrinkage and the ability to form stable alginate hydrogel are affected by the concentration of calcium ions present in the cross-linking gelation process.

And the third one is the use of external detachment force. If you are using an external force, it will hasten the detachment process, due to which what happens is you will get the premature size. which is smaller than that generated by natural dripping. And the speeding droplet enters the gelation bath with high kinetic energy.

So it potentially leads to a change in the shape. The next important parameter is the dripping tip diameter. The diameter of the dripping tip influences the size of the droplet and also the size of the beads. The size of the droplet influences the relaxation time necessary for the droplet to reform into a sphere prior to gelation. And the next important parameter is the sterilization of the alginate solution.

If the alginate solution is sterilized, it may lead to the deterioration of the alginate structure. Thus, it will affect the size and form of the beads. And the next parameter is the bead size. The curing time of the bead in the gelation bath is very important. The degree of bead shrinking is mainly affected by the duration of contact with calcium ions.

And the next one is The MG ratio of alginate. The MG ratio refers to the ratio between the mannuronic acid and guluronic acid in alginate. This MG ratio is very important. The degree of bead shrinkage is affected by the MG ratio of alginate.

The next parameter is bead shape. The bead shape is affected by the viscosity of the gelation bath and the collecting distance between the needle and the gelation bath. Another important parameter is the pre-gelation step. The pre-gelation phase helps retain the droplet's spherical form before it hits the gelation bath surface. The next one is the viscosity of the alginate solution.

The viscosity of the alginate solution is crucial for maintaining the spherical form. The next one is the stirring rate of the gelation bath. The vortex force caused by a rapid stirring rate may distort the beads. The last one is the surface tension of the gelation bath. The surface tension of the gelation bath is essential because it reduces the impact force on the droplet when it collides with the gelation bath surface.

Let us see the overview of the alginate bead-based 3D cell culture model. To prepare the alginate bead-based 3D cell culture model, The first step is to weigh the desired concentration of sodium alginate powder and add it to the cell culture medium to get the exact weight-to-volume ratio. Stir it until the powder is completely dissolved. Then, the third step is, once the cells are grown to 70% confluency in the tissue culture plate, prepare the cell suspension by following the ATCC guidelines.

So, once the cell suspension is ready, the fifth step is to count the number of cells and add the desired amount of alginate-containing medium to get the exact number of cells per alginate bead. Once you mix the cell-alginate suspension thoroughly, transfer the cell-

alginate suspension into a syringe or any extruder. Hold the syringe tip above the cross-linking solution. Here, the cross-linking solution is 100 millimolar calcium chloride.

Dispense the cell-alginate solution and allow the cell-containing alginate beads to crosslink in the solution. So, once you get the alginate beads, remove the calcium chloride solution and wash the cell-containing beads with cell culture medium. Make sure that all the calcium chloride is removed. Then, you can replate each alginate bead in individual wells and add medium to them. For that, we have to use a 12-well plate.

So once the cells are grown in the 5% CO2 incubator after incubation, you can visualize the cells directly under the microscope or stain them to confirm the viability of the cells. So this is the overview of the alginate bead-based 3D cell culture model. I hope you got the overall idea about the 3D cell culture. Let us go to the lab and learn how to prepare an alginate bead-based 3D cell culture model.

To perform the in vitro 3D cell culture experiment, we need to prepare the alginate-containing cell culture medium. First, we will UV-sterilize the biosafety cabinet along with all the required materials. Next, we will prepare a 2% sodium alginate solution in 50 mL of cell culture medium. Start by adding 50 mL of complete cell culture medium to the bottle. Set the temperature to 37 degrees Celsius and adjust the RPM to 500.

After that, carefully add the sodium alginate powder into the bottle and switch on the RPM. Allow the solution to stir until the sodium alginate powder is completely dissolved in the medium. Once dissolved, pour a small amount of the alginate medium into a centrifuge tube for the experiment. And store the remaining solution in a 4-degree refrigerator for future use. At the same time, warm all the necessary materials to 37 degrees Celsius.

To perform the 3D cell culture experiment, we will need the following materials. Complete cell culture medium, DPBS, that is the Dulbecco's phosphate buffered saline 0.25% tripsin-EDTA, 2% sodium alginate containing cell culture medium, empty centrifuge tube for waste disposal. After removing the flask from the incubator, we will examine the cells under the inverted microscope. to assess the confluency of the A549 cells that is the lung cancer cell line.

As you can see, the cells are grown more than 70% of the T25 flask, so we can proceed to the next step. The cells are well adhered to the flask and now we will move on to the trypsinization and the centrifugation process. A detailed demonstration of these steps will

be covered later in another lecture. After centrifugation, we observe the cell pellet at the bottom of the tube. We carefully discard the trypsin containing media without disturbing the pellet.

Next, we add fresh media to the micro centrifuge tube and slowly re-suspend the pellet before dispensing it into the new media. To count the cells, we add an equal volume of 0.4% trypan blue dye to the media containing the cells and mix thoroughly. Then we will add 10 microliter of the mixture onto both sides of the cell counting chamber slide. After inserting the slide into the automated cell counter, we press the count cells button. Once the count is complete, we save the data to an external disk and open it on the monitor to review the exact cell count.

Here we can see that there are approximately 22 lakh live cells per mL. For the 3D model cell culture experiment, a total of 40,000 cells are required per well for both the control and test wells with the experiment being performed in triplicates. To prepare the cell suspension for the control wells, add 100 microliter of the cell suspension to 9.9 mL of complete media, which will result in a concentration of 20,000 cells per mL.

Then add 2 mL of this prepared cell suspension to each of these 3 control wells, ensuring each well will contain a total of 40,000 cells. Next, for the 3D alginate model, we will add 5 mL of 100 millimolar calcium chloride to each of the three wells. Now we will prepare the cell alginate suspension. We will need 10 beads per well, each containing 4000 cells.

These beads will be dispensed into the wells containing calcium chloride, which will act as a crosslinker. When you use a 10 mL syringe to prepare bead then each bead will be approximately 10 µL. So we need to dispense a total of 100 µL which will contain 40,000 cells into each well. We will centrifuge 200 µL of cell suspension to collect the pellet and then resuspend the pellet in the alginate containing cell culture medium of 1 mL.

This will result in a final concentration of 4 lakh cells per mL in the alginate medium, which will result in 4000 cells per 10 microliter, which is ultimately one alginate bead. Now we take the solution into a sterile syringe and hold the syringe with the needle tip above the surface of the calcium chloride solution. Dispense the alginate beads into the solution by moving the syringe in a circular motion. Dispense at a rate of 1 bead per second to prevent clumping.

Allow the alginate beads to cross-link in the calcium chloride solution for 10 to 15 minutes. Once the beads have formed, we will remove the calcium chloride solution and wash the beads with DPBS followed by the complete cell culture media. Finally add the same volume of the media as in the control wells. Now we will incubate the beads for 48 hours and observe the results after this period.

After the incubation, we will examine the cells under the microscope and stain them with acridine orange ethidium bromide that is AOEB stain. Here we can observe the cells in the control well as well as in the 3D model where the cells are embedded in the alginate beads, arranged in stacks. For the AOEB staining experiment, we will remove the medium from one of the control wells and add 200 microliters of AOEB stain. After incubating for 3 to 5 minutes, we will remove the dye, wash the well gently with DPBS and observe the cells under the microscope.

Next, observe the cells under the microscope while they are still in DPBS. Here, you can see live cells in the control well with no dead cells visible. We will repeat this process for the remaining control wells, followed by the test wells. We will save these images. For the test well, remove the treatment medium, add 200 microliters of AOEB stain,

incubate for the same duration, and wash the well before observing under the microscope. In the test wells, the cells are also observed to be alive, indicating the biocompatibility of the alginate model. Here, you are also able to see the cells present in different stacks of the alginate model, which is the three-dimensional arrangement that allows cells to grow and interact in all three dimensions. We will save these images as well.

We will repeat this process for the remaining test wells. In this experiment, we have learned the preparation and analysis of a 3D cell culture model using alginate beads. The cells exhibit healthy growth within the 3D environment, showcasing the viability and suitability of alginate for 3D cell culture applications. As a summary, in today's lecture, we learned about

What is 2D cell culture, and what is 3D cell culture? And what is the difference between 2D and 3D cell culture? We also learned about the importance and applications of 3D cell culture. We also learned about the types of 3D cell culture and the alginate bead-based 3D cell culture model. Finally, through practical demonstration, we also learned how to prepare the alginate bead-based 3D cell culture model.

Thank you for your kind attention. I will see you in another interesting lecture.