Experimental Nanobiotechnology

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Lecture 19: In vitro Methods to Study the Apoptotic Potential of Nanomaterials

Hello everyone, today we are going to learn in vitro methods to study the apoptotic potential of nanomaterials. In today's lecture, we are going to learn what is apoptosis and what is the difference between apoptosis and necrosis. We are also going to learn various types of in vitro apoptosis assays. At the end of the lecture, we will also have a practical demonstration to understand the apoptosis assay in detail. Let us see the difference between apoptosis and necrosis.

Apoptosis is a normal or programmed cell death and apoptosis is a physiological process. Whereas necrosis is an accidental cell death, and it is a pathological process. You can see the difference between apoptosis and necrosis in this picture. When a normal cell undergoes apoptosis, membrane blebbing will occur, then the cell breaks into several apoptotic bodies, which are then phagocytosed.

There is no inflammation in the case of apoptosis. Whereas in the case of necrosis, cell swelling happens, followed by the plasma membrane rupturing and the cellular contents leaking out, which leads to inflammation. Most anti-cancer drugs kill cancer cells through this apoptosis pathway. Some nanomaterials also have the potential to induce apoptosis in cells.

In today's lecture, we are going to learn about the various in vitro methods and assays to understand the apoptotic potential of nanomaterials. Let us see how we can detect apoptotic cells. Apoptotic cells can be detected by analyzing surface morphology, cell membrane integrity, mitochondrial function, nuclear events, and DNA cleavage.

In today's lecture, we will learn more about this in detail. There are several types of in vitro apoptotic assays, and in today's lecture, we will learn some of the important in vitro assays. Under in vitro assays, we can broadly divide them into colorimetric, microscopic,

flow cytometry, and molecular analysis. Under colorimetric, we will learn the ELISA technique.

Under microscopic analysis, we will learn acridine orange, ethidium bromide staining, and Hoechst rhodamine staining. We will also cover SEM analysis—how we can use scanning electron microscopy to understand apoptosis. Under flow cytometry, we will learn the Annexin/PI assay as well as the ROS assay. Under molecular analysis, we will learn RT-PCR. Let us learn about acridine orange and ethidium bromide staining.

So, this is one of the simple methods for understanding apoptosis. Here, we are going to use a combination of two dyes. One is acridine orange. The other one is ethidium bromide. Acridine orange is a fluorescent DNA-intercalating dye, and it is permeable to all cell nuclei and emits green fluorescence.

You can see here the live cells appear green. And when the cells undergo apoptosis, during the early stage of apoptosis, what happens is there is a change in the cell nucleus and cell organelles, due to which the cells at the early apoptotic stage appear bright green. They emit bright green fluorescence.

And when we add this combination of acridine orange and ethidium bromide, when the cells are undergoing apoptosis at the late stage, what happens is this ethidium bromide enters. You can see that here the cell membrane is intact, and here the cell membrane has some kind of perforation. So, due to that, this ethidium bromide stain can enter and it stains the nucleus. That leads to the combination of this green plus red, which gives the orange color.

That will be like orange red. So if you are seeing the cells which are in the like emitting the orange red fluorescence that means cells are in the late apoptotic stage. And if the cell is undergoing necrosis, what happens is the cell membrane is ruptured whereas you can see that the nucleus is intact so this ethidium bromide will stain the nucleus

and cell nucleus appear red color from this data you can clearly understand the figure a this is a untreated okay and whereas the figure b, c, d these are treated with the different concentration of nanomaterials and you can see that the bright green fluorescence, this bright green fluorescence is the early apoptotic cells and this orange color

as I told earlier, these are the cells at the late apoptotic stage and here there is no necrotic cell, there is no red color fluorescence. So, when you treat with these nanomaterials, you can simply stain with this combination of these two dyes and we can easily identify the

cells which are in the early apoptotic and the late apoptotic stage. But there is one limitation in the AOEB staining that is like that staining assay can be done at the end of your experiment. And if you want to do the time dependent study, you have to go for the alternate dye

That is Hoechst and Rhodamine B. Let us learn about Hoechst and Rhodamine B staining. Here, the Hoechst stain is a membrane-permeable nucleus-staining dye that emits blue fluorescence when attached to double-stranded DNA. It is used to differentiate condensed pycnotic nuclei compared to normal nuclei. During apoptosis, what happens is we observe nuclei with condensed chromatin. We are going to use another dye, which is Rhodamine B.

This is a membrane-permeable dye that stains the mitochondria and cytoplasmic compartment. You can see here the cell nucleus appears blue in color. The cytoplasm appears orange in color. When cells undergo apoptosis, what happens is the cytoplasm will shrink, and the nucleus will also condense.

That is what we are going to observe under the microscope. From this data, we can clearly understand. So, this is the untreated. In the untreated cells, we can see here the orange-colored cytoplasm and the blue-colored nucleus. When the cells undergo apoptosis, we can see that

You are seeing the bright blue color. So that is due to the chromatin condensation. And you can also see that the cytoplasm also shrinking. So you can see that the orange color is also very less when compared to the untreated cells. So that is due to the cytoskeleton compaction.

We can also use this scanning electron microscope to understand the morphology of untreated and treated cells. You can see here the untreated cells. This is a breast cancer cell and this is a lung cancer cell. The untreated cells are like spindle shaped and well attached to the surface and intact membrane morphology. Whereas you can see that the nanocomposite treated cells, these are shrunk in size and rounded in shape.

And also it is showing that membrane blebbing. You can see these are the membrane blebbing. So these are the hallmarks of apoptotic cell death. Let us learn about another important technique that is ELISA. By using this ELISA, we can quantify the cellular DNA fragmentation.

So during apoptosis, cellular DNA will be fragmented, and that can be detected and quantified using ELISA. Here, we are going to use sandwich ELISA. For that, we have to coat the microplate with the anti-DNA. That means this antibody is against the DNA.

So, the DNA can bind to this antibody, and before that, we have to label the DNA of the cells with BrdU. BrdU is bromodeoxyuridine. It is a thymidine analog, and this will be attached to the DNA of the cells and once the cells are treated with the nanoparticle, they undergo apoptosis. During apoptosis, what happens is the cellular DNA will be fragmented into small fragments and

this can be detected using antibodies specific for this DNA and once you do the microwave treatment, it will convert the double-stranded DNA into single-stranded small DNA fragments and we already have this antibody on the microplate, so this DNA will attach to the antibody Then, we have to add another antibody, which is anti-BrdU antibody, and it has an enzyme called peroxidase (POD). And this will bind to your DNA.

So, as I told earlier, this is a sandwich ELISA. So, this is your microplate where you have the antibodies specific for your DNA. labeled with BrdU, and we are going to add another antibody, which specifically binds to the BrdU-labeled DNA. It is also carrying an enzyme called peroxidase, so your DNA is sandwiched between the two antibodies. That's why it is called a sandwich ELISA. Then, you have to add the substrate.

That is the TMB, which is tetramethylbenzidine. Once you add this TMB, this enzyme will convert the TMB into a colored product, and this color can be quantified using a spectrophotometer. In this way, we can quantify apoptosis. From this slide, you can clearly understand how we can use ELISA to quantify cellular DNA fragmentation. If you are getting higher absorbance, that means more cellular DNA fragmentation, which indicates more apoptosis.

Let us see another simple assay for understanding apoptosis: apoptotic DNA laddering. During apoptosis, what happens is caspase-3 activates CAD, which is the caspase-activated DNase enzyme, and that leads to cleavage of chromosomal DNA. Once you isolate the genomic DNA from untreated and nanoparticle-treated cells and run this DNA in agarose gel electrophoresis, you can observe the DNA fragments. These DNA fragments will appear like a ladder, so that's why it is called DNA laddering.

you can see here in the lane 2 this is the untreated one in the untreated cells when you isolate the genomic DNA you are getting a single band it's a intact the genome DNA is intact whereas in the nanoparticle treated cells you can see that the DNA is fragmented into small small pieces and when you run in agarose gel electrophoresis you get this laddering pattern. So, that is why it is called as DNA laddering.

Let us see how we can quantify the upper process using flow cytometry. Flow cytometry measures the light scattering properties of cells and fluorescent emissions of molecules attached to cells. Here, once the cells are treated with the nanoparticles, then cells can be dissociated and washed and diluted using PBS that is phosphate buffered saline. Then you can stain the cells with the suitable dye.

Once the cells are stained with the suitable dye, you have to strain the cells using the cell strainer to remove the cell clump. Once you do the flow cytometry analysis, what happens is The cells which are stained with for example, the cells are stained with the green fluorescence and some cells are stained with the red fluorescence. So, that can be detected using the detector system and the data can be analyzed using the software in the computer.

Let us learn about the principle of Annexin-PI staining for understanding apoptosis using flow cytometry. The membrane phospholipid phosphatidylserine in apoptotic cells is translocated from the inner to the outer leaflet of the plasma membrane. Thereby, it exposes the PS to the external cellular environment. That means, during apoptosis, the PS will be exposed to the external cellular environment.

And when you use the annexin stain, it will specifically bind to the And if this annexin is conjugated with fluorescein isothiocyanate, FITC, it will enable the sensitive detection of cells undergoing apoptosis by flow cytometry. In addition to this annexin, if you add another dye, such as propidium iodide, it will be easy to identify early apoptotic cells.

In early apoptotic cells, what happens is you will have an intact cell membrane. So that will exclude the PI, whereas the membranes of late apoptotic and dead cells are permeable to PI. So only the late apoptotic cells and dead cells will allow the PI to stain the cells. In this way, we can easily detect cells which are in early apoptosis and which are in late apoptosis. So from this picture, you can clearly understand.

So by using this FITC-conjugated annexin, we can detect the early apoptotic cells and the late apoptotic cells. Whereas this PI will be helpful for understanding the late apoptotic

cells and the necrotic cells. From this picture, you can clearly understand that, as I mentioned earlier, this phosphatidylserine (PS) in the normal live cell, you can see that it is inside the cell. But during apoptosis, in the early stage of apoptosis, you can see here that this PS is

exposed to the external environment, and when you add this annexin, which is conjugated with FITC, these early apoptotic cells will appear green. And when you use the combination of another dye, which is PI (propidium iodide), you can see that during the late apoptotic stage, there is membrane rupture, and due to that, the PI can easily enter and stain the nucleus.

So when you use the combination of this annexin and PI, we can easily quantify the apoptotic cells in the early stage and the late stage, and also we can quantify the necrotic cells. When you analyze the data, you can see here, when you compare it to the control, the treated one shows a higher apoptotic percentage, and each dot represents a cell. So, each dot represents a cell.

Most of the metal nanoparticles induce apoptosis by producing ROS that is reactive oxygen species that can be detected using ROS assay. We can use the flow cytometer to detect the ROS. For that we have to use a dye called DCFHDA which is a non-fluorescent dye. When you add the dye to the cells and this non-fluorescent dye will be converted into another form that is this DCFH DA will be converted into DCFH by enzyme called cellular esterase.

And if there is a more ROS then this non-fluorescent dye will be converted into DCF that is the fluorescent. That means if you have more ROS then you get high fluorescence. The ROS levels can be quantified by determining the percentage of fluorescent cells using a flow cytometer. Here R2 is non-fluorescent cells.

We can get the population of cells as R2 and R3 and R3 is the fluorescent cells where ROH production is there. From this picture you can understand this is a breast cancer cell and this is a lung cancer cell and this is treated with the folic acid conjugated nanoparticle and when you are treating these cells with this nanomaterials you can see this untreated one there is no fluorescence that means

there is no ROS no reactive oxygen species whereas when you treat the cells with the particular nanomaterial you can see here the ROS production is high so that is the fluorescence also high and as I already told you in one of the lecture that is IC 50 IC 50

means the concentration required to inhibit 50 percentage of the growth of cell and when you use the 0.5 IC50 you can see the percentage of the fluorescence is less and

IC50 you are getting approximately around 24 percentage and when you are using the double the concentration of IC50 you can see that the fluorescent intensity also high that means the ROS production is very high here and from this picture you can clearly understand we can also do the time dependent study with respect to the time also we can see the ROS production is high For example, in this case, so this is a folic acid functionalized Fe3O4 nanoparticles and that generate the reactive oxygen species.

And we can see here with respect to the increase in the time, the fluorescence is increasing. That means the nanoparticle is released from the nanofiber and it is inducing more ROS. That is why you are able to see the more fluorescence. And you can also see by using the flow cytometer, we can also quantify the fluorescence. So by using this assay, we can do the time dependent as well as the concentration dependent studies

For understanding the reactive oxygen production by the nanoparticle. Let us see a simple assay for understanding the mitochondrial membrane potential. To analyze the fluctuation in the mitochondrial membrane potential, we have to use rhodamine 123. This is a cationic dye, and it can stain the mitochondria. During apoptosis, the early stage of apoptosis, there will be a change in the mitochondrial membrane potential.

That can be detected by using this rhodamine 123 stain. You can see here in the untreated cells, there is no loss of red fluorescence. The fluorescence is intact. Whereas in the nanoparticle-treated cells, you can see here the significant decline in the fluorescence. That is due to the loss of mitochondrial membrane potential.

This confirms that there is the induction of apoptosis after treating with the nanoparticle. When compared to the ROS assay, if we have more fluorescence in this assay, that means the cells are alive, and the mitochondrial membrane potential is intact. Whereas if there is less fluorescence, that means the cell is undergoing apoptosis and that is why there is a loss of membrane potential.

Finally we can also confirm the apoptosis through gene expression analysis. Here we have used semi-quantitative RT-PCR for analyzing the apoptotic signaling genes and these two genes BCL-2 and BCL-XL these are anti-apoptotic genes and here this is the untreated sample and this is the nanoparticle treated sample. You can see here in the nanoparticle treated

the gene expression is down regulated that means these anti-apoptotic genes are down regulated and whereas these are the apoptotic genes so you can see here these are up regulated that means the nanoparticle is inducing apoptosis based on the various assay we can propose the mechanism of apoptosis induced by the nanomaterial you can see here when you are using this nanomaterial it is inducing the ROS So, which can be detected by using this DCFHDA dye and that leads to induction of P53.

So, that is a P53 mediator apoptotic pathway. So, that can be studied by using the RT-PCR. And we can also understand the mitochondrial membrane potential by using this rhodamine 123 staining. And finally, what happens is, so this will trigger the caspase 3. And this caspase 3 will induce the DNA cleavage and it breaks the DNA into small fragments and that leads to the apoptosis of the cell.

I hope you got the overall idea about the various assays for understanding the apoptotic potential of nanomaterials. Let us go to the lab and learn this technique in more detail. We have seeded 10,000 cells for the AOEB assay. Now we will view the cells under the microscope to confirm their presence in both the control wells and the wells where the test material is to be added.

Next, we will place the 96-well plate in the 5% CO2 incubator for 24 hours. Yesterday we seeded the cells for the assay; today we will prepare the desired concentration of the test material for treatment. Similar to the MTT assay we performed in the last lecture, the test material must be diluted in the cell culture media. Once prepared, we will take the seeded cells from the incubator and treat them with the test material. Here you can see that the cells are well adhered to the 96-well plate.

We have observed that the cells are adhered to the 96-well plate, and now we will treat them with the test material. Right now, we are going to test the material in three wells for the AOEB stain and another three wells for Hoechst staining. In this, the first and the third columns are the control wells, while the second and the fourth columns are the wells where the test material will be added. Now, we will add 100 microliters of cell culture medium to the first and third column wells.

Next we will add the test material to the remaining wells. We will incubate the cells according to the treatment period which will be 24 hours and after incubation period we will observe the results. For the AOEB staining experiment, we will first remove the medium from one of the control wells and add 10 microliter of AOEB stain. We will

incubate it for 3 to 5 minutes and then observe the cells under the microscope for imaging.

Next, observe the cells under the microscope While they are still in DPBS, we will use three filters, Trans GFP, Texas Red and Bright Field Imaging to visualize the live and dead cells. Here you can see live cells in the control with no dead cells observed. We can now save the image.

We will repeat this process for the other two control wells. After completing the control wells, we will now move on to the test wells. For each of these wells, we will remove the medium containing the treatment material and add 10 microliters of AOEB stain. The incubation time should be the same as for the control wells. In the test wells, we observe bright green fluorescence, indicating early apoptosis.

This suggests that the test material has the potential to induce apoptosis. We will save these images as well. We will repeat this process for the other two test wells. In this step, we will perform Hoechst staining on the seeded cells, similar to the AOEB staining experiment. We will begin by removing the medium from one of the control wells and adding 10 microliters of Hoechst stain.

Incubate the wells for 3 to 5 minutes. After incubation, remove the dye and gently wash the well with DPBS to remove any excess stain. Ensure that the cells remain in DPBS to prevent them from drying out. Next, observe the cells under the microscope. While they are still in DPBS.

Hoechst stain specifically binds to the DNA in the nucleus, causing it to appear blue. In this image, you can see the typical nuclear pattern, indicating proper staining. This is a sign of healthy cells that are not undergoing any process such as apoptosis. We will save these images. Repeat this process for the other two control wells.

Once the control wells are completed, move on to the test wells. For each test well, remove the medium containing the treatment material, and add 10 microliters of Hoechst stain, just as done with the control wells. The incubation time should be the same as for the control wells. In the test wells, no fluorescence will be observed in the trans-GFP and Texas red channels,

confirming that the Hoechst dye is working properly. Additionally, we observe fragmented and condensed nuclei under the DAPI filter, which is characteristic of apoptosis, indicating that the cells are undergoing apoptotic cell death due to the

treatment. Capture and save these images as well. In these two experiments, we have performed an apoptosis assay using both AOEB staining and Hoechst staining methods.

As a summary, in today's lecture, we learned various in vitro methods to assess the apoptotic potential of nanomaterials. Thank you for your kind attention. I will see you in another interesting lecture.