Medical Biomaterials
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Lecture - 17
Analytical Tools

Welcome to the course on Medical Biomaterials. We will continue on the analytical tools we have been talking of the various physical, chemical and mechanical techniques for analyzing a biomaterial. Today, we will spend some time on the tools, which will determine the biological response, because when the material is placed inside the body, there are going to be lot of biological responses and we need to analyze some of them.

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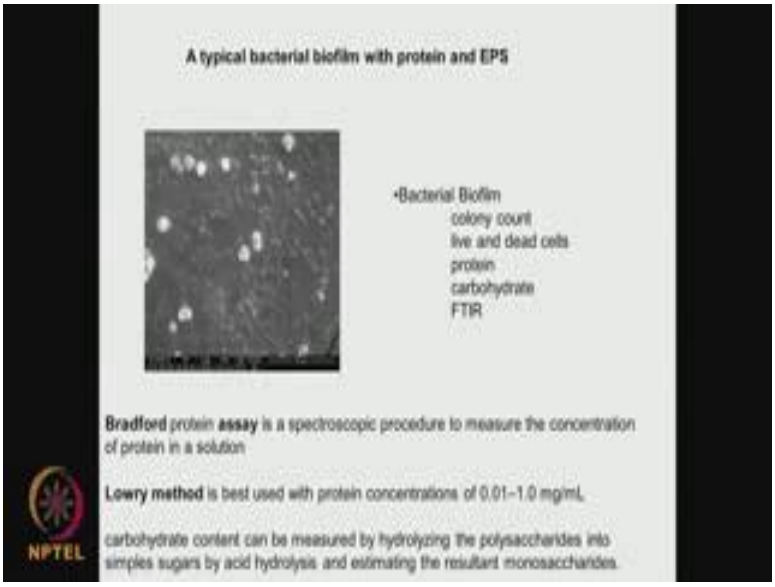

So, today we are going to talk about that. This picture shows a typical biofilm on a material, we talked about biofilm quite a lot, we can see the cells here, we can see some proteins exopolysaccharides all these. So, one is interested to know the details about the bacterial biofilm. So, what are the various techniques by which I can determine the bacterial biofilm one is called the colony count we can count the number of live and dead cells using this technique, sorry we can count the number of live cells not the dead cells that is wrong. So, we can count the number of live cells that is called a colony count.

We can measure the percentage of live and dead cells using a fluorescent technique. So, I will spend more time later on these two. We can measure the protein amount; we can

measure the carbohydrate amount. So, all these are quantifiable biochemical assays. We can also do a FTIR - Fourier transform infrared spectroscopy which talked about in the previous class to determine whether there are amides present, whether there are OH group presence and so on actually. So, this is how we analyze the biofilm on a biomaterial surface.

So, we will talk about this colony count method, live and dead cells later. How do you do the protein and carbohydrate there is something called the Bradford method to determine the protein the amount of protein that is present. It does not differentiate between what type of protein it is or molecular weight. This tells you how much amount of protein is present; it is called the Bradford method. It is a spectroscopic procedure and it can give you the concentration of the protein in the solution. So, what we can do is we can scrap the biofilm from the material, take it into your solution and then using the Bradford method, we can find out how much is the concentration the protein.

There is another method, it is called Lowry's method this is good if the protein concentration is very, very low this is quite accurate, whereas Bradford we can up in higher concentration. So, it gives you a bulk property the amount of protein that is present in the biofilm say of say 1 centimeter by 1 centimeter dimension by using the Bradford or the Lowry's method. If I want to know the carbohydrate, carbohydrate is nothing but sugar various type of sugars what we do is we hydrolyze the polysaccharides into simple sugars by acid hydrolysis, and we can estimate the amount of monosaccharides that are present in the biofilm.

So, this is what you do we hydrolyze the polysaccharides into simple sugars by using acid hydrolysis then we find out how much monosaccharides are there. So, the amount of carbohydrates, amount of proteins that are present in the biofilm can be measured by this particular biochemical tool different biochemical assays. Colony count live and dead cells we will talk about it later.

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If you are interested in looking at the antibacterial activity the antibacterial activity of a material there is something called the disk diffusion method or zone of inhibition method? So, it is also called agar diffusion test, antibiotics sensitivity test against bacteria. So, the different names are there. So, what we do here in this method is we can have disk containing either antibiotics, antibacterial, nano particles, they are placed on an agar plate where the bacteria is growing.

So, we make the bacteria to grow on the agar plate, no we actually plate the bacteria on the agar plate, then we can play keep this disk containing antibiotics and antibacterial or nano particles and then we will incubate this antibiotics will stop the bacteria from growing or kill the cell. So, there will be an area around it, where this bacteria is not growing. As you can see here, there will be area around it where the bacteria are not growing. So, this tells you how strong is the antibiotic or the antibacterial activity of the material which you are testing.

So, larger the diameter of the zone - clear zone then we can say it is got a higher antibiotic activity, smaller the diameter of the zone then it means the antibiotic activity is very low. So, how do you do this? We take an agar plate, we will put in the bacteria spread it all over and then we take disk containing the antibiotic or antibacterial material or nanoparticle which we are trying to test. And the activity we will place it here then we

will incubate the whole plate for say 24 hours and then see whether there is a zone clear zone produced.

Clear zone indicates that the bacteria have not grown near there, because of the action of the antibiotic. Larger the diameter as you can see here larger the diameter that means, more is the antibiotic activity; smaller the diameter then less is the antibiotic activity. This is a very simple and fast way of checking if you are designing any antibacterial surfaces or antibiotic surfaces. This is called a disk diffusion method or zone of inhibition method or agar diffusion method and so on.

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So, there is another way where you do it based on colony count method that means, you can find out how much is the live colonies present on in a biofilm or in a solution. So, colony forming unit that is CFU that is represented in different way is a unit used to estimate the number of viable bacteria or fungal cells in a sample. So, here we are measuring viable bacteria, we are not measuring dead bacteria only viable. So, what we do is culture the microbes on a plate, and then counts the viable cells that have grown. So, if you find too much dense population, we dilute the solution so that for example, when you dilute, and again plate it and then culture it, we can very clearly see the separate colonies because here we are not sure that whether it is only so many colonies or whether there are clumps.

So, what we do is we dilute it. So, we can dilute it 10 times 1 fold we call it that means, a one-tenth dilution or we can do 2 fold that means, one-hundredth or 3 fold – one-thousandth until you get very clear like this. Until you get very clear single colonies which you can count. And then you can multiply by the number of times you have diluted. So, instead of counting in this particular plate, because you do not know whether there is only single colony or there are multiple colonies keep diluting it diluting it until you get it very clearly and well separated out colonies. This is called the colony count method. In this method, we can find out the number of live bacteria. So, here we cannot find the number of dead bacteria.

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If you want to find out the live and dead cells in a biofilm for example on a surface, we use two different dyes we have two dyes they are fluorescent dyes, one is called the green fluorescent nucleic acid stain SYTO9, another is the PI propidium iodide. So, this particular dye green fluorescent dye - SYTO9, it penetrates live and dead cells, whereas the propidium iodide penetrate propidium iodide penetrates only dead cells. So, whereas, the green fluorescent penetrates both live and dead cells.

So, propidium iodide penetrates. This is the fluorescent method. So, when we look under a fluorescent camera, whatever is green we can say it is the live cell, and whatever is red we can say it is the dead cells because propidium iodide penetrates only the dead cells, it sort of neutralizes the green that is present inside. So, wherever you have green we can

say it is live, and wherever it is red we can say dead. So, we can get a ratio of live to dead.

So, if I have a biomaterial and I add these two dyes, incubate it, and then look under a fluorescent and then I can see how much green is there, how much red is there, I can do a surface modifications and then see whether I can reduce the amount of live cells and so on actually. So, dead cells are present because may be the surface is able to kill the bacteria. So, this is also a very useful technique, it gives you both a idea of ratios of both live and the dead; whereas the colony count method tells you how much live cell is there. So, both these methods are complimenting each other because if the bacterial at the biomaterial surface is having antibacterial property, then you will see lot of dead cells also which can be seen using this particular technique.

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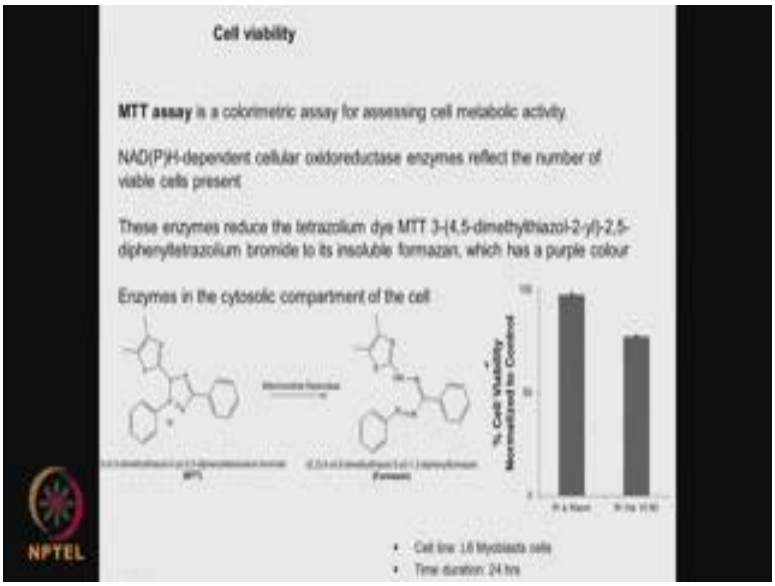

So, far we talked about bacterial we are also interested to know, what is the response of a animal cells to a biomaterial. So, we need to know the cell viability of the animal cell. So, if the animal cell comes in contact with the biomaterial what will be the percentage cell viability. There is an assay called MTT assay, which assesses the cell metabolic activity. So, if the cells are live, they have certain metabolic activity which can be assessed using this particular assay. What is this MTT? So, MTT is a tetrazolium dye this is the name for it is a dye methyl thiazolyl diphenyltetrazolium bromide. So, what happens is when the cells are metabolically active there are some enzymes like cellular

oxidoreductase enzyme which work on this particular dye. So, these enzymes are NAD P H dependent cellular oxidoreductase enzymes and these enzymes sort of reflect the number of viable cells present. So, if the viable cells are very high then the membrane enzyme activity will also be high very high. So, these enzymes reduce this particular dye that is called the MTT to insoluble formazan like this you know. So, this is the MTT and the oxidoreductase enzyme, they reduce to insoluble formazan and which is measured using a colorimetric technique.

Now, these enzymes are present in the cytosolic compartment of the cell. So, by incubating the biomaterial with the animal cells and then later on you add this dye and again incubate it and then the formazan crystal which you produce you sort of solubilize it and then using a colorimetric you find out how much it is. So, that the amount the extent of enzyme activity is the measure of the amount of the viable cells that are present. So, if the biopolymer or biomaterial is toxic, you will have less viable cells. So, you will have less of this enzyme, whereas if the material is not toxic, we will have more of the viable cells and hence the enzyme activity will be also more. So, this is how the assay MTT works.

For example, if you look at this graph, I am testing two polymers and seeing which one is cytotoxic and which one is not against say L 6-myoblast cells L 6 myoblast cells are present in the human tissues and muscles. So, for 24 hours, what you do is you take these cells and you take this particular polymer, incubate it, and then you later on you may add this dye and you get the formazan crystals. Then with respect to the control, you see how much of this is formed and from there you can say- what is the cell viability. So, percentage of cell viable with respect to control, control is without any polymer, just the cell is growing.

So, here we see 100 percent that means, this particular polymer is not toxic to the cells that mean, cell viability is maintained that is why you get 100 percent. Whereas if you take this polymer we get around say about 80 percent; that means, 20 percent of cells have died because of this polymer, so this polymer is toxic with respect to killing almost 20 percent of cell. So, this is how we do this particular assay and it is very useful because a polymer material when it is placed inside the body it should not be cytotoxic to the cells the animal cells.

So, the first step is we do this type of experiment to see whether it is showing 100 percent cell viability or less generally 90 or 100 is ok, even 85 to 100 is also ok. If the cell viability comes down below 85, then you can say the material is cytotoxic. So, we need to reduce the cytotoxicity of the material, so that the cell viability increases further. So, the viability is calculated with respect to the control, control does not have any biomaterial placed. So, it is the normal cells growing. So, this is called MTT assay which is looking at the activity of this oxidoreductase enzyme which is the reflection of the viable cells present.

There is another assay that is called the MTS assay, this is also a colorimetric assay, and it also measures the proliferation in cytotoxicity of the material. So, this is based on the reduction of the MTS tetrazolium compound.

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So, here we use in the previous case we used that MTT bromide here we use m t s tetrazolium compound this compound is reduced by the cells there are some enzymes called dehydrogenase enzymes in metabolically active cells. So, if the cells are active you will find these dehydrogenase enzymes which will reduce this particular material to generate formazan. And again we dissolve the formazan and measure the amount in the previous case we are looking at the cellular oxidoreductase enzyme which tells you the cell metabolic activity that is the MTT assay. Whereas in this MTS assay we are looking

at a reduction of MTS tetrazolium by the dehydrogenase enzyme, again it is a metabolic activity of the cells.

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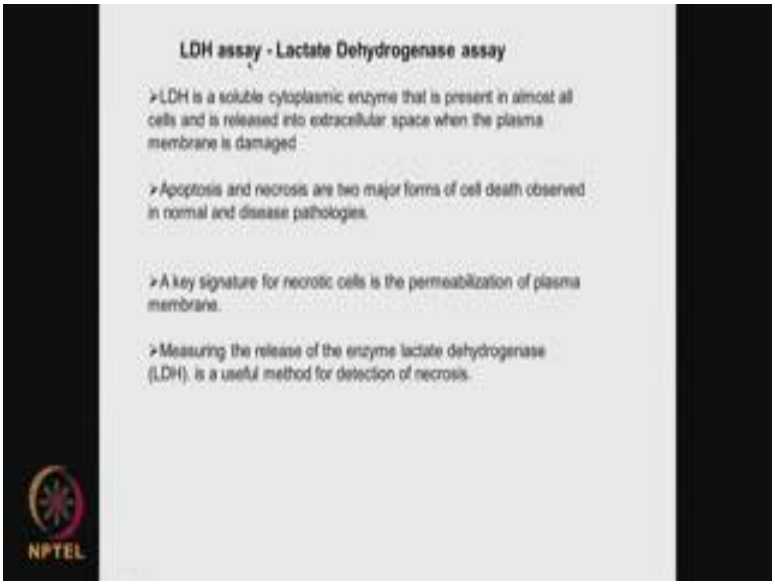

So, two different techniques gives you an idea about the metabolic activity of the cells; that means, they are alive, alive cells produced produce enzymes, and we are monitoring that enzyme then there is something called LDH assay is the lactate dehydrogenase assay. Now, this is the soluble cytoplasmic enzyme, that is present in almost all the cells and when there is a cell membrane damage or plasma membrane damage, this is released into the extra cellular space and you are measuring that. So, the measure of that is an indication that the membranes of the cells are damaged. So, how do the cells die cells die because of two different mechanism one is called apoptosis another is called necrosis.

Apoptosis is a normal cell death that is a programmed cell death all cells are supposed to die through apoptosis necrosis can happen because of a certain toxicity cancer and so on. It is generally happening because of disease pathology apoptosis is the normal. So, when the cells die because of necrosis, there is a permeabilization of the plasma membrane, and this particular enzyme lactate dehydrogenase is released. So, we measure how much is released in the extracellular media which tells you there is necrosis. So, your biomaterial may be causing certain necrotic reaction. So, this is also a very useful assay to find out, whether the cell death is because of necrosis. So, you are monitoring the presence of that enzyme in the extracellular medium.

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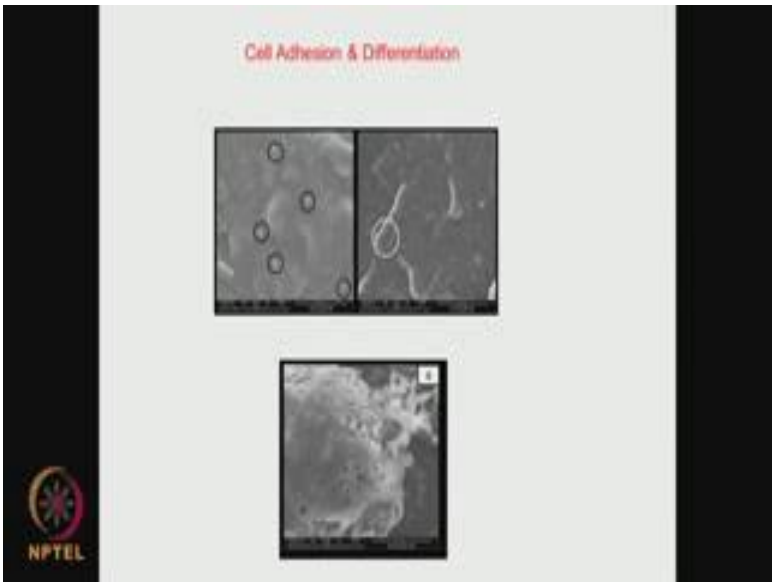

Then if you are doing tissue engineering you want the cells to grow differentiate adhere all those things. So, you can monitor the cell morphology as a function of time, may be 7 days. And see how the cells are growing, whether they are differentiating into spindle like form or whether they are not growing or whether they are dying and so on, so that is called the cell adhesion. This is more a qualitative in nature you can monitor them using scanning electron microscope over a period of time up to 7 days and so on actually.

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Now, we can also look at the biocompatibility of cells on a surface using a dyes like NucBlue this is called a NucBlue stain. So, this dye it stains the nucleus and we can see whether there is a condensation of the nucleus under the fluorescence microscope. So, if there is apoptosis and the nucleus condenses from this dye we can see whether the nucleus is condensed; that means, the cells are dying because of apoptosis which is called the normal death. So, there are different ways of looking at how the biomaterial whether the biomaterial is cytotoxic to the animal cells and sort of what type of mechanism through which the cytotoxicity happens.

So, a first two approach is this MTT assay then comes MTS assay. So, we are trying to look at the metabolic activity of the cells. So, if the cells are metabolically active or live, we can look at the presence of enzymes oxidoreductase and other enzymes so which is monitored using certain stain dyes like MTT or MTS, which is converted into formazan. Then we can look at whether the cells are dying through apoptotic or necrosis by looking a particular enzyme that is called the lactate dehydrogenase. So, if the cells are dying because of necrotic cell membrane damage, this particular enzyme is released, so that is the indication of necrotic death not apoptotic death.

And then we can look at how the cells grow on the surface how they differentiate into spindle form and so on, whether the cells are growing or the material is cytotoxic or creating adverse reaction to the cells. Then we can also look at using a particular dye called the NucBlue stains, what is happening to the nucleus whether they are condensing and because of apoptotic or they are not condensing because of necrosis, so different methods by which we can monitor the cytotoxic effect of a material on cells.

So, for past 3 or 4 lectures, we have been spending lot of time looking at various tools huge number of tools in the area of biomaterials you can see its mind boggling set of tools analytical tools instrumentation tools for looking at the material surface, looking at material character, physical characteristics looking at strength of materials. And then going into thermal properties of the material, then if it is a polymeric type of material you may be interested to know the molecular weight then we have a microscopic techniques, where you are looking at the surfaces of a materials, you are looking at the biofilms that are formed; the morphology of the biofilms using different types of a microscopy.

Then spectroscopy techniques like a UV visible spectroscopy, Fourier transform infrared spectroscopy, which tells you functional groups present type of compounds or organic that are present on the surfaces. So, many different types of techniques we have looked at. And as we said biomaterial requires lot of analytical tools and analytical techniques and you cannot be expert in all these, but we need to have get support from different experts. Then came the biological techniques one is related to bacterial. So, we looked at the biofilm that is formed, the characteristics of biofilm like number of live colonies, ratio of live dead cells, the protein that is present the carbohydrate that is present.

Then finally, we looked at response or interaction between animal cells and the biomaterial whether the biomaterial is causing cell death is there a change in the viable cells, what type of a effect it is producing whether it is necrosis or whether it apoptycis and so on. So, lots of tools have to be used, if you want to be very successful in the area of biomaterial research.

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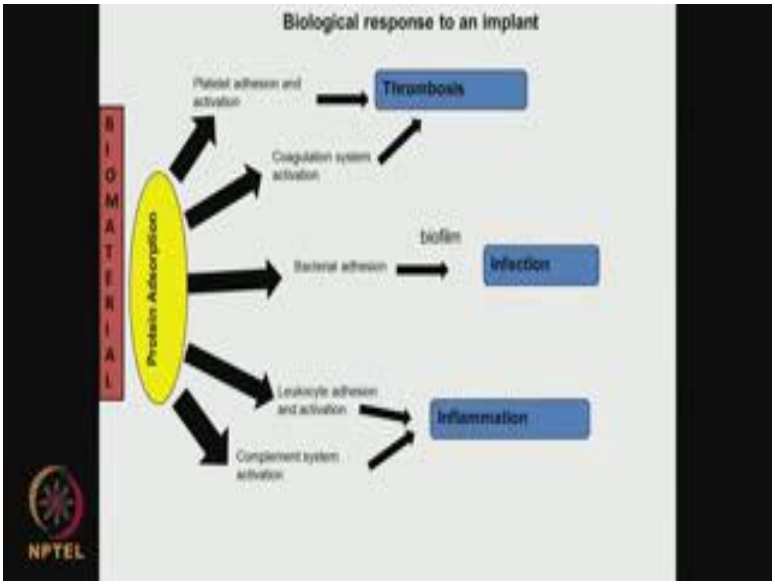

Now, let us proceed further as soon as the material is placed inside the body, there is a biological response that is happening. I did introduce little bit of it and of course, I think in a next couple of or 3 lectures, we are going to talk little bit more biology. And without biological details you cannot become a real expert in the area of biomaterials. It is not like you just synthesize a metal or a polymer and then give it away not bother about what is happening what is the response between this material with the biological fluid,

biological component we need to have little bit of understanding of that. So, I am going to spend the next two three lectures on that and you are going to come across many biological terms. So, you do not have to get scared about it, but you need to know that.

So, as soon as the biomaterial is implanted there is protein absorption that is taking place that we could call it the initial the fast response that takes place. Then we have platelet adhesion that means, all related to the blood components and adhesion and activation, some platelets may get activated which may lead into thrombosis. There could be coagulation activation that also could lead to thrombosis. So, platelet adhesion or coagulation all related to the blood. So, one need to know in details these aspects if the biomaterial is a blood contacting device. For example, a small diameter vascular graphs, or large diameter vascular graph or cardiovascular stents or diaphragms or heart patches. So, all these are blood contacting devices. So, you need to understand some of this platelet adhesion as well as coagulation activation.

If we are talking about infection, infection can happen irrespective of the location of the biomaterial. So, we need to understand about the bacterial adhesion. I did spent some time on biofilm formation which leads to persister cells and lot of infection. Now, if we look at the nucleus side addition and activation complement system activation all this leads to inflammation. So, a material which is placed also because of may be platelet ization, thrombosis infection and end up having inflammation, and finally the material could get rejected there could be systemic effect and not only the local infection, but even the systemic inflammation could happen.

So, these factors lead to inflammation. So, we have many things happening, blood contacting device undergoing thrombosis, infection happening at all parts of wherever the biomaterial is placed, where you can have inflammation which is slightly at a later date, inflammation having a local inflammation as well as systemic inflammation. So, so many biological response responses happen because of the placement of the implant, whatever be the type of implant that has been placed. So, the protein absorption takes place here large number of proteins take part in the early stages of the protein absorption, some of the absorption is favorable and some of them are not desired at all.

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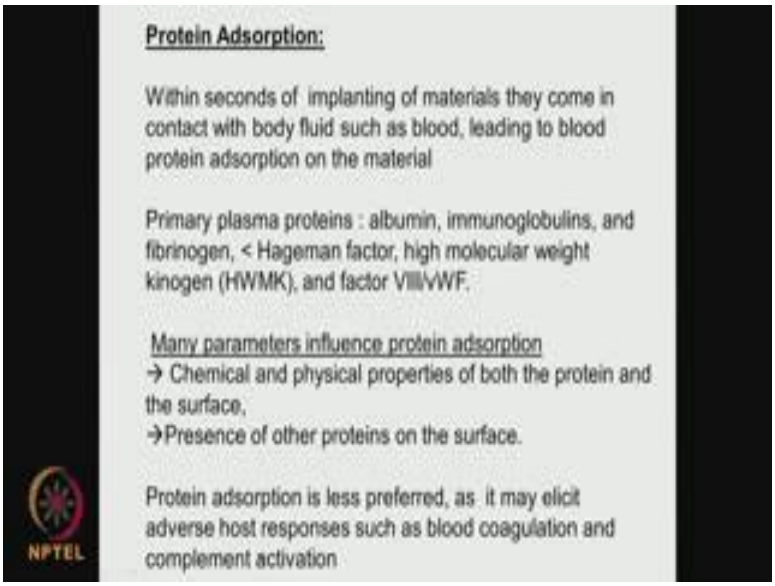

So, protein absorption, what is this protein absorption, within seconds of implanting the material they come in contact with the body fluid that means, the material coming in contact with body fluid such as blood leading to all the blood protein getting absorbed on the material. I did talk about fibrin fibrinogen and so on little bit in detail in the previous lecture, so within seconds that happens. So, these primary plasma proteins like albumen human serum albumen immunoglobin fibrinogen and little bit of hageman factor, high molecular weight kinogen, and factor 8 vWF. And all these proteins come in contact with your material and they start interacting with your material get absorbed. So, many parameters influence this protein absorption one of them is the chemical physical properties of both the protein and the surface that is the biomaterial surface and presence of other proteins on the surface. So, are there any hydrophobic proteins present hydrophilic any charged proteins and so on actually.

Generally protein absorption is less preferred because it may illicit adverse host response such as blood coagulation and compliment activation. And also in some cases I did mention bacterial attachment also increases if there are some proteins and that are already present on the biomaterial. So, we would like to prevent that also in some situations. So, we will talk about all these more in detail in the next class.

Thank you very much for your time.