Drug Delivery Principles and Engineering
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Lecture – 27
Protein Adsorption – III

Hello everyone, welcome to another lecture for Drug Delivery Engineering and Principles. I am going to talk about further on protein adsorption.

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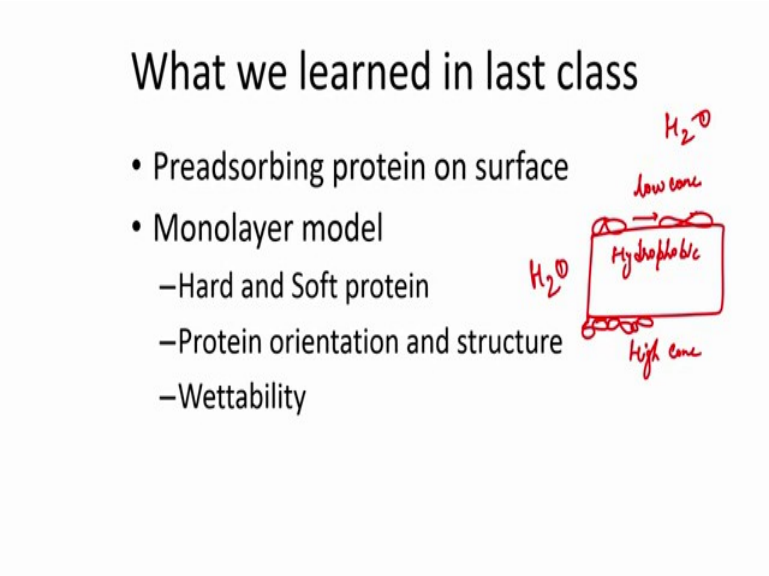

So, let us just do a quick recap of what we learned in the last class. So, in the last class we talked about pre-adsorbing proteins on surfaces, which basically means that if I want a certain kind of response from a surface, let us say if I wanted to bind those cells, then what I can do, I can add ligands proteins onto the surface even before I put it with the cells, so what will happen is, these proteins will adsorb on the surface and will directly start interacting with the cell with their certain cognate receptor.

So, the advantage here is, I get a lot more control over my surfaces because now they are interacting the way I want them to interact. And then the certain there are lots of applications of this that you can vary to sort of modulate this response that you are getting. Then we talked about a model that is being used to study protein adsorption one of that was a monolayer model. So, what it is? It means that let us say if I have a surface, it just means that there will be only one layer of protein, that will adsorb on the surface to

cover whatever is exposed area and once that layer has a adsorb no more preabsorption will happen.

So, that is the assumption in this monolayer model and again we will discuss today that this is actually not true, but for the sake of this model, it will be an orientation like this there will be no more protein that is going to come in adsorb here. And then at the same time we discussed about hard and soft proteins. So, what we are saying is, there are some proteins which are fairly hard meaning with a fairly rigid they do not change the structure very easily the structure is very stable and then there is some proteins which are very prone to denaturation, their structure is easily changeable. So, these are called soft proteins.

And then we talked about protein orientation and structure essentially saying that if I again have a surface which is exposed to low concentration of protein. So, what will happen is, in the protein that is initially getting adsorbed will have time to expand on the surface because it still has this open side next to it. So, it will keep on expanding until either the site gets filled or the protein reaches its happy state in terms of the equilibrium.

So this is a case in a low concentration setting whereas, if I have high concentration then what will happen is, the protein that gets initially adsorbed will not have space left because of the high concentration as several other proteins have also adsorb and it will tend to be at a higher amount on a given unit area or the given sort of the structure of the protein will also remain more or less rigid or it will not really change a whole lot.

And then we talked about wettability of the surface itself. So, what we were saying is if we have hydrophobic surface, meaning it has low wettability; then it will tend to form very strong bonds with proteins, because the outside water environment is not competing with it and the hydrophobic domains will tend to interact with this hydrophobic surface as well the surface also does not want to interact with water. So, there is an energetically driven process, which causes a lot more interaction between protein and hydrophobic surface compared to let us say this is a very hydrophilic surface and then the proteins will cell adsorb to it, but their affinity to the surface will be much lower.

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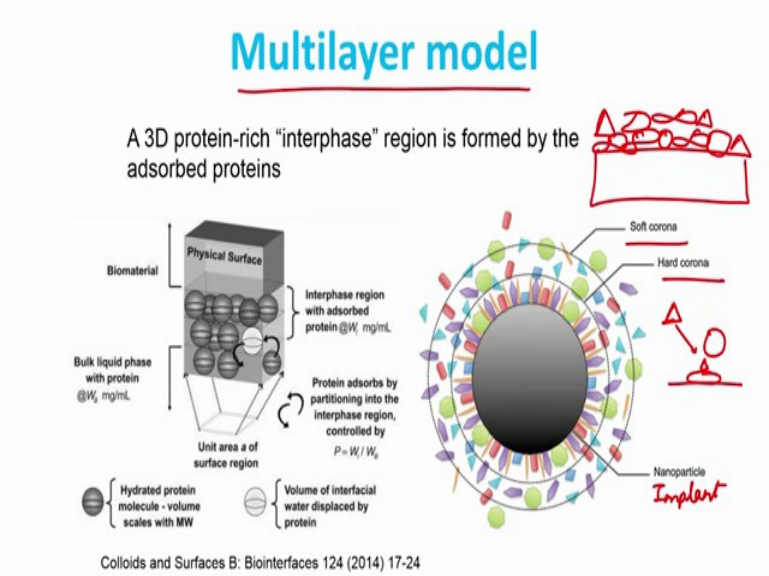

So, now having done all this, as I briefly mentioned this monolayer model is a model that is not really well accepted at this point of time because more and more research has shown that there actually does form multiple layers of protein on a surface. So, to explain this its simply another model is proposed this is a multilayer model.

And what it essentially means is that there is a 3D structure to a protein this interface. So, even if I have a surface, there could be several layers of proteins that is going to come and attach to it. And essentially what will happen is its defined as sort of a hard and a soft corona. So, a hard corona is defined as something that is fairly stable on a surface, it is very difficult to rip this protein out from the surface. So, something it is directly interacting with the material and it is very close to the material is typically called a hard corona and something which then comes and then interacts with this hard corona is called a soft corona.

And the whole reason for the soft corona to happen and this could be nanoparticle or the implant, it does not have to be a particle it could be a bulk device and the reason that this soft corona start interacting the hard corona because this hard corona has actually a change in the structure. So, this is not depicted in this image, but let us say this circular protein has now become elliptical where its adsorb. So, it has now exposed new sites to the body which were never exposed earlier. So, now, there could be an another protein that comes and sort of attaches to these exposed sites and has some affinity to them. So,

that is why you get the soft corona and may not be a very strong interaction at that point, but they cell some interaction to it.

So, as again is depicted here also, so you have a physical surface, then you have some amount of protein that comes and then there is some other protein that comes on top of those proteins and this essentially creates a whole corona of protein around a particle.

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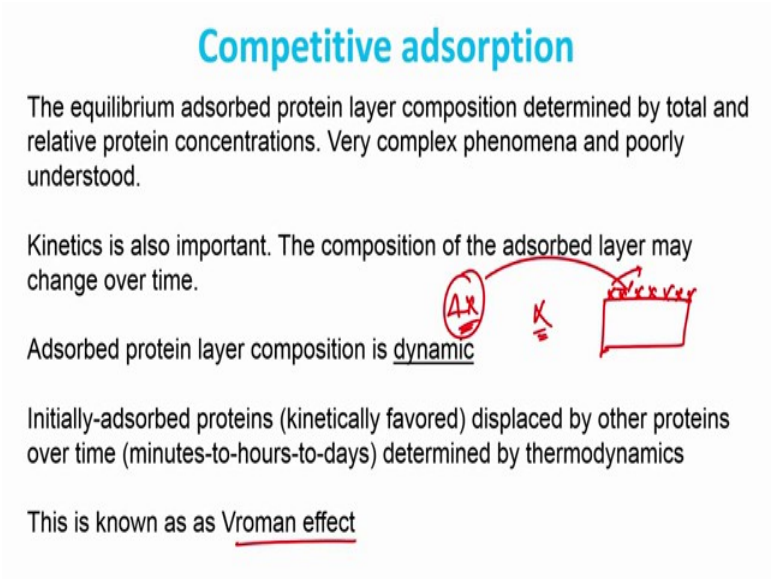

And then there is another concept called competitive adsorption. So, what this is, is the equilibrium adsorbed protein in composition is defined by how much is the total protein first at the site and then what is the relative protein concentration. So, this is very complex phenomena and very very poorly understood for most of the materials that we use and then the kinetics of this has also become important. So, what it essentially is saying is, is again if I have a surface, first of all the amount of protein that is coming in depends on the total protein amount that is getting adsorbed.

It depends on how many types of proteins are there, then it will also depend on what are their concentrations. So, all of course, not all proteins will have the same concentration - some will have very high, some will have very low and then the kinetics also becomes important. So, if let us say one protein even though it is a lower concentration, it gets completely adsorbed on the surface very very quickly, then those will dominate or otherwise vice versa will happen for other kinds of situations.

So, the kinetics of these protein adsorption and again the kinetics of different proteins will be different and then it is also dependent on time and location of the implant and why am I saying that is because what will happen over time is let us say the environment is changing, there more cells coming in, they're secreting more proteins and the concentrations of protein is changing, the types of proteins are changing and because of that what will happen is this equilibrium which is being maintained is going to constantly change or if the implant actually is moving or let us its a particle which is traversing through the body and then of course, you will have different effects taking place at that time.

So, essentially saying that the adsorbed layer of this composition is fairly dynamic and typically as I previously described the hard corona is still fairly stable, but it can be dynamic and what can also happen is that initially the proteins that are adsorbed can be displaced by other proteins over time with very high affinity. So, let us say if I have a protein adsorbing on a surface with a certain affinity x and then I have another protein with an affinity of 4 x, even though this protein is at a lower concentration and maybe has taken time to diffuse to the surface and x is already adsorbed to it, what will happen is this protein will eventually be able to slowly and slowly be able to displace this and get adsorbed there because a equilibrium says the higher affinity will dominate over the lower affinity. So, to this particular phenomena where essentially the higher affinity proteins get to replace the lower affinity proteins from the surface is also known as Vroman effect -you will see that we used quite a bit in the literature. So, just something to remember.

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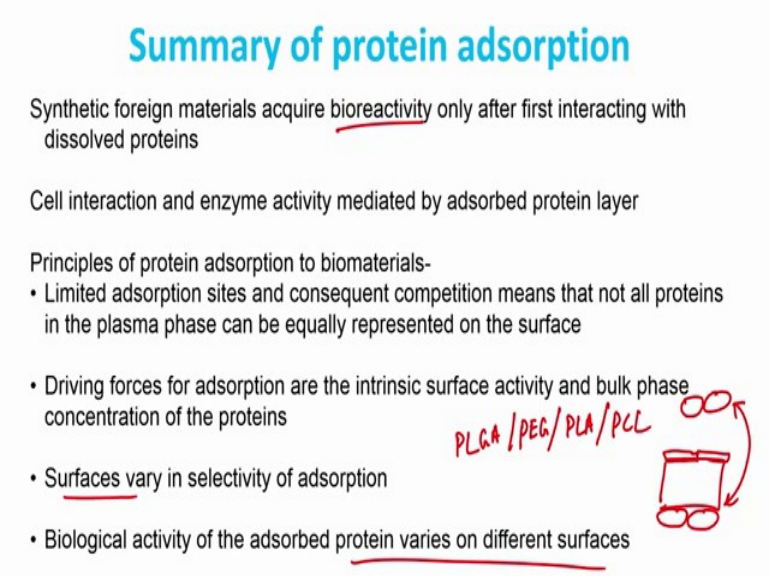

So, again just to summarize this protein adsorption, so we are saying that synthetic foreign materials acquire bioreactivity only after first interacting with dissolved proteins. So, I mean even if I have a material that has no sites that can bind to the cell, what will happen is the proteins will adsorb to it, these proteins will have sites that can bind to cell and that is how they can mediate cell interaction and gives bioreactivity to the surface.

And any kind of cell interaction or enzyme interaction that is going to happen will be mediated by this protein layer. And then there are some principles of protein adsorption. There are limited adsorption site and so, there is a lot of competition that happens between the proteins to sort of get represented on the surface and then there are driving forces this could be several things- could be the hydrophobicity and all.

The surfaces themselves are going to change; I mean if I am using PLGA versus PEG versus let us say PLA versus PCL, all of these have different hydrophobicity, hydrophilicity, have different surface groups, have different interaction with the proteins. So, the type of proteins that can absorb on these surfaces is going to be very different and then again the biological activity of the adsorbed protein varies on different surfaces as we just discussed.

So, once the protein adsorb, the more it changes conformation the lesser will be its activity. So, if a protein that has a structure like this, gets absorbed and becomes a structure like this, it has changed quite a lot of structure. So, its activity may get severely

damaged or might not be even active at all whereas, if the same thing becomes like this its fairly similar. So, you can think of a scenario where this may be still 70-80 percent active than when it was in solution, so these things can also vary.

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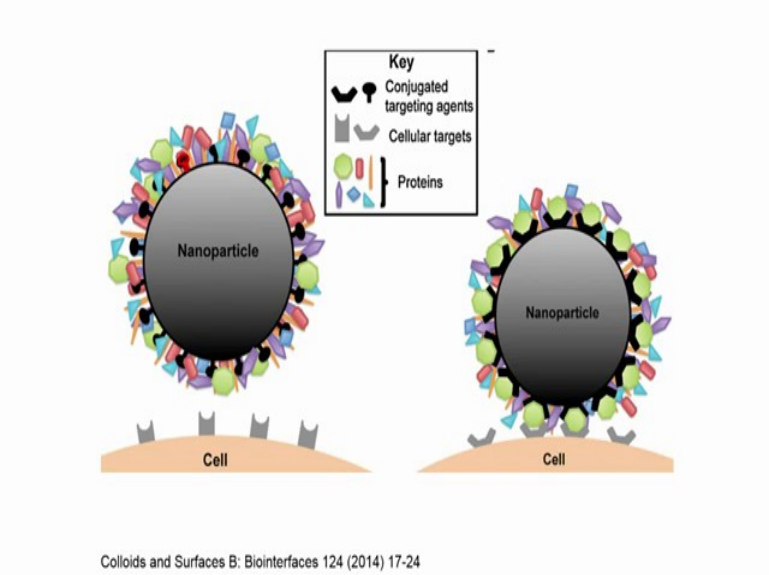

So, again this is just showing how a nanoparticle actually interacting with the cell, the cell is not actually seen the nanoparticle surface directly because it is already adsorbed protein in the surface within less than milliseconds and its actually interacting through the adsorbed protein. So, it becomes very important to study protein adsorption.

And then another interesting thing is, you can conjugate a particle with various ligands you think that they may be used to interact with the cell surface, but look what happened once you put it in the media there is a whole lot of protein adsorption layer that has come in and this particular targeting ligand is not even accessible to the cell. It may be accessible in some cases depends on the surface and the ligand itself, but this is something to consider while you put these ligands that they may not be actually directly interact with the cell.

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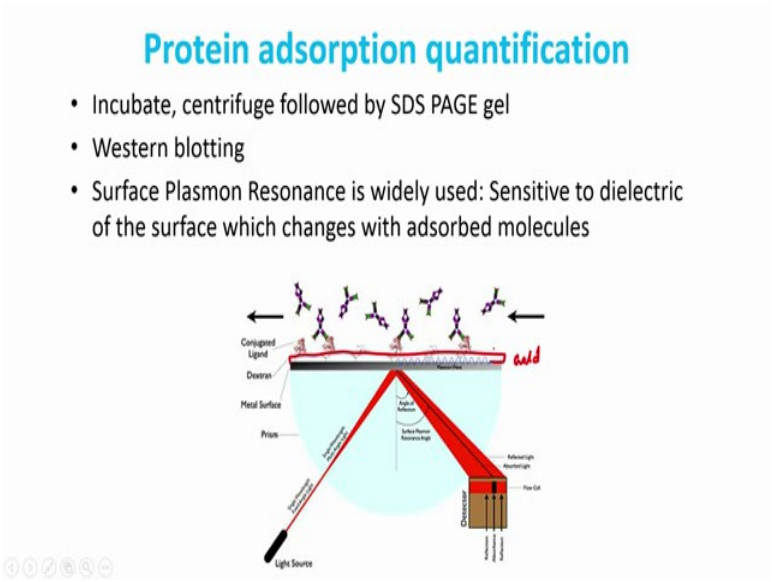

And let us talk about how can we study this quantification of protein on the surface. So, one thing is to do an SDS PAGE. And so, it is fairly simple what you do is you put your implant or particle and incubate with let us say serum and so, what will happen is, what whatever different proteins are there will come and adsorb and serum is just an example you can put it in some other fluid, maybe if you are going to give it to lungs to innovation, you are going to put it in lung fluid or lavage, if you are going to give it through orally you can maybe put it with saliva.

So, there is all different kinds of biological fluids that can be used - depends on the application. So, once these proteins have coated on this implant, you take this implant I mean again as I said this is a matter of milliseconds we are talking about. So, you can leave them for a few minutes and take the implant you centrifuge to remove anything that is external and resuspend it let us say in water and once you have done that, you can use an SDS PAGE.

So, SDS is again a very strong detergent. So, what it does is this completely denatures the protein and interacts with the protein in such a high affinity that it comes off from this implant both the hard corona and the soft corona. And then you can just run a PAGE gel which is nothing, but as gel that separates the protein out on the basis of its size or molecular weight and in that regards then you can study what all proteins are there through western blotting.

So, I hope everybody knows western blotting - all it is to transfer it to a membrane and then once its transferred to a membrane let us say now this is the membrane itself you can come with antibodies against known proteins. So, let us say if I am predicting fibronectin is one of the protein that is getting involved, I will come in with the fibronectin antibody and incubate with this. So, if fibronectin is one of the protein that was on the implant, then this empty body is going to go and bind to fibronectin and I can tag this antibody with some fluorophore initially, so that is going to start fluorescing or I can put some enzyme and allow some color around this band.

So, that way I can know that fibronectin present in this milieu or its not present in this milieu and similarly you can do it for several other proteins. So, again this way is fairly semi-quantitative, its not very quantitative. Another good way to do this is using something well a surface plasmon resonance which is again very widely used. So, its highly sensitive and it depends on the dielectric of the surface. So, what you can do is, you can take your surface that you want to test. So, that is here; you can then coat it with some metal layer. So, in this case let us say if we have coated with gold.

So, once the proteins adsorb to it, the dielectric of this particular protein layer is going to be different from your initial surface and because its different you can shine a light - its going to scatter differently and you can read it through a detector and that is going to give you a surface plasmon effect because the dielectric is changed and through that you can then quantify by running some standards of known amounts, you can then quantify whether the protein is first of all adsorbing and interacting with the surface.

And then secondly, if it is then what is the amount of protein that comes on the surface because this dielectric will be directly related with the amount of the protein that has come and adsorb on the surface. So, that is one way you can study the protein adsorption.

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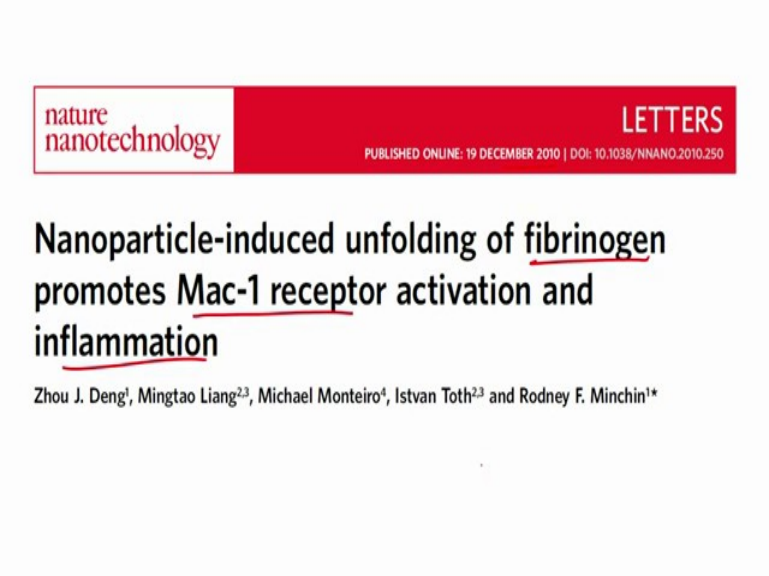

So, what we will do is, we will we will take an example from the literature - this is just to sort of give you an idea of how the research is happening. So, this is a paper that was published quite a while back now in 2010 and it is about how nanoparticle can induce unfolding of a protein called fibrinogen and this unfolding then results in signalling through a receptor called Mac- 1 receptor and that causes inflammation. So, here is just an example of how a foreign material that is introduced causes inflammation. So, its more a mechanistic table.

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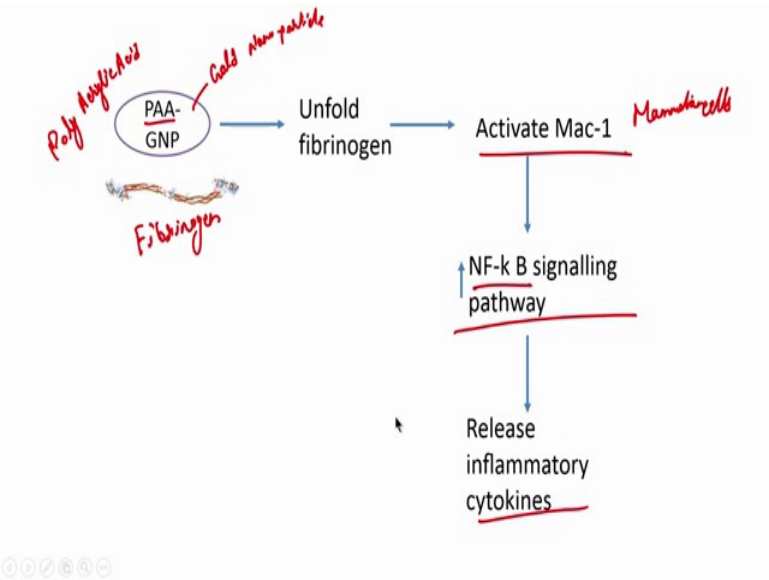

So, what the authors have done in this paper is they have taken gold nanoparticles. So, this is gold nanoparticle, they have coated this gold nanoparticles with polymers- during synthesis it uses various polymers and one of them is PAA which is Poly Acrylic Acid. So, what they've done? They have coated the gold with poly acrylic acid and then they have incubated it with a protein called fibrinogen- its very widely available protein and it is found quite an abundant amount in our serum as well. So, and what they have shown in this paper is well this adsorption happens, it activates Mac-1 on immune cells or actually on other mammalian cells.

And once it activates Mac- 1- Mac- 1 goes into the nucleus that signalling goes into the nucleus and causes the up regulation of NF-kB pathway which is a very known to be a master regulator for inflammation and that causes the release of lots of inflammatory cytokines and that can cause some reactions in the body, you can start getting fever- very similar response to when we get sick.

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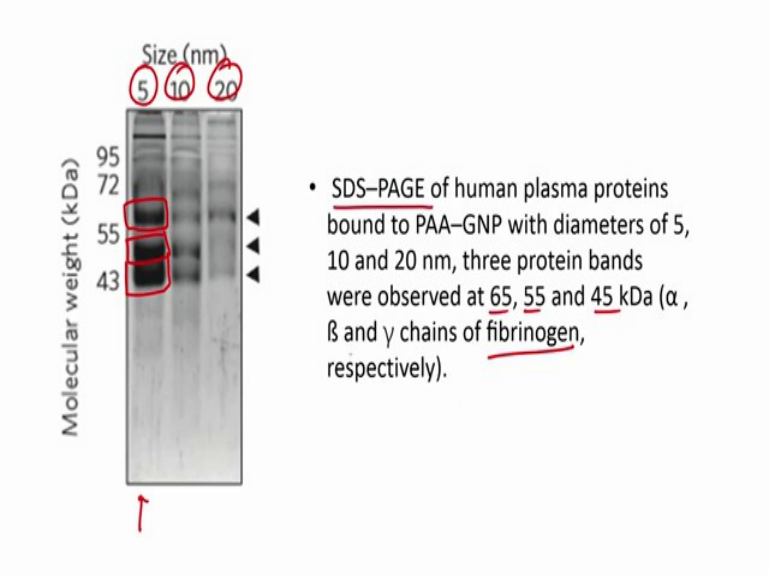

So, let us go a little more deeper, so what the did is they make three different sizes of particles. So, 5,10 and 20 nanometers in size -this is the diameter and then what they did is, they incubated this with serum and then the same process that I just described with the SDS-PAGE. So, this is just a SDS-PAGE. So, what you can see here is several types of proteins have come in and adsorb on these ones and what you see here is 5 nanometer has quite a bit of protein and then 20 nanometer has less protein for the same amount of

the gold. And what they primarily observed was that the 65, 55 and 45 kDa chains of fibrinogen were very abundant.

So, essentially when I talk about this, I am looking at 65 which is this guy, the 55 which is this guy and then the 45 which is this guy. So, these three bands were very prominent every time they did this experiment. So, then they found out that this is actually nothing, but three different chains of fibrinogen.

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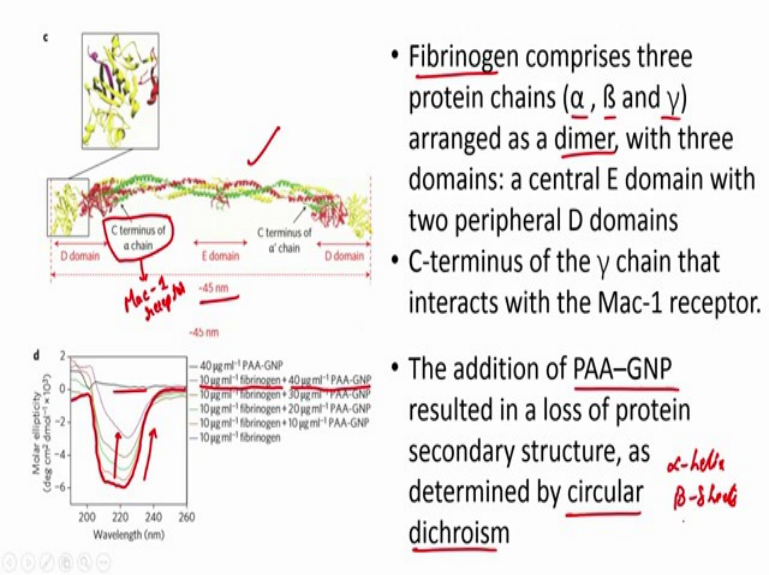

So, that is what they are describing here. So, fibrinogen comprises of three protein chains the alpha, beta and gamma and this is the confirmation - fairly large molecule 45 nanometer and it's a dimer. So, what they are further shown is that this C terminus which is fairly covered in a normal protein structure as you can see here has a binding site for a receptor called Mac- 1 on a cell.

So, Mac- 1 receptor can come and bind to this site although the site is not typically exposed. So, the Mac- 1 does not bind to fibrinogen by itself unless the fibrinogen structure is somewhat damaged or denatured, so that this Mac- 1 site is exposed. And then what this showed is that the addition of these PAA coated gold nanoparticle resulted in the loss of the protein secondary structure. So, as you can see here, so what they have shown is, this is a circular dichroism its a its a method that measures the secondary structures in the protein. So, proteins when they fold they have structures like alpha helix

and beta sheets and others as well. So, these alpha helix and beta sheets have a certain wavelength through which they give signals.

So, if this is a normal protein structure which the C in this case. So, this red line is a normal protein, so which means that if you are getting this red line the protein is in this particular configuration; but as you add more and more gold nanoparticles to your solution what you find is that the structure is actually changing and you can see this signal that you get from the secondary structure is actually decreasing as you go forward. So, this is supposed to be the highest concentration. So, at this point you are talking about 10 microgram per ml of fibrinogen and give it to this 40 microgram per ml of your gold particles.

And just to show that the gold itself does not have any secondary structure they will just run the gold alone. But so, what you can see is there is quite a bit of change in the structure or loss in the secondary structure, which is actually proving the point that once this protein goes on to the particles, it changes structure.

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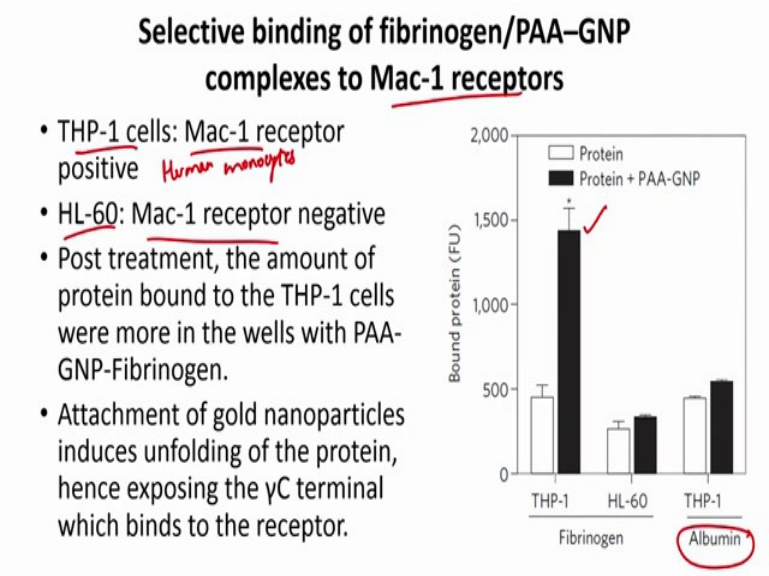

Then they have shown that this fibrinogen has selective binding for your Mac- 1 receptors. So, what they have shown is they have used THP-1 cells which are a human monocytes; and this THP-1 has a Mac- 1 receptor. And then another one they have use HL-60 which does not have a Mac- 1 receptor.

So, what this see here is if you just put protein, the amount of protein that gets bound to the cell surface is fairly low, but once you put protein plus gold nanoparticle you get quite a lot of protein that goes on the cell and the reason for that is now because the Mac-1 receptor site is now accessible, the Mac- 1 receptor on the cell surface now can bind to this.

Whereas, in the negative cell line you do not see that and if you incubate with albumin also you do not see that, because already the albumin has coated the particle. So, the particle cannot bind to the fibrinogen. So, just describing what I just said that causes the unfolding and that is why it can interact with the Mac receptor.

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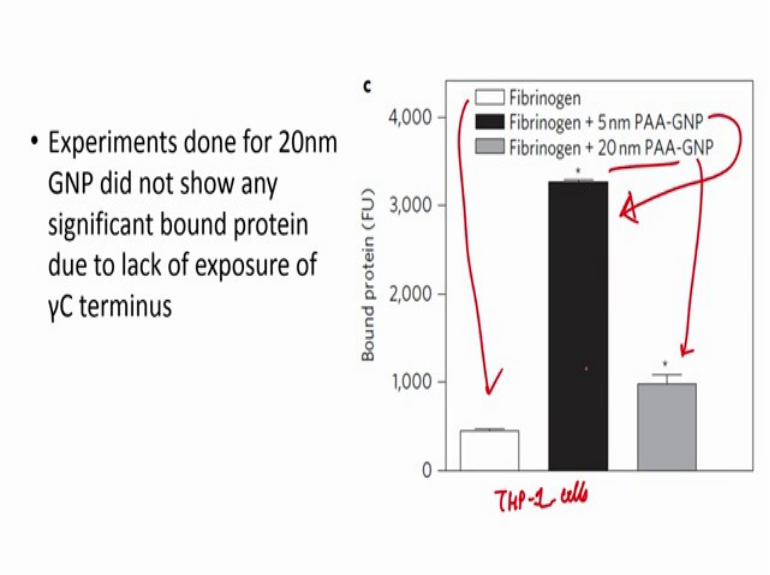

And then these guys further went ahead and show that the experiments it would done with 20 nanometer did not show any significant bound protein. So what they have done is this is again your bound protein on these THP-1 cells and what to see if you have only fibrinogen its structure is preserved. So, that does not cause anything. If you have 5 nanometers just like the last figure it causes a lot of binding of the protein. If you use 20 nanometer, which we showed earlier, it does not bind and change the structure that much, you see again there is a reduction in that protein that is getting adsorbed.

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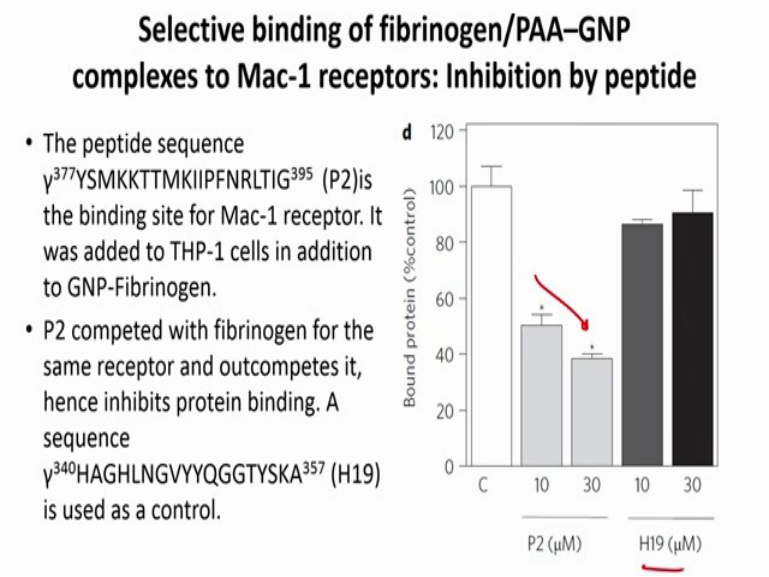

And then they showed that you can use some inhibitor of this particular Mac- 1 receptor and so, if you put receptors that binds to the Mac- 1, then your fibrinogen coated protein on the particle cannot bind to this and so that is what they have shown here. So, the bound protein is actually decreasing whereas, if you use another peptide which has no affinity, it does not do anything. So, it is a further confirmation that this is Mac-1 receptor that is actually interacting with your protein.

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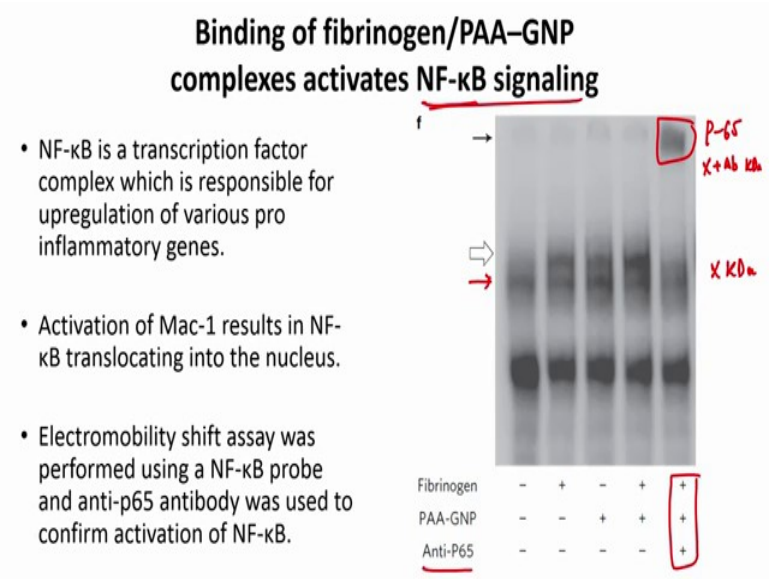

And then they have gone ahead and showed that the binding of the fibrinogen activates NF-kB signalling. So, if you take this last example what they have done is they have added all fibrinogen ,PAA gold nanoparticles, as well as antibody that binds to the NF-kB P65 domain and so, what you can see is because of that you get a big shift.

So, if NF-kB let us say was for an example x kDa and let us say in a normal gel the x kD will come here. Once it binds to the antibody now its molecular weight is increased by x plus the antibody molecular weight, so that has now gone and shifted up there. So, further confirmation that actually this gold nanoparticle bound fibrinogen not only is activating Mac-1, but that are causing further signalling in is actually in causing a NF-kB expression.

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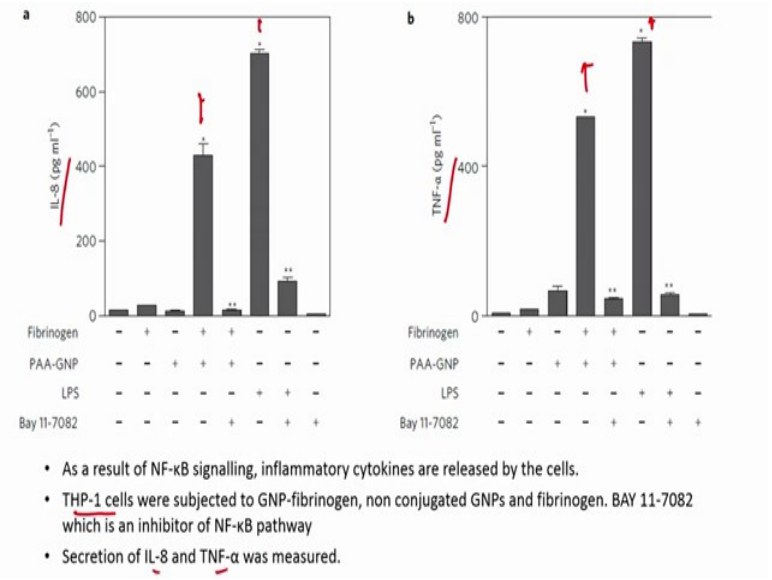

And then again as I said NF-kB is a master regulator. So, it will secrete lots of cytokines. So, in this case you are seeing IL-6, IL-8 and TNF alpha which are both inflammatory cytokines. So, their expression level goes up when you have NF-kB signalling and which is again a major cause of the inflammation that is happening. So, again the THP-1 cells were used here which showed that the secretion of IL-8 and TNF alpha.

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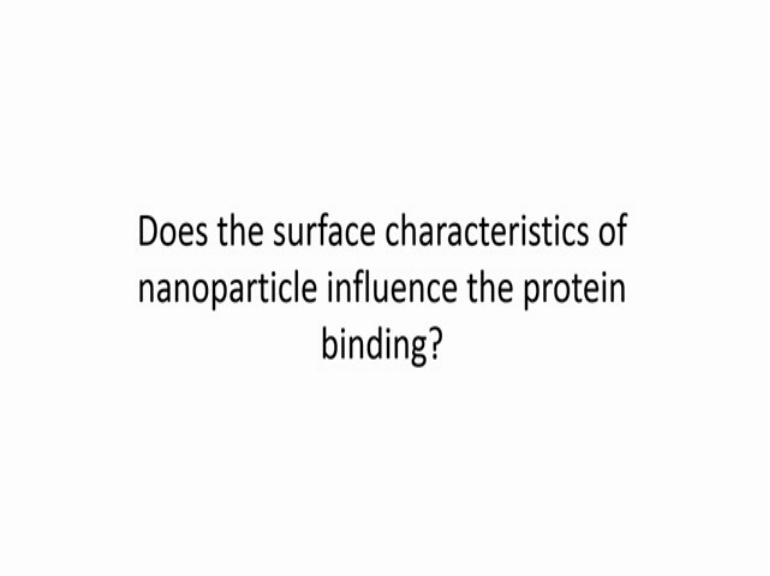

And so, does the surface characteristic actually influence the protein binding? So, what they did is, now the change the surface.

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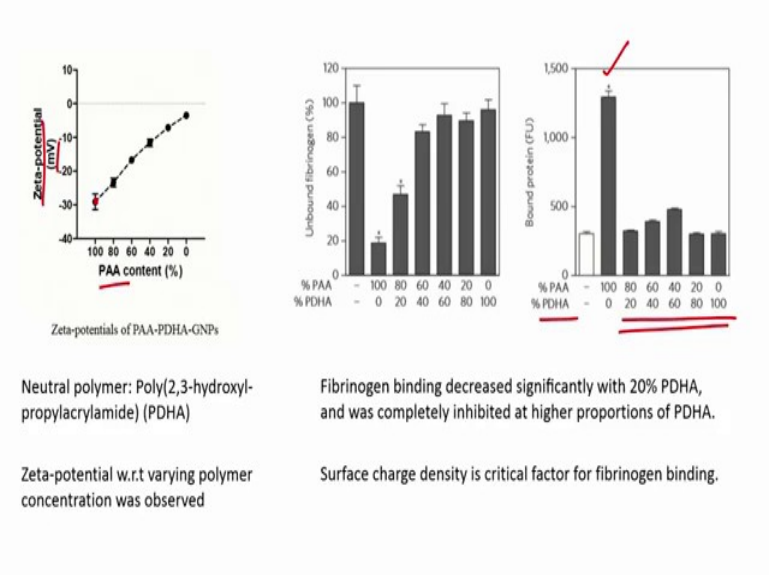

So, in this case what they did is when they made these gold particles they kept on decreasing the PAA concentration, the PAA, as I said is Polyacrylic Acid, which is highly negatively charged. So, when use 100 percent PAA you get a fairly highly negative charged particle the zeta potential is a way to measure the charge and as you decrease this PAA amount the charge increases because this negative charge is going

down and so, what they further show now is that your protein binding is actually decreasing to the gold particles as you are changing the charge.

So, they have replaced PAA with this PDHA and they are further showing you that charge is what is responsible for binding of this protein to the to these cells and if you have lower charge, then your fibrinogen is either not binding to your gold particle or even if its binding, it is not exposing Mac- 1. So, this as a surface charge density is critical or fibrinogen binding in this case. So, I think that is where we will stop and we will take it further in the next class.

Thank you.