Biomaterials for Bone Tissue Engineering Applications
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Week- 03
Lecture- 13

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Welcome to this module on bacteria structure and properties. In last several modules I have already covered various aspects of eukaryotic cells i.e. (truly nucleated) truly nucleated cells, particularly their structure as well as different cell fate process and so on. So in this module I will concentrate more on the bacteria.

Now why bacteria is equally important as like cells in the context of bone tissue engineering applications because that whenever any implant which goes into that human patient, there are always chances for prosthetic infections which is largely attributed to the colonization of the bacteria on an implant surface or colonization of the bacteria leading to the bio film formation on a implant surface.

So therefore some of the things that I will be discussing in this, I will be discussing in this module is that how the bacteria structure is different from an eukaryotic cell structure and second thing that how this bio film formation takes place which is preceded by (bac) bacteria material interaction. So, bacteria material interaction, bacterial growth on a material surface leading to bio film formation that will be also central theme of the of this particular module discussion.

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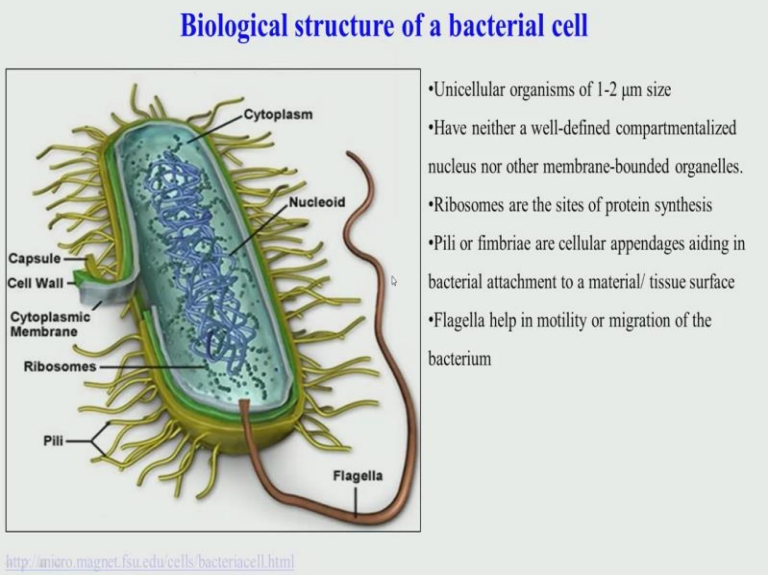

So this slide essentially shows you more widely known bacteria that is that E coli, Escherichia coli and what you see here, it is a very unique cellular organism as you know that (prokaryotic may) this typical prokaryotic cells and it is a size is 1-2 micron. This fundamental difference between the structure of a bacterial cell (as well as) and then that of that eukaryotic cell is that bacterial cell does not have a well defined compartmentalized nucleus or other membrane bounded organelles and this typically, this, they, they do not have any well-defined nucleus. As a result you can see all through the, all through the cytoplasm or cytosol. Your nuclear (ba ba) this DNA is dispersed and the ribosomes, just like eukaryotic cells, they are also present in the bacterial cell and this is the site for the protein synthesis.

Now in the context of the cell migration I have mentioned certain podia that typically eukaryotic cell, they have this podia (if) are filopodia and lamellopodia. So this filopodia and lamellopodia, they help in the cell mortality or cell locomotion on a biomaterial substrate and I have compared them in a more simplistic terms, it is like a cell migration is somewhat analogous to that baby crawling on a floor and then baby when crawls then baby uses both hands as well as legs so similarly in a biological cell, eukaryotic cell you have certain podia like filopodia, lamellopodia, those podias they help in the cell migration.

Similarly here in the context of the bacteria you can see that it has a certain large tail like structure which is known as the flagella and (this) this is also there are small hair like structure, you can see, that is coming appear as an outgrowth to the, from the cell, bacterial cell wall or cell membrane, they are known as pili.

So pili and flagella, there, they help in bacterial attachment on a biomaterial substrate and particularly flagella help in the mortality, flagella is this longer longer structure which you can see very clearly, longer tail like structure that particularly helps in mortality or migration of the bacteria.

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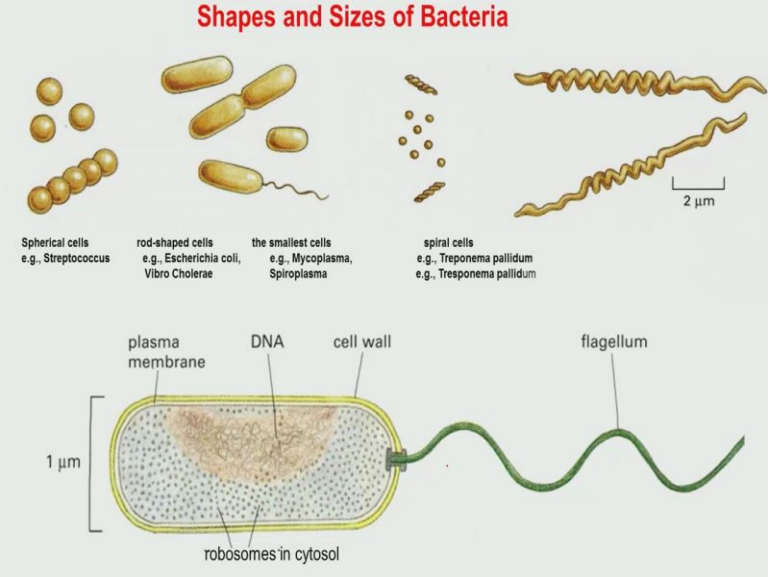

Okay, depending on different shapes and sizes you can classify the bacteria in terms of this cocci that is a spherical cells like stuff streptococcus, then you have the rod shaped bacteria that is the Escherichia coli, vibrio Vibro Cholerae and you have the spiral cells and also you have, this is the typical rod shaped bacteria, I have already mentioned in the last slide.

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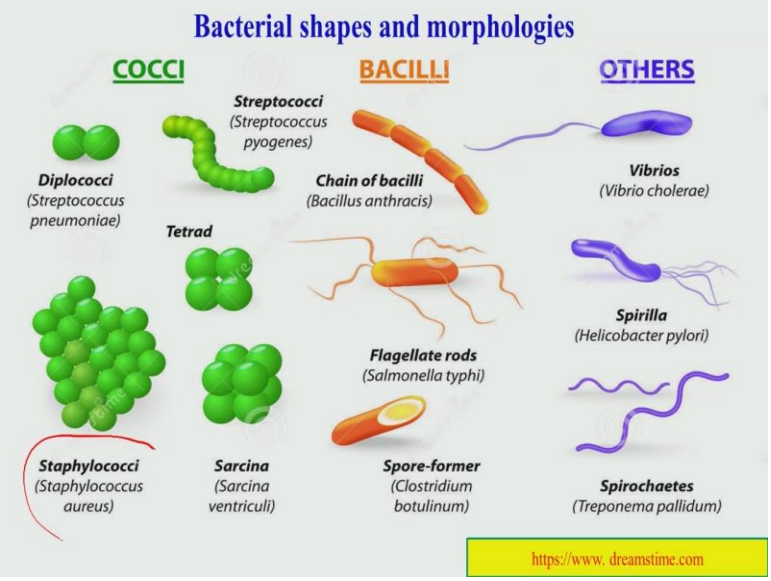

So this cocci particularly staphylococci, these are more pathogenic bacteria that means these are like staphylococcus aureus and there is another strain called staphylococcus epidermidis, so these staphylococcus aureus and staphylococcus epidermidis, these are 2 bacterial strain. They actually cause most of the bacterial infection. So you have that other cocci species like streptococci and so on.

So, those are not that much used in the biomaterials research to assess the bacterial infection or the possibility of bacterial colonization on the material substrate, but most widely used bacteria which are used in biomaterial research is this one that is E coli, Escherichia coli that which is gram negative bacteria, and the spherical shaped like s epidermidis or s oreous, these are like 2 other gram positive bacteria which are used widely in research on biomaterials.

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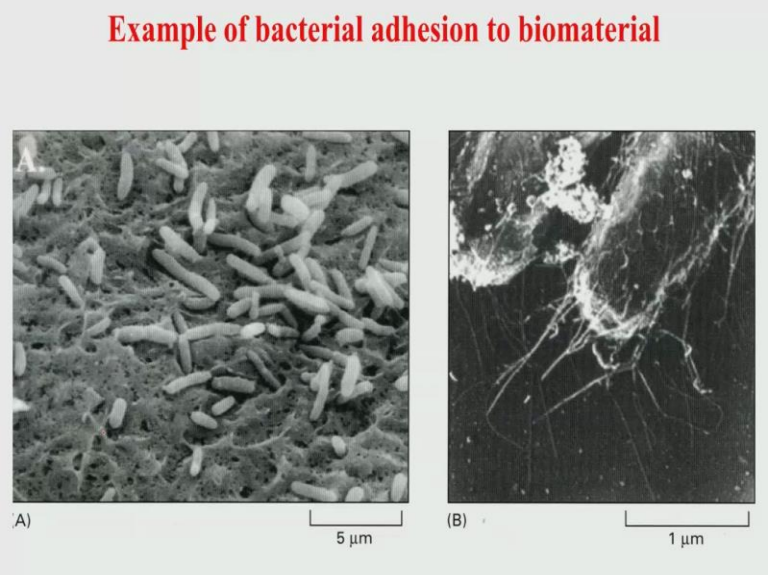

So this is the summary of what I have said just now and now this slide essentially shows you that how this E coli bacteria, they adhere on a material substrate. So this is your porous material substrate here, so this is your material substrate. And on a material substrate then this E coli bacteria they are adhering. And you can see this is the signs of flagella from this E coli bacteria and this flagella are actually the sides of the form attachment of a bacteria on a biomaterial substrate.

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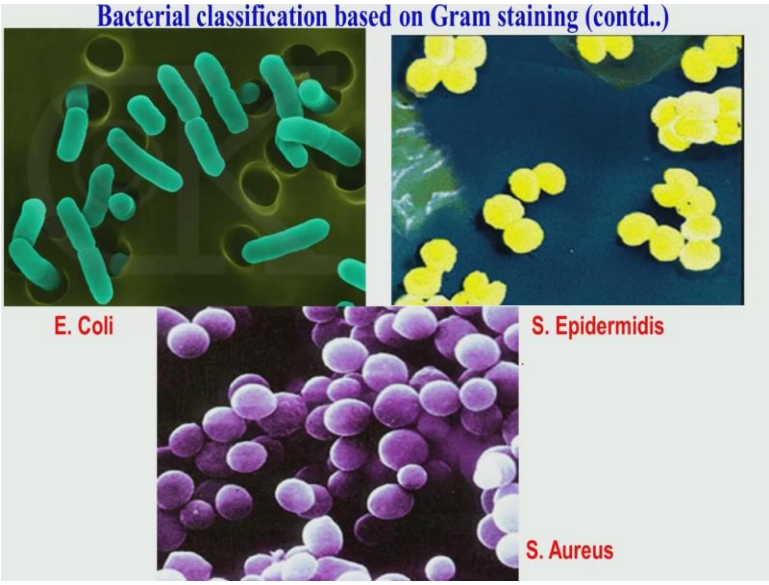

So this is again, if you remember your that school biology, this is the, depending on the gram staining, (you have) you with the bacteria can be classified to 2 classes. That is one is that gram positive and another one is called gram negative. So gram positive and gram negative, these are

the most widely used classifications in the micro biology. The examples of the gram negative bacteria is that E coli and examples of this gram-positive bacteria I mentioned just a few minutes ago it is the staphylococcus that is oreous and (ap ep) epidermidis. They stain certainly differently than from the E coli and also shape wise E coli is very different for that of the 1 staphylococcus species.

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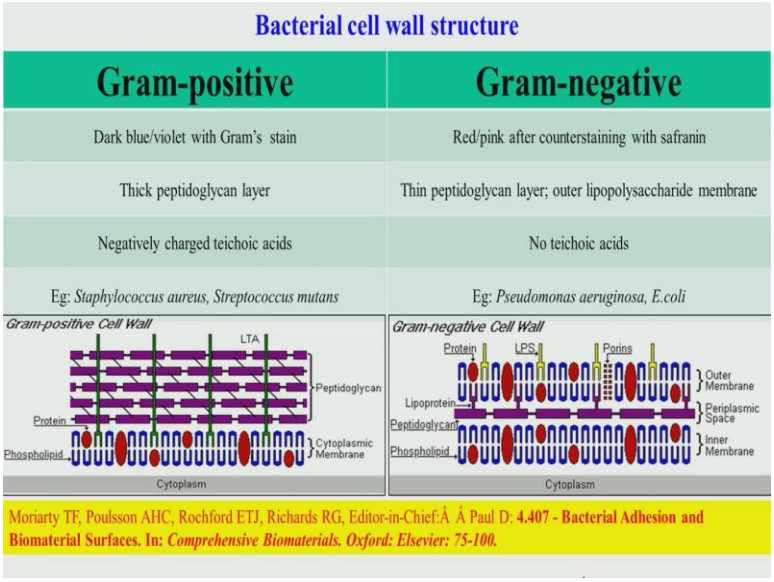

One of the things that distinguishes these 2 classes of bacteria, gram positive and gram-negative is the way their cell membrane or their membrane is been built up. So this is (bashr) this slide compares these 2 type of bacteria, gram positive and gram negative, so gram positive always appears like either dark blue or violet whereas, (gram neg) with gram staining agent and when stained these with gram staining agent, counter staining (with ga ba) safranin, then it appears gram negative like E coli, they appear more like red or pink. So in the gram positive you have a thick peptidoglycan layer and in gram negative you have extremely thin peptidoglycan layer.

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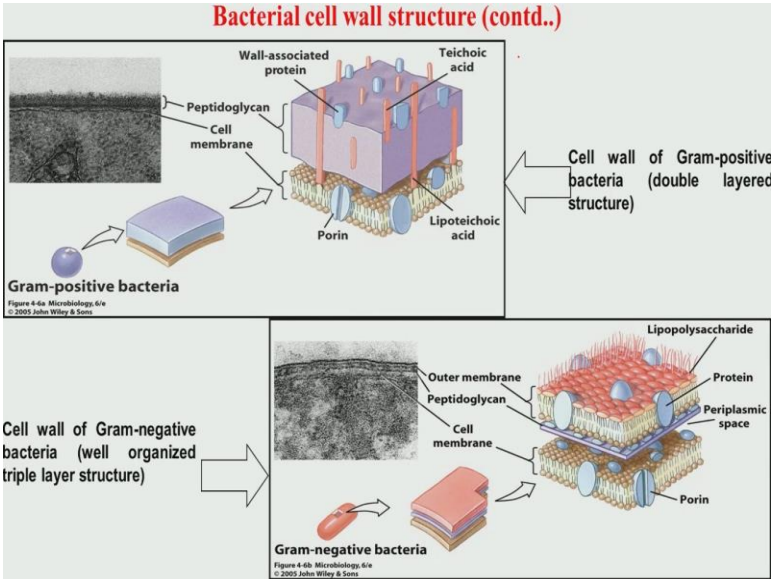

So let me discuss this with clarity here in this particular slide. So this is your gram positive bacterial cell membrane, so you can see just like a eukaryotic cell you also have a double layer kind of a structure the ,and you can also see there are a lot of proteins (which are) (which are at) which are attached to the inner wall and you have a reasonably thick peptidoglycan layer.

So you can see this peptidoglycan layer, if the thickness is t1, if the peptidoglycan layer which is which is thickness, this particular thickness, peptidoglycan layer is t2, certainly t1 is greater than t2. So that means peptidoglycan layer in the gram positive bacteria like staphylococcus species is much thicker than the peptidoglycan layer in the gram negative bacteria.

Ok so that is the number 1 point. Number 2 point is that gram negative bacteria like E coli, they represent a very characteristic 3 layered structure with peptidoglycan layer being sandwiched between the 2 layers, 1 is the top layer and 1 is the bottom layer. And as usual like with eukaryotic cell your, there are several trans membrane proteins also which you can see very clearly, here in that gram negative bacteria. So in a way the cell bacterial wall in the gram negative is much more thicker and also it is quite different from that of the gram positive bacteria.

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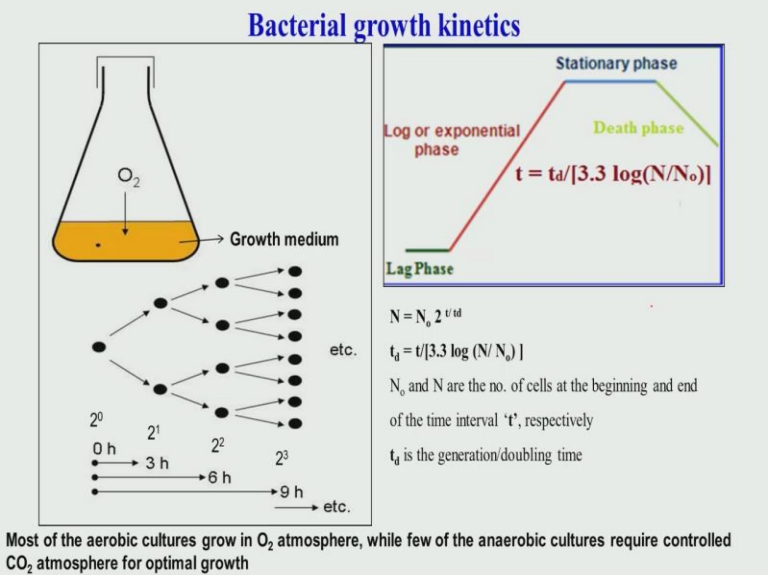

Now once the bacteria starts growing in a growth medium, so typically in that cell biology literature eukaryotic cells, for the eukaryotic cells people tend to use as a culture medium which is mostly DMEM or alpha MEM based culture medium, in case of the bacteria culture people use growth medium it is a more (())(11:26) growth medium. So this growth medium and culture medium somehow this terminology is more segregated in the micro biology and cell biology respectively.

So bacterial growth also follows similar kind of growth pattern like, in the, in the case of other cells so 1 to 2, 2 to 4, and so on so you can see that how fast, only difference between the eukaryotic cell and bacterial cell is that; here growth kinetics is much faster compared to eukaryotic cell. Typical doubling time in bacteria is somewhere between 30 to 40 minutes, you remember in the last to last module I have mentioned that typical doubling time for eukaryotic cells is almost like few hours like human cells, mostly the cell doubling time is 11 to 12 hours. Here in case of bacteria this is half an hour, so less than 1 hour that is the typical doubling time for the bacteria.

So second thing that has been (mentioned) that has been shown here, so, ok, depending on that, the necessity for the oxygen in the growth medium you have aerobic bacteria and anaerobic bacteria, microbes essentially. The third thing is that, that how this growth takes place, so essentially you have a very initial period of lack phase. So lack phase is more like incubation kind of period where the bacteria has not yet started to grow in a normal manner. You have a log

or exponential phase, here the time, the generation time is defined as the td divided by 3 point 3 log N divided by N not.

So this comes from a very simple equations, the simple equations has been written here that is the number of bacteria at any given time point t is equal to N not that is the initial bacterial concentration at the beginning of the cell , at the beginning of the bacterial growth conditions and td is the generation time or doubling time. So essentially, I repeat, this simple equation essentially tells you the number of bacteria at any given time t N, capital N is equal to N not multiplied by 2 to the power t by td where td is the generation or doubling time and t is that any given time and N not is the number of bacterial cells at the beginning of the growth conditions.

Now after this log or exponential phase, bacteria reaches the steady state phase that is the growth stationary phase and if you grow it further longer and longer then it enters into the death phase. So the way this kind of a bacterial growth conditions bacterial growth kinetics, they follow this kind of this kind of qualitative description of this bacterial growth kinetics to some extent is little different from the way the eukaryotic cells, that grow in culture.

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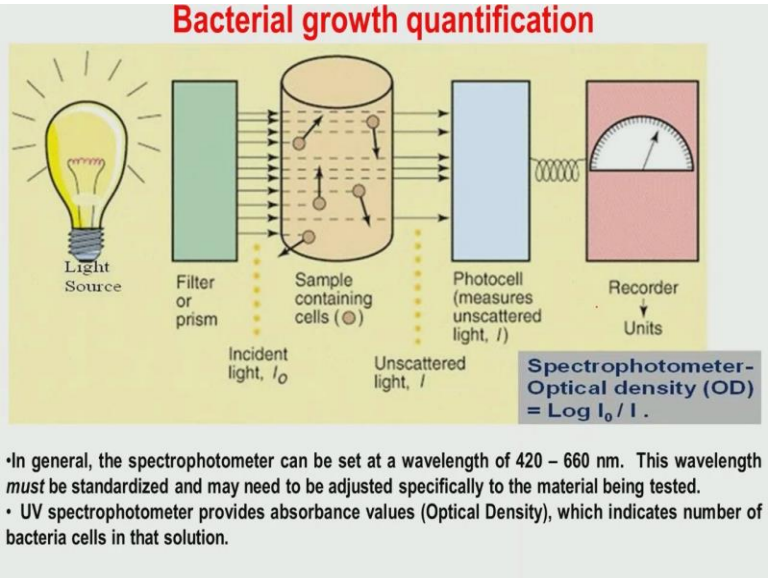

Now how you can quantify these kind of growth kinetics, 1 of the most standard and widely used method is Spectrophotometer, as the name suggests that means that there is certain light where (ba ba) light ray of certain wavelength will be made to pass through the bacterial growth medium. Once without bacteria and once the blank solution like that is the blank solution and 1 case with bacteria, actual growth medium containing bacteria.

In both the cases you measure the intensity of the transmitted beam. And (s) optical density is typically noted as is typically measured, determined by logarithmic of the ratio of the I not by I ok. So from there you can find out that what is optical density of any bacterial growth conditions, okay.

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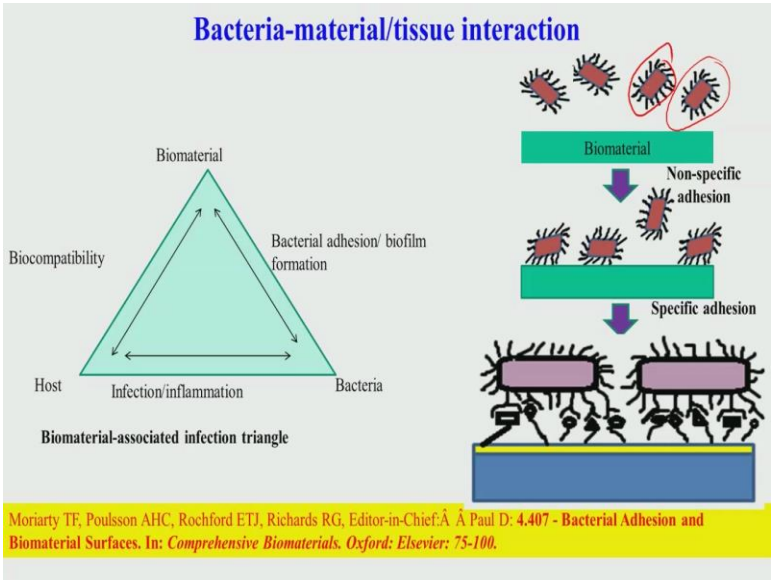

Now, so coming to this, how this bacterial bacteria will attach to the biomaterial substrate. So this is your individual bacteria which is shown here. Now this bacteria will come to the biomaterial substrate, now they (eb) they will essentially form specific adhesion complexes here. And this mechanism is been discussed in a little but more details in a stage wise manner.

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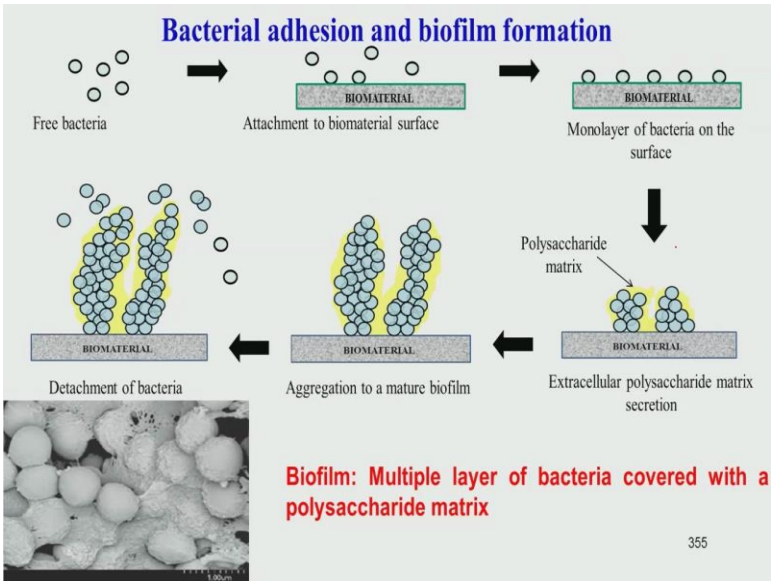

So you have a freely suspended bacteria in the growth medium and the same growth medium you are (implant) you are placing your biomaterial substrate. So individual bacteria will come and will attach to the bacteria then it will form a mono layer concentration. Once this mono layer forms then subsequent bacteria will come here and will get attached to the single bacteria. So what I am trying to show you here that the single bacteria which initially has come and adhered on the material substrate is capable of forming an individual colony on the biomaterial substrate.

I repeat a single bacteria, suppose it is shown here 5 bacteria has been attached to the biomaterial substrate, so what I am saying that ideally single bacteria can form single colony, that means the single bacteria can generate now 5 different colonies on the same biomaterial substrate. Out of these, just for illustration purpose, we are showing here 2 such colonies, but actually it can potentially from 5 such colonies, okay?

Now in this colony formation essentially is favorable or its colony (form) colony is more stable if the bacteria now can secrete and form some polysaccharide matrix which is shown here like some yellowish kind of substance. This yellowish matrix, polysaccharide matrix help the bacteria colony to glue among the constituent bacteria and that actually helps bacterial colony to grow further in size, as you can see this in 1 colony, this is second colony so this is growing in size.

But this growth cannot take place in an indefinite manner. In other words after the number of bacteria goes to a large number then this individual bacteria can fall off from the colony and (this means) this means that this is that concentration that has reached that is the bacterial colony that more than that this individual colony cannot contain more number of bacteria. Ok. The, now this colonization of the bacteria and bacteria colony formation is the first step.

Now if you grow this bacteria for a sufficiently longer time period then what will happen, that this this total (sh k) sample is total biomaterial substrate will be covered with full of bacteria. Now intentionally we are showing here this spherical bacteria, that staphylococcus, staphylococcus species. It can be either oreous or epidermidis. Now why we are showing, I have mentioned few minutes ago that s oreous or epidermidis are the most pathogenic bacteria, they are most responsible for prosthetic infection.

So, therefore, showing this bio film formation also; which is (insti b) which is instigated by this colonization and then extra cellular polysaccharide matrix formation to a mature bio film. So bio

film formation you can observe or is possible only when this bacteria is cultured for an extended time period in growth medium.

Now this, this earlier 1was (this) more qualitative discussion based on that our understanding that how bacterial adhesion and bio film forms and the way the bio film has been mentioned or bio film has been defined, it is the multiple layer of bacteria covered with a polysaccharide matrix. So that is how bacterial bio film is being defined here.

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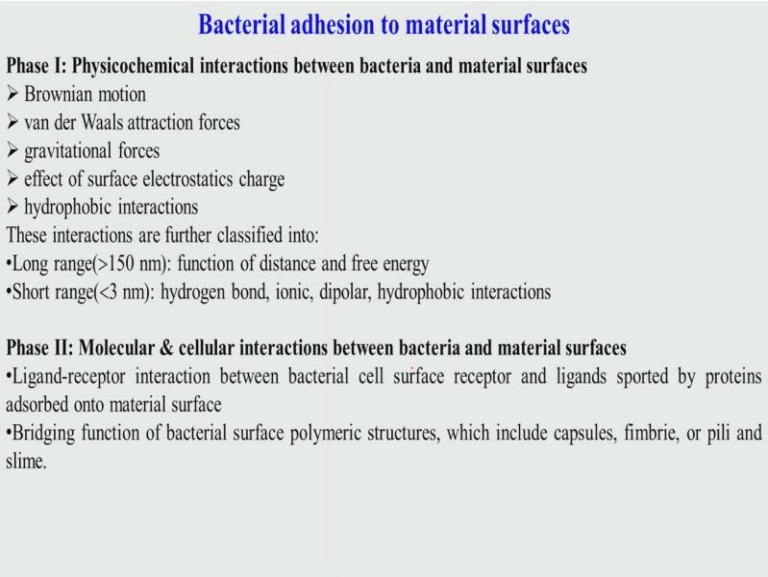

Okay, now this is little bit more theoretical considerations of the different stages of the bacterial adhesions to material surfaces. The first 1 is the physiochemical (ton) interactions between bacterial material surfaces. You have several forces of attraction like you have the van der Waals forces of attraction, you have the gravitational forces and also you have a hydrophobic interactions.

Now these interactions mostly falls under the 2 category, 1 is the long range and 1 is the short range. Short range essentially means within a few nanometer from the material substrate, these interactions are essentially facilitated by dipolar and hydrophobic interaction as well as weak hydrogen bonds. But in the long range interaction which is taken place more than 150 nanometer away show this is the function of distance and free energy.

Second 1 is that phase 2 that is molecular and cellular interactions between bacteria and material substrate. So here again it is the same type of interaction like Ligand-receptor interaction between bacterial cell surface receptor and ligands supported by proteins absorbed into the

material surface. So this mechanism is similar to that of that eukaryotic cell adhesion on a material substrate and second 1 is the breaching function of bacterialsurface polymeric structures which include capsules, fimbrie or pili and slime. So this is also another level of, (nebi) another reason for quicker adhesion of bacteria on material substrate.

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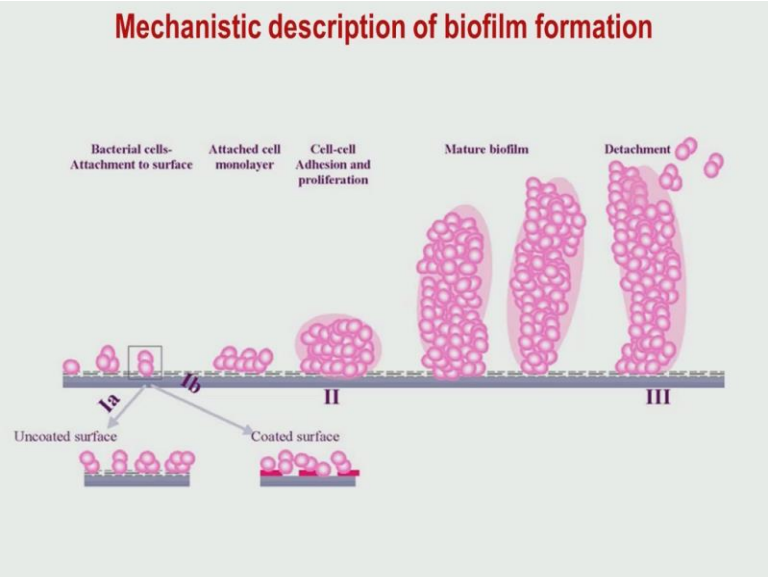

So this is how has been shown here little bit more clearly. So you have that bacterial adhesion and attachment to the surface then it is the attached cell mono layer, then cell cell adhesion, we had this polysaccharide extra cellular matrix deposition around the bacterial colony that helps as a glue. Then this colony grow in size to a critical number and then subsequently when it exceeds the critical number then it detaches. And that is how it happens that is how it leads to a more mature bio film formation.

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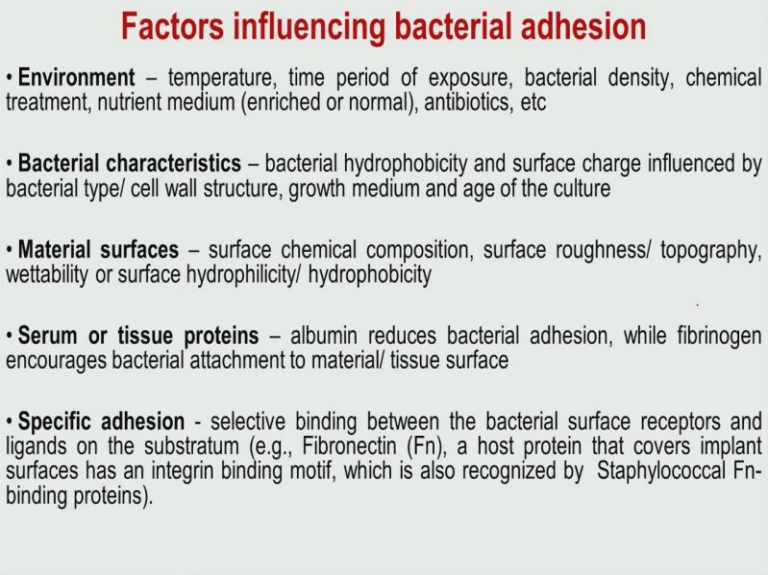

Now having said this let me explain you or let me at least mention some of the important factors which influence the bacterial adhesion. This factors include environmental factors, environment means that bacterial micro environment like I have mentioned in the last few modules that cellular micro environment plays an important role, the way cells will decide that which fate processes cells will adopt. Similarly, environmental parameters like temperature, time period of exposure, bacterial density, chemical treatment or nutrient medium or antibiotic. So these things are equally important.

Second 1 is that, bacterial characteristics like what is the cell wall structure whether it is gram negative, gram positive; what is the bacterial hydrophobicitial surfaces that is important. Material surface characteristics these are kind of similar to that of the cell material interaction. Essentially what is the surface roughness or topography or what is the weight ability or surface hydrophobicity or hydrophilicity, these are important.

4th 1 is the Serum or tissue proteins mostly if you put if you coat the (bacterial) biomaterial with certain albumin proteins and all that typically reduces the bacterial adhesion. But if you coat them with fibrinogen, that essentially encourages the bacterial attachment to material surfaces. So it does not necessarily mean that all proteins have some repelling properties towards the bacterial adhesion, in fact some of the proteins can essentially encourage more and more bacterial attachment which should be avoided.

Fifth 1 is that specific adhesion like selective binding between bacterial surface receptors and ligands on the substratum that a fibronectin that is a host protein that covers the implant surfaces, that also important. Because if you remember (on a) when a biological cell adheres to a material substrate, just like if you go back to this slide and if you recall the way a eukaryotic cell is attached, is attached to the, is attached to a biomaterial substrate is quite different to some extent and then this cell signaling processes take place and then another cell comes.

So these, this multiple cell that comes and then attach, it takes lot of time for the eukaryotic cells to take place but here in case of bacteria being physically much smaller that the eukaryotic cells, so typically the eukaryotic cell size is 25 to 30, micron whereas these kind of bacteria, it is 1 to 2 micron so it is much much lesser than that of the eukaryotic cells.

And also different dynamics involved in this biological medium, it is also kinetically much slower because it involves lot of signaling processes to take place in case of eukaryotic cells whereas in bacteria this process somehow takes place faster particularly the doubling time is also much faster than eukaryotic cells so that this colonization and mature bio film formation also takes place even greater than 4 hours of culture.

Now greater than 4 (s) 4 hours of culture in case of eukaryotic cell, you will not be able to see many of the cells on the material substrate because mostly the culture period is in case of eukaryotic cell, is greater than 24 hours. So 24 hours is minimum culture period and then in some of the faster growing cells you can 24,48 hours but some of the slower growing cells you start seeing the cells on a material substrate only after 48 hours then you have to grow the cells for a much longer time period.

Now this kind of comparison between the way eukaryotic cells interact on a material substrate and the way (s) eukaryotic cells adhere and makes the colony that this kind of comparison is very important for you to understand and assimilate.

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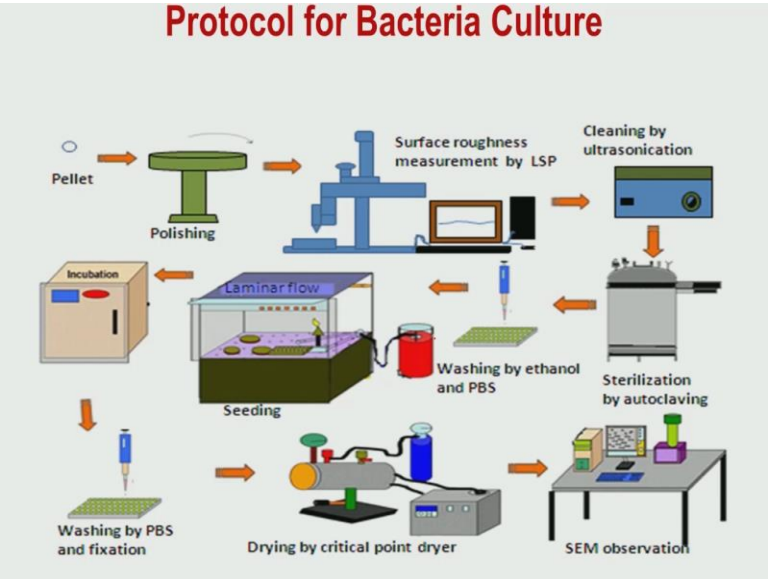

Now this is the snap shot of this how this entire protocol of the bacteria culture as well as that in the context of the (ba) investigation with biomaterials is followed in the lab scale, so you have that any of the sample, or test sample, or material or what we call pallet, you do polishing after that you ultrasonication and then do sterilization. Then after sterilization you do several stages of the washing with ethanol and phosphate buffer saline.

Then you seed the bacteria (b) then you seed the bacteria on the material substrate in a typical bioseptic cabinet or laminar flow depending on the type of bacteria you are dealing. Then after that you have to put it in the incubator, this bacteria, bacteria along with the materials. Now remember here in the bacteria you do not require the continuous flow of CO2 like you require for the eukaryotic cell culture. So bacteria growth takes place in the normal aerated environment, normal ambient (ef en) environment.

Only thing that you have to maintain is 37 degree Celsius temperature and ambient environment is what you need to (wha) what you need to ensure in a, in a bacteria culture room. Then after the bacteria is grown for (wo) 1 to 4 hours or (diff dep) different time period then you can wash by PBS and fixation and then you do either critical point dryer or some of the reagent for drying and then after that you can use the trusses microscope or scanning lit microscope to see how the bacteria adhere on a material substrate.

So this (ba) (cal ba) this, after this bacteria culture is over, then you can count that how many bacteria they have grown on a material substrate and how kinetically it changes their growth

pattern compared to when the similar bacteria is grown (on) in an isolation like without any material substrate. So I will stop it now and then we will go to the next 1.