Tissue Engineering
Prof. Vignesh Muthuvijayan
Department of Biotechnology
Indian Institute of Technology, Madras

Lecture - 15
Material Characterization - Part 3

Good morning everyone. So, in the previous classes, we have learned about what are surface properties and bulk properties and how to characterize the surface properties and Mechanical Characterization of the materials.

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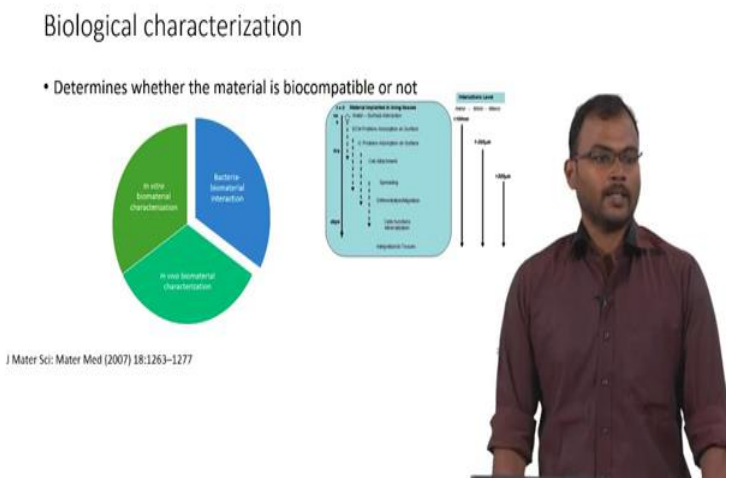

So, coming to the final characterization, which is biological characterization. This is the end game that determines whether your material is biocompatible or not. Whatever implant or devices you are introducing will be taken by the host system or will be rejected by the host system. In biological characterization, there are three major categories. One is in vitro biomaterial characterization, and another is in vivo biomaterial characterization, and apart from this, there is a separate section, which is bacteria biomaterial interaction.

In vitro is where you will do all the related biological experiments on a lab-scale environment. In vivo, where you will experiment with the material in animal models like mice, pigs, canine species. Bacterial interaction is mainly needed when you are looking for infection on the material surfaces. When you are implanting material into the body, so within a nanosecond, the proteins will get adsorbed onto the material surface. Based on

the protein adsorption, it determines how the cells are adhering to the material surface, which will lead to the host cells, and material interaction. If the cell attachment continues, it will lead to the spreading of the cells and then which will lead to differentiation and migration.

So, all these steps will lead to the complete integration of material into the body. Based on the application, you have to decide what type of interaction you need. For example, if you need an interaction between the cells and the material for the application such as orthopedic implants, wound dressing, so you need your material to be integrated with the host cell. But, if you are looking into cardiovascular devices where you do not need any cells to adhere to the surfaces. Here, you should avoid the cell attachment on the material surfaces.

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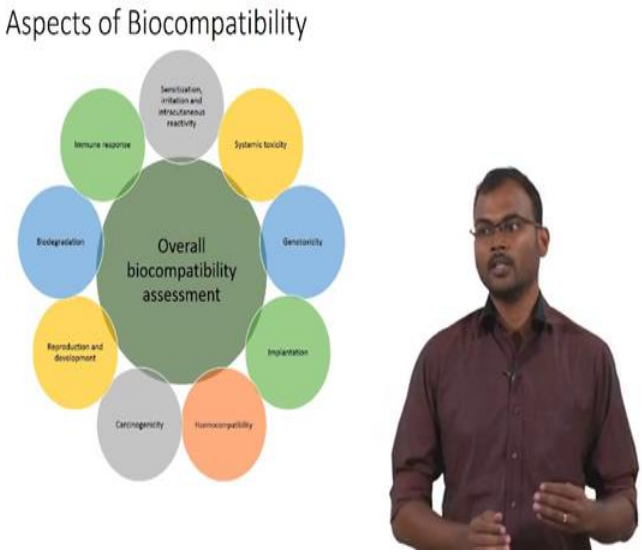

Coming into what are the aspects you have to look for the material to be biocompatible. There are a lot of aspects based on again application. Until now, whatever the surface property and the bulk property you have analyzed or characterized, those can be changed based on the results you are getting. But for this biocompatibility, it is entirely dependent on how the material is interacting with the cells. So, what type of cells you are using and what type of experiment you are doing, based on that, it will interact with your material. Whatever material you have characterized and made suitable for your application, it should be attached to the cells based on how effective it can be integrated into the system.

Coming into aspects first is sensitization, irritation and intracutaneous, reactivity. This is mainly applicable to wound dressing materials. So, what happens is when you are putting a wound dressing on a particular wound, it should not cause irritation, or it should not produce any allergic reaction on the surfaces. We have to test for that. Then systemic toxicity involves what duration of toxicity you are going to observe over the period of time.

Toxicity, which is a commonly used term for studying the biocompatibility of any scaffolds, hydrogels, or materials; so, what you are basically going to do is, whether the material you have produced is leaching out any compound or it is interacting with the cells and killing the cells. We have to check that using systemic toxicity. Genotoxicity represents how material causes damage to the genes; Thereby, it leads to mutation.

Implantation mainly observed when you are surgically implanting a material, which can affect the nearby tissues and organs also. If you are using orthopedic implants, it can have an effect on nearby bones and nearby muscular tissues also. Based on where you are implanting, you have to find out the factors affecting that. Haemocompatibility is primarily involved in cardiovascular applications, where you need your material to be fully blood compatible.

Carcinogenicity is again linked to genotoxicity or the mutagenicity where, if there is a mutation, it can lead to causing cancer-causing effects, which will lead to further rejection of your implants. Reproduction and development, it is not primarily used for all the materials. But if there is a possibility that your material leachate or your nanoparticles or the molecules, which you are using can affect the reproduction system or the embryonic development stage, it has to be checked for that.

Biodegradation it is used for a lot of application such as vascular sutures and staples where you are using that material, and it should be degraded over the period of time, so you have to check those things and all. How the cells will degrade those materials and all you will check in that biodegradation. The immune response is basically involving the total immune system, whether the introduced material has any antigenic effects so that it will trigger an immunogenic cascade. So, that it will lead to inflammation of the site of implants. If you are introducing a metal orthopedic implants if it is causing leach out immunogenic responses, that place will have an inflammation occurring. So, these are the major aspects.

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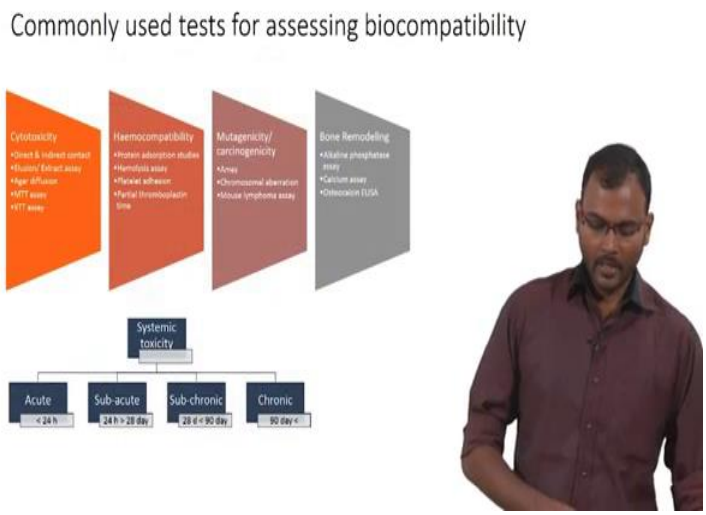

These are the commonly used tests for assessing biocompatibility and cytotoxicity, which is the initial and mandatory test we have to do for all the materials. Whether that material is killing the cells or it is having an inert effect, or it is promoting the cell growth and so on. Then, haemocompatibility where you have a blood-related application. Mutagenicity and carcinogenicity, where you will check for any change in mutations that occurred in the system. Then bone remodeling, it is a separate topic where you can estimate how much of the material is affecting the bone integration or bone growth, so that is bone remodeling.

For cytotoxicity, I will explain in detail all the assays. It has direct and indirect contact and a couple of other experiments MTT assay and XTT assay, which is used for calculating how much your material is toxic to the cells. Haemocompatibility, where these experiments will tell you whether your material is not causing any damage to the blood cells or it is leading to any platelet aggregation or blood clot formation.

In mutagenicity and carcinogenicity, Ames test and chromosomal aberration and mouse lymphoma assay. So, in the Ames test, we can do it in a bacterial study, where a bacteria usually a salmonella species will be there. In that one, we will have a mutant that is having a lack of histidine gene. So, if there is no histidine supplied in the media, the bacteria cannot grow. You will introduce your compound, so if it is causing a mutagenic reaction on the gene, it will convert the histidine, and it will grow; so, that is the basics of the Ames test. By this, you can observe whether the compound you are using is causing a mutagenic

response or not. For all these tests, that would be a control where you will have a normal bacterial gene, which has a histidine utilizing gene. So, if your compound is not enhancing that, it can suggest that your compound is only changing the gene of the histidine lacking species.

In chromosomal aberration, we will check how the chromosome is after mitotic division, we will check with the compound and without compound. If there is a chromosomal abbreviation, there would be either an insertion, deletion, or translocation observed on the chromosomal structures, so that can be observed in chromosomal abbreviation.

Mouse lymphoma assay is similar to Ames assay, but we will do it in mouse lymphoma cells. In the cells which will have a thymidine kinase enzyme, that would be knocked out in a knocked out mouse. They will supply thymidine, so if your compound can mutate the cells, then it can use that thymidine. By that, you will understand that your compound is causing any mutagenic responses or not.

In bone remodeling, the main factors we will look up are alkaline phosphatase assay. When you are using a dental implant, orthopedic implants, bone cement, they had to integrate into the host cells, osteoblast cells for remodeling. This leads to the formation of increased calcium content, phosphate content, and osteocalcin is the major molecule for the bone cells.

If there is an increase in these factors, you can actually estimate them using these assays. Based on this, you can identify whatever material introduced has an effect on improving the bone cell integration within your scaffold.

Student: What is the role of the ALP enzyme in bone growth?.

So, ALP enzyme what it does is, it converts phosphates to inorganic phosphates. Other phosphate derivatives into inorganic phosphates, those phosphates, are the major content of bone. They are the basic components of calcium phosphates, so there are a lot of phosphates; based on that, you will understand if there is a higher activity, then there is a faster growth of bone tissue.

Systemic toxicity determines how long the implant you are going to use it in a system. The acute system is where you will use it less than 24 hours, such as catheters, to do angioplasty

and other treatments. Sub-acute is 24 hours to 28 days. Then, sub-chronic 28 days to 90 days urinary catheters, it would be within 2 months, or 3 months and then they will remove it. Then chronic, which is above 90 days. You have to test whether the material is not having any effect on the cells surrounding it, so those chronic will involve all the bone implants and cardiovascular implants used permanently. Based on this duration, we have to check whether the material up till that time, it is not having any adverse effect on the host system.

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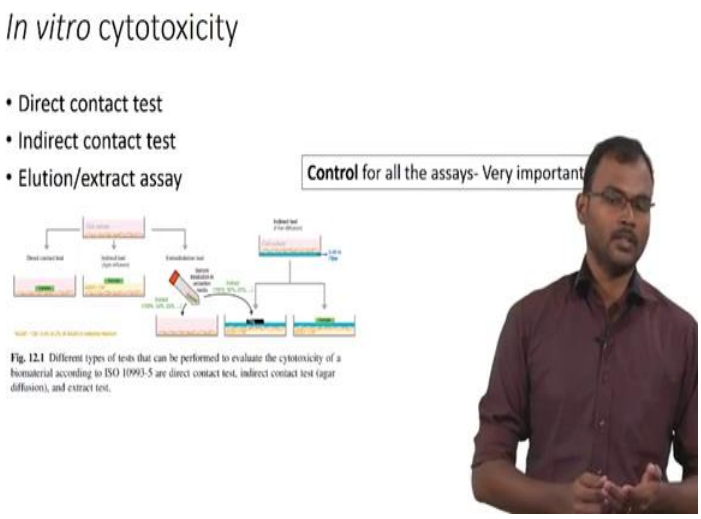

Coming into toxicity, this is a primary test we will do for all the material, either it is a hydrogel, scaffold, or nanoparticles, whatever the material. We have to check whether it is accepted by the cells or not. In general, this can be done based on three different methods; direct contact, indirect contact, elution, or extract assay. All this variation is based on what is your sample and what type of sample you are having.

Direct contact is where you can see the figure. The first one where you have grown cells on the tissue culture plate, and you put your sample on the top of it and check whether it is affecting any of the cells underneath it. So, that can be done for your nanoparticles or sample having not too much load. If you have too much load, it can destroy your cells usually.

An indirect test, which is done using an agar layer on the top of the cells, and your sample would be there. In that, if your sample is leaching out some compounds or some erosion

or some other drugs are releasing from the material, then that can leach out and affect the cells underneath it, which is indirect contact assay.

So, indirect contact can be done based on how it is interacting with the cells also. As you can see in the right-hand side top figures, where you grow the cells on a filter membrane, so then after the cells adhere to the surfaces, you invert it, and then you will put the sample on the top. So, your sample is not exactly in contact with the cells, but the leachate of your sample would be going into the cells and observed if it has any effect, it will kill the cells, or if it promotes the cells, it will have that effect.

Extraction or elusion assays are used for soft materials, where you can keep that material in a media for 24 hours or 48 hours based on your application, then you have to extract the media. If there is a leach out from these compounds, you can take that media and culture it in that with the cells or with the indirect test method. So, you have the cells; you have the extracted media; you can test whether that leachate has any effect on the cells. All these things are based on the sample size and how effective the leachate is coming out of the sample.

If there is no leachate coming out of the sample, it will not have any effect. For that, you can move on to the next one of the tests, which involves cell adhesion and proliferation. All these experiments can be done for those assays also where you keep your sample at first; then, you seed the cells on top of them. At this one, toxicity measures how much of your material is toxic. If you found out that material is not toxic, not causing any effect, for all these assays, then you can go for cell adhesion experiments.

Cell adhesion is very important when you are integrating your material with the host system. For that, you will have the sample at the bottom, and the cells would be seeded at the top of the material. Over the period of time, if the cells adhere based on your material surface, that can also be estimated. For all the assays while involving biological characterization, control is very important; you should have a positive control and negative control. Negative control there would be a standard material.

If you are testing orthopedic implants, there would be reference material like stainless steel. So, you have to test that material for cytotoxicity. The negative control will not have any adverse effect on the cells; there would be a positive control also. The positive control will have a maximum killing effect on the cells. Based on those you can identify the range

or concentration, you can decide whether your material is cytotoxic or not. Usually, according to literature, if your material causes below, below a 70 percent decrease in the cell viability, then that material is not suitable for the biological application.

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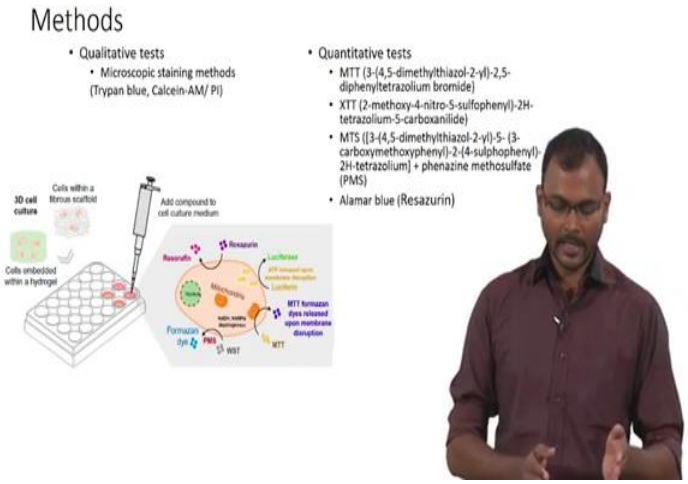

Now we learned how to experiment and identify your material is toxic or not. So, how exactly you identify that thing? That can be done by qualitative and quantitative assays. Qualitative involves you observe them visually using a microscope so that you can use a normal dye like trypan blue or calcein-AM and propidium iodide.

Calcein-AM will go inside the cells, and it will bind with the cytoskeleton, and it will give a green fluorescence; and propidium iodide, which will go and bind to the nucleus and it will give a red color. If there is cell death, you can use these stains and identify whether the cells are alive or dead, you can observe them under the fluorescence microscope. So that is a qualitative test.

And the quantitative tests, there are a lot of assays available. These are the commonly used ones. MTT, XTT, and MTS all these are tetrazolium salts. So, what it does is, so using electron receptors will convert into formazan. So, the formazan will give a different color. In MTT, what happens is, it will go inside the cells and mitochondrial dehydrogenase, it will reduce the MTT, and it will form formazan crystals.

In MTT, it is a final step of the reaction, where you will kill off all the cells. But in XTT, MTS, and Alamar blue, all cells would be alive. In MTT, the formazan crystal formed is inside the cells. So, you have to estimate how much quantity of formazan has been formed. So, you have to rupture the cells and dissolve them using a solubilizing agent like DMSO and all to check the absorbance of the formazan crystals formed.

Whereas, in XTT and MTS, which are water-soluble tetrazolium salts. It happens outside that cells itself with the help of phenazine methosulfate, PMS, it involves a plasma membrane electron transport, which converts these tetrazolium salts into formazan dye. And without rupturing the cells, you can directly dissolve it in a culture media or the buffer and check the amount of formazan crystals formed.

Student: That formazan formed is not insoluble.

In MTT.

Student: No in XTT and MTS.

No, it is water-soluble.

Student: So, can you use the same cells again for some other experiment?

Yeah. You can wash those cells, and you can observe it, but if you keep on repeating that, the cells will obviously die. You can immediately takeout, and you can use that.

Next is the Alamar blue assay, which involves resazurin dye. This dye involves a cytoplasmic redox reaction, it goes into the cells, and due to the redox reaction, it will convert into resorufin, which will give a pink color. Compared to these above three methods, this is a highly sensitive method where you can estimate even a very small amount of cells also. And it has another advantage that it has a fluorescence property. So, using a fluorescence spectrophotometer, you can calculate even a minor change in the cell quantity.

So, for all these things, there would be control, where you do not treat your cells with any of your samples. And there would be another control where there will not be any cells. Whatever the material you are using will be in the media because those controls are very important. All this involved colorimetric assays so, if your material is changing the media

pH or adding colorimetric value to the system, then, the results obtained can be of a false positive. To avoid that, we should always have control over all of the assays. So, these are the methods for cytotoxicity.

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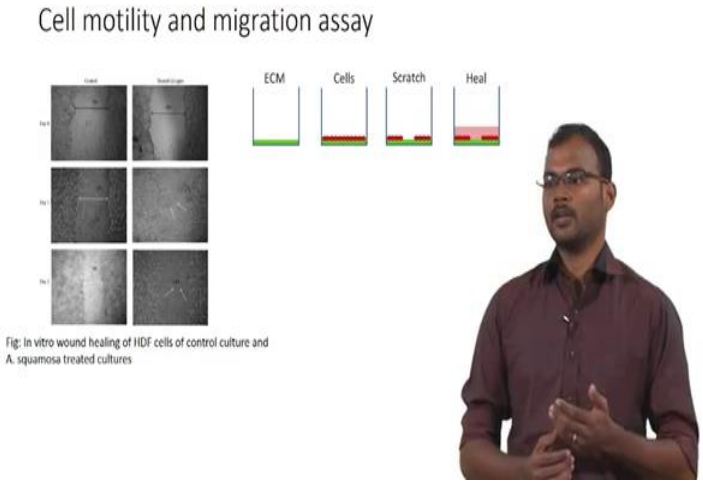

After cytotoxicity, if your material is not toxic, you can go for the cell adhesion. Cell adhesion involves the same list of procedures, where you can put your sample underneath the cells before seeding. In cytotoxicity, what you do is, you seed the cells, you grow them. For all these animal cell lines, it has to have a substrate to attach on to the surface. If you have a tissue culture plate, your cells would be properly spread on that tissue culture plate, and then you put on your material if it is causing toxicity that would be observed.

In cell viability or the cell adhesion assay, what you do is, you put your material, and then you seed the cells on to the material surface. Material means any flat surface material or scaffold. Based on your surface properties, whether it is hydrophilic or it has any ligand for the cell receptors, the cells will attach, and you can estimate the amount of cells attached to the material you are having.

Coming into cell motility and cell migration assay, these are the next stages of characterization, where it is predominantly used in wound healing application. In wound healing application, what you have is, there would be a wound separating two tissue; you have to join them. That can be done using this scratch assay.

You have a material you seed the cells on to the material, and you scratch them and introduce whatever compound to check for the healing property. So, you put that compound and check whether how the wound is getting closed. As you can see in the figures, the left side is control samples, and the right side is treated samples. So, they have first grown cultures. Then, using a small tip or spatula, they create the wound. On the left-hand side, there will not be any treatment. On the right-hand side, they have used the Annona squamosa extract, which is an extract of custard apple leaves. They have used it and observed the wound closed within two days. So, it has a wound healing property. By this, you can identify how fast your wound can be healed using a different concentration of the compound and different compositions.

Student: What are those HDF?

So, HDF is Human Dermal Fibroblasts. So, it is a skin cell, dermal fibroblasts. Also, whatever the material you are using, if it is non-toxic to one type of cell does not mean it is non-toxic to all types of cells. Each cell has a different property and a different mechanism.

Based on the application, if you are going for the bone application, you have to check with the osteoblast cells. If you are going for the vascular application, you have to check with the endothelial cells. If you are going for a wound healing application, you have to check with fibroblasts cells. Each cell has a different attachment property and different effects on material surfaces, so that you have to always think before choosing the studies.

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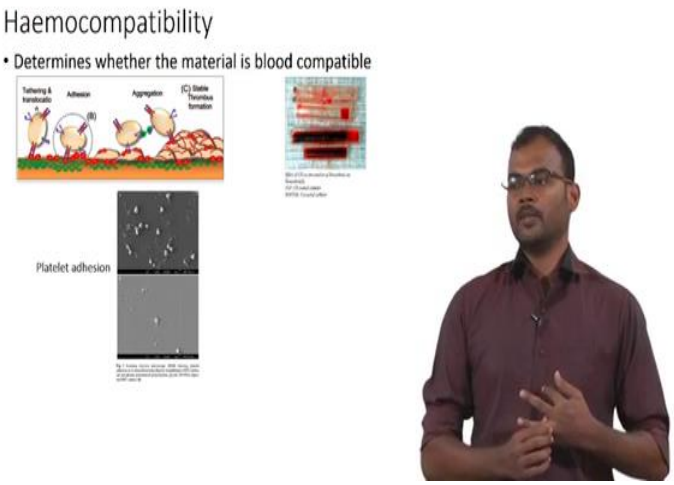

Another important segment in biocompatibility is haemocompatibility. Haemocompatibility is specific to the blood-related applications. If the material is haemocompatible, it has good blood compatibility. For example, if you consider a heart valve, vascular grafts, there you have continuous contact with the blood throughout the system and the time; At the time of implantation and at the time of removal so, there would be a continuous contact of the blood cells with the material.

What is thrombus formation? So, a thrombus is a blood clot, which is formed by adhesion of platelets and fibrin mesh activated on to the surfaces. Initially, the platelet will adhere to the material surface then aggregation happens. If one platelet gets adhered to, it will send out extracellular signals which will activate the surrounding platelets. It will act as a homing beacon for making the other platelets to come into its nearby area, and it will form an aggregate that is called platelet aggregation. When that stable platelet aggregate has been formed, there would be a parallel reaction; coagulation cascade happens, which forms a fibrin mesh.

That fibrin mesh acts as a network, which will trap these platelet aggregates and form a blood clot. If you have a vascular graft, and if a blood clot is formed on its surfaces, it will lead to stroke and other complications. In the right-hand side, it is an example of how surface modification can improve the haemocompatibility.

So, the top one they have used the CTI coated catheter. CTI is a corn trypsin inhibitor, which will inhibit the clot formation, and the bottom one is without any coating. As you can see, based on this study, you can identify a material that can avoid clot formation or not. What are the experiments you can do for haemocompatibility? The main experiment is platelet adhesion because that is the initial step of thrombus formation.

That can be observed using SEM; you incubate your sample with platelets, you extract healthy platelets from human volunteers, then you can check how the platelets are adhering to the material surface, and you can observe it under SEM. Or you can identify quantitatively using previous cytotoxicity experiments; you can do MTT assay also. There is an assay called lactate dehydrogenase activity assay, which involves the live platelets using the reductase enzyme; Using a reductase enzyme and forming a colorimetric product, which will lead to quantification of this platelets.

For all these things, you should have a standard; you have to count the number of cells and what is the number of cells having that effect. Like in MTT, for the particular absorbance, what is the number of cells? So, you have to make a standard; from that, only you can calculate the actual number of cells on your material surfaces or how much cells been reduced or increased from the control. Standard is very important for all these biological aspects.

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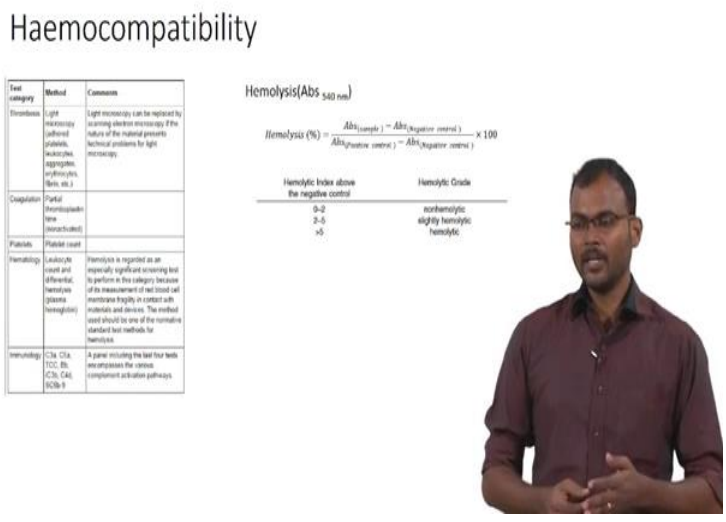

Other than platelet adhesion, there is a commonly used assay, which is called hemolysis. Hemolysis, as the name suggests the lysis of the erythrocytes. Whenever your RBCs are broken down, it will release hemoglobin that can be estimated using UV visible spectroscopy. So, based on the standard international organization for standardization, the hemolysis index should be below 2 for a non-hemolytic material; it should not have any clot formation on the material surfaces and all. If it is slightly hemolytic, the range is 2 to 5, and if it is highly hemolytic, it is above 5, which is not at all recommended for any applications. Hemolysis is calculated based on

$$Hemolysis\ (\%) = \frac{Abs\ of\ sample - Abs\ of\ negative\ control}{Abs\ of\ positive\ control - Abs\ of\ negative\ control} \times 100$$

So, what are the negative and positive controls are negative control is a normal saline solution which does not have any effect on the RBCs. A positive control can be either water or triton X, where it will lysis the cells immediately on contact with RBCs. And the sample is where whatever sample you are testing to check whether it will have any effect on RBCs. Based on this, you will find out the hemolytic index and identify a material is blood compatible or not.

Other than these methods, there is a partial thromboplastin time, which is to estimate the amount of the time required for the thrombus formation. So, you have your control material, and you have your sample material. You put on your blood sample in both of them, and you add a coagulation factor in both of them. This coagulation factor will lead to thrombus formation. So, if your material is non-hemolytic, which is avoiding thrombus formation, there would be a delay in thrombus formation. Whereas in this reference material, it would be immediately formed, based on that, you find out the partial thromboplastin time. And you can look into markers of coagulation cascade like C3a, C5a. So, all these are there is a series of a pathway involved in the coagulation cascade.

So, you can check for the particular markers, whether it is an inactivated or activated state. From that, you can identify that the material which you are inserting is activating the blood cells and causing this coagulation cascade. Because, it is a serious issue that if a small clot forms, if it gets detached from the surfaces, it can go and block somewhere in the arteries where it is called emboli; that movable clot is called emboli.

So, that can block the arteries, and it can lead to serious issues. When you are looking for a blood-contacting application, you should always do the haemocompatible studies.

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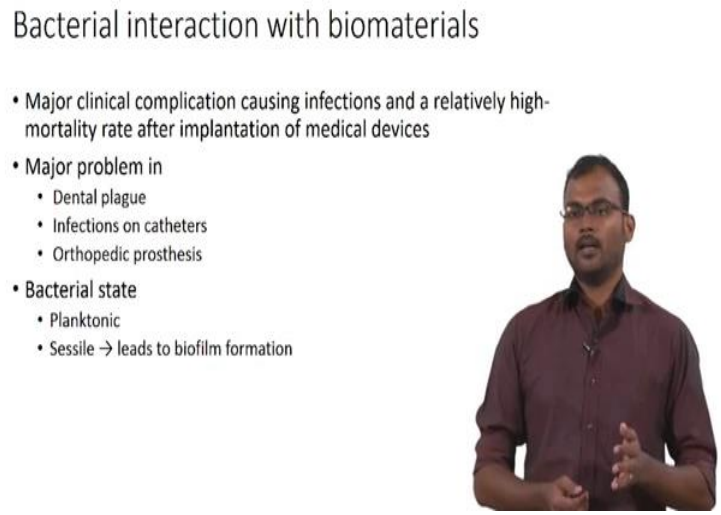

Coming into bacterial interaction with the biomaterials, one of the major complications immediately arises when you are introducing a material into the system is an infection. If there is an infection, there would be an immediate rejection; because, if there is a bacterial infection on the material surface, it can lead to immune responses and all other toxic to the cells, so, it would be immediately rejected. We have to modify the material as much as possible to avoid any bacterial adhesion and forming a bacterial adhesion on the surface.

This is majorly observed in dental plagues, where you have dental implants in which you have a lot amount of bacteria exposed to the material. And infection on urinary catheters, so urinary catheters, there would be a lot of bacteria excreted. Orthopedic prosthesis; there also, if there is a little bit of bacterial interaction, is there, leads to infection and serious issues. In normal conditions, the bacteria would be as a planktonic state, which is a suspension in the liquid solution. But when this bacteria is adhering to the material surface, it becomes a sessile state which will lead to the formation of biofilm.

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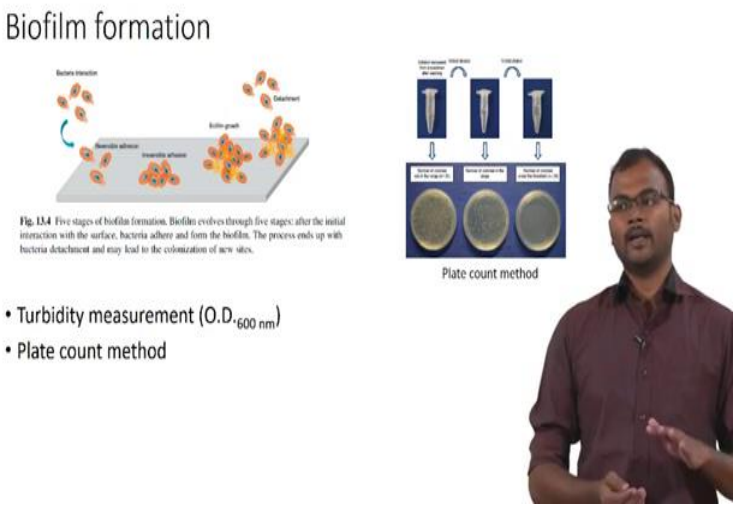

What is biofilm? So, as a normal bacterial adhesion, it is reversible. Due to the shear force, it can be easily removed off from the surfaces. But, when a bacteria adhere, and it forms aggregates and all it then changes its metabolism and secretes exopolysaccharides. These exopolysaccharides what they do is, they cover these bacterial aggregates and form a thin biofilm. So, these exopolysaccharides will protect them from the immunogenic cells, and thereby it will be there for over a long period of time. And whenever the host immune system is low, these can come out of the exopolysaccharides, and it can infect the nearby tissues and cells.

Biofilm is a major problem, and your material should be avoiding biofilm formation as much as possible. For biofilm estimation, there are two basic methods; one is turbidity measurement, and another one is a plate count measurement. In turbidity measurement, you measure the number of bacterial cells based on the turbidity observed at O.D of 600 nm; it is a standard for observing bacteria, you can estimate the number of bacteria.

You have the control sample, you have your testing sample, you inoculate the control with the bacteria, and you have your sample and inoculate with the bacteria. And after a particular amount of time, when the bacteria reaches its log phase, you will check for how much of the bacteria lead to adhere to the material surface. From that, we have to wash out the bacteria adhered to the surfaces and estimate using a UV-visible spectrophotometer.

Student: Does adhesion is mainly by gram-negative bacteria? Because only they have the lipopolysaccharide membrane.

It's not exactly by gram-negative bacteria, it depends on the material properties also, however, predominantly it is on gram-negative bacteria. But, if your material has very rugged surfaces, bacteria can go and bind into the pits and cones available. It can bind to the depth of the material surface, and it would stay there for a longer period of time. So, when it leads to aggregation, then it forms a biofilm, both gram-negative and gram-positive.

Student: They do not study the interaction of a fungal with biomaterials.

Fungi infection it is not widely studied; because for fungi infection, we need different environmental condition than bacterial condition. But there are some fungi infections that still affect the materials. And it can be easily killed off fungi. Bacteria, even if you sterilize it, it can form a cyst and be available for a longer period of time, even they study the sterilization techniques for the materials. After sterilization, how much sterilization residual is still there, that also has to be studied according to the standards. So, bacteria are the major problem rather than fungal.

You are coming into the plate count method, where you estimate the number of bacteria adhered using a colony-forming unit. The procedure involves similar to turbidity measurement; in the plate count method, you inoculate your sample with the bacteria, and you take out the sample, and you wash off the biofilm using solubilizing solution, you dilute those samples, and you spread it on an agar plate. Based on how many colonies formed and every single cell will form a single colony. Based on how many colonies it has been formed, you can estimate the number of bacteria adhered to the material surface.

The optimum plate count should be around 30 to 300. As you can see on the left-hand side, its a very dense one where it's not an accurate method, and on the right-hand side only below 10 below 30 is there, so that is also not a standard one. It should be in the mid-range between 30 to 300 so that it will give you the correct value of bacteria adhered to the material surfaces. For all these things, you have to characterize your materials before that surface characterization and bulk characterization so that you should have the idea that it should avoid the bacterial adhesion, and it should promote the cell adhesion; It should

avoid the platelet adhesion. So, all those things you have to do before the biological characterization.

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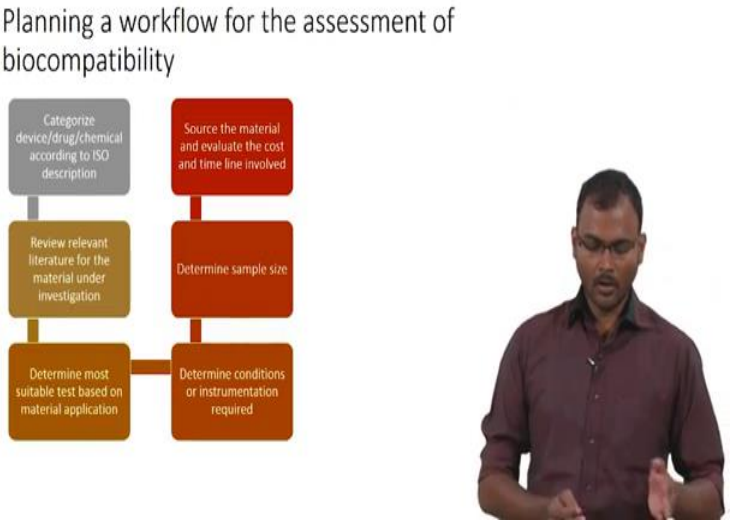

Overall flowchart for how you can plan for producing material. First, you have to categorize what type of device either an orthopedic implant or you are going for a wound dressing material or hydrogels or the nanoparticles. Based on the application, there is a lot of standard descriptions given already on ISO, ASTM, or FDA. They have proper standard protocols; you have to do all those tests for your material. Then based on the relevant literature, you have to identify whether that will have the effect based on your particular application. Then you have to understand what are the testing condition and instrumentation needed. Then the sample size. Based on the sample size, also, the cytotoxicity varies.

There are certain guidelines given by ISO that, if you are using a nanoparticle ratio, how many, how much of the amount of nanoparticles you can use for how many numbers of cells available. Because when you are taking it to in vivo studies, you will consider for body mass ratio. If you are taking a capsule or drug, that would be based on how much bodyweight. So, the sample size is important for materials also.

If your material has toxicity, if you have a bigger sample, you will not use that much big sample when you come to the application so, you have to reduce the sample size. Then, you have to finally go for the cost and time involved in developing that material.

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So, these are the standards given by ISO. Each of these standards has a different set of guidelines for the performing different applications, such as 109935 which only involves in vitro cytotoxicity; whatever the toxicity experiments I have mentioned earlier, it will give you the clear guidelines of whether if your material is flat surface how you can do; and if your material is scaffold or hydrogel, how you can do. Like that, each of these standards will give you what are the parameters you have to follow for the characterizing the biological compounds.

If all this characterization has been done, then you have to finally take into in vivo. In vivo also similar to in vitro only, but after implanting into the animal model, you have to sacrifice the animal and take out the sample again out and check for the histopathology surrounding the implanted devices. So, all those things you have to do again, how much of cells are adhered to the surfaces, whether it has caused any immunogenic responses — all those things you have to do for that.