Introduction to Proteomics
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Lecture - 05
Lab session – Protein-protein interaction using label-free biosensors

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As you know that there are detection systems like in case of the protein estimations or the kinetic studies, you people perform the ELISAs. Like in ELISA, it is endpoint detection. You are attaching a kind of a tag to that, but there is the other UV spectrophotometer based like where exactly you are measuring at a 280 nanometer due to the tryptophan excitation automatic excitations.

Or sometimes you are attaching a fluorescent tags. Those are called as labeled based, even in case of your iTRAQ or ICAT based in mass spectrometry, you are attaching a tag right there. Here label-free detections, where exactly label-free detections comes is, there is a different concept solution-solution interface, solution solid interface. These are the 2 techniques. You heard about ITC techniques, isothermal calorimetry based titrations.

It is where exactly if there are 2 proteins are interacting together, what is the energy absorbed or released into the reaction system? This is we call it as a solution-solution interface. I think in the last this one, there is a one technology called as a NanoTemper. It is a kind of once

again solution based, solution-solution interfaces. Here I am going to discuss about the solution-solid interface. What exactly this solution-solid interface?

So one of the protein say suppose 2 proteins are interacting together, one protein we have to put it on a solid support and another should be in the solution form that is what we call it as a solution-solid interface okay. I am going to discuss about this. What exactly this BLI technology is? BLI is nothing but the bio-layer interferometry. SPR technology, it is a surface plasmon resonance.

Here we have a like a BLI technology. The technologies are different. The output from the SPR or from the BLI is the same okay. So how exactly this technology was? The word interferometry once again light has come into pictures, the optics.

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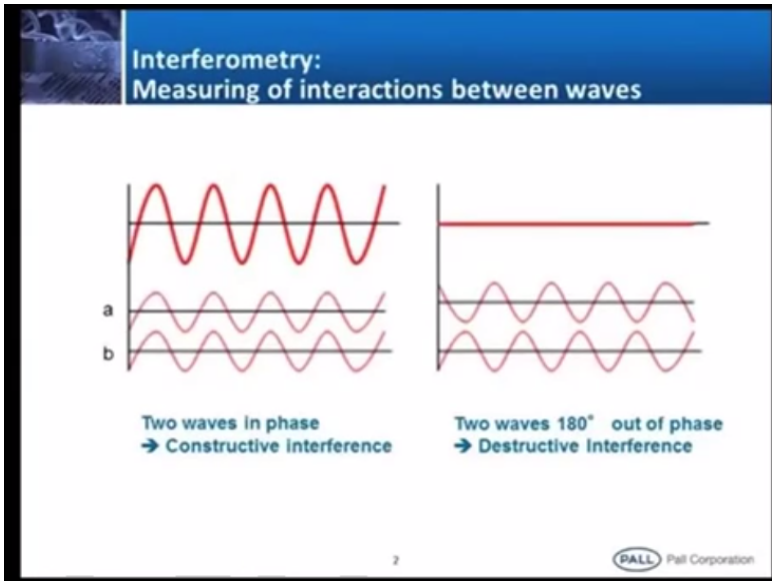

So as you know that light, it consists of waves once again. When it propagates in a same direction, we call it as a constructive interference. When it is in out of phase at 180 degree, we call it as a destructive interference. So constructive as well as the destructive interference makes the signal pattern here. How exactly?

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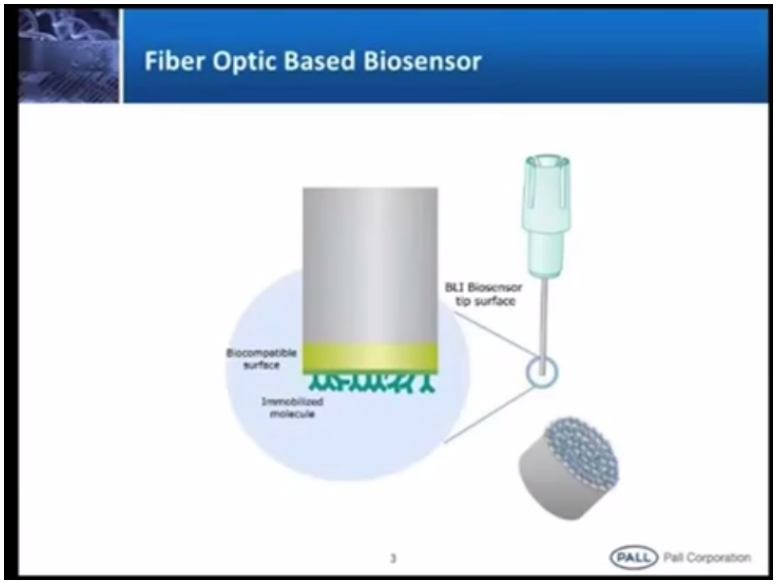

So we are using a sensor based device here. If you look at this, this is a sensor this is made up the plastic. This is the glass consist of a fiber optic inside. So if you look at this diagram, at the tip of this we have some kind of a coating we call it as a biocompatible matrix to that we are going to attach a protein of your interest. We call it as a ligand. That is what we are going to immobilize. This is what we call it as solid support.

So in case of ELISA, you are also coating one of the protein to the plate right. Similarly, we are coating on to the sensor.

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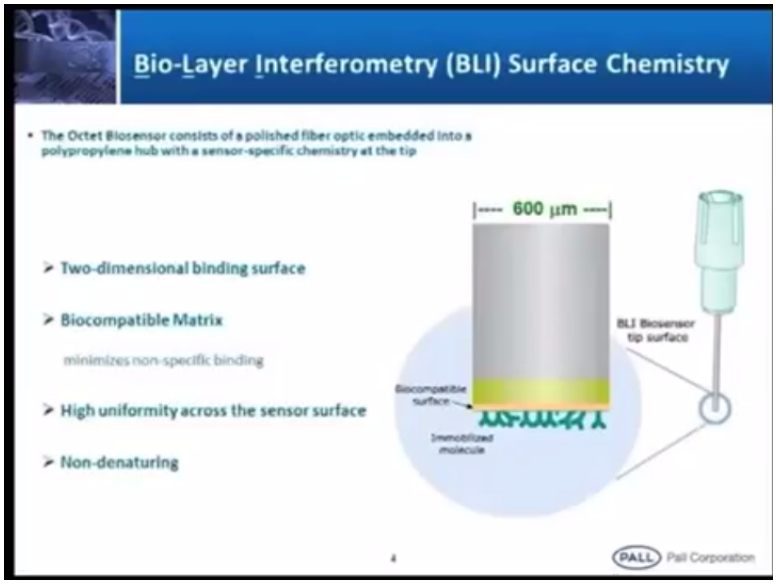

If you look at next diagram, look at the diameter of this. It is just only a 600 micrometer, it is a 2-dimensional binding surface when protein immobilize onto a solid support and the biocompatible matrix is something like it is inert in nature, you can use any kind of (())

(03:27) buffer systems, pH from 1.5 to pH 9 we can easily work. Most of these biomolecular interactions we are working at the physiological pH.

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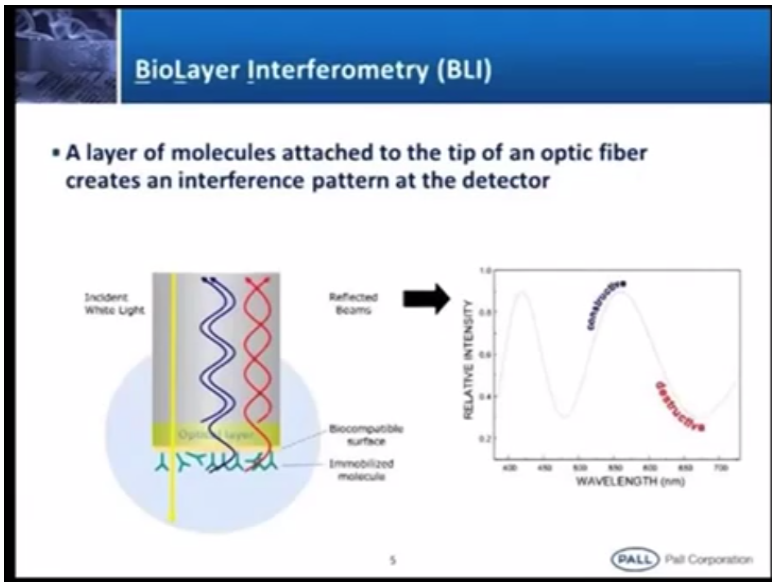

So how exactly this technology works? As I told it works on the interference pattern. Interference patterns make signals here. I am passing a light, it is a white light, it is just only from 400 to 800 nanometer, it is not a UV based. So I am passing the light, light get reflected back. It is a reflection based phenomenon. So one of the light, which is coming from the internal optical layer.

So suppose when we say that there is an interference coming say suppose I am putting a 1 stone into a water, you see that there is waves right. If I put a 2 stones at simultaneously to water, what happens? Waves will be going that side but this side there is like each are mixing together. Then they call it as interference. Here due to this biocompatible matrix, always there is a kind of the block, some light gets reflected back.

It is not like that only a 5% of the light only gets reflected. Rest of the 95% will be transmitted in the well. So 1 light which is going through the ligand due to your ligands, there is a kind of obstacle here, once again light get reflected back. Constructive interference makes the signal rise; destructive interference makes the signal drop.

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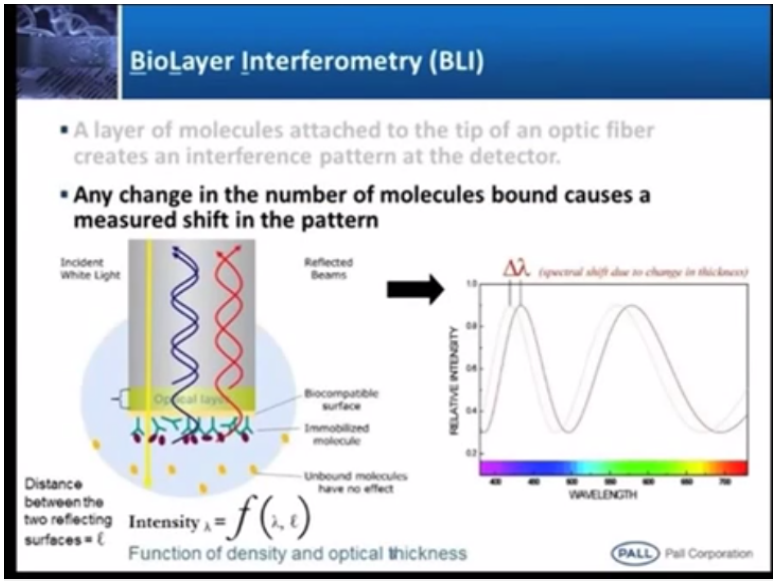

So suppose when same sensor when I dip into the corresponding binding partner and I am giving an example. Here I am coating a sensor with a protein A matrix, protein A has strong interactions with monoclonal antibody okay. So if you look at this diagram, the antibody starts binding to this ligand. Now earlier always there is a constant from the internal optical layer. Now there is a reflection coming from here.

If you look at this, there is a path length get increases, earlier the light was coming from here, now the light is reflecting from here. So this optical path we are measuring in terms of the optical thickness, that optical thickness we are measuring in terms of nanometer binding. Say more the molecules starts binding on to a sensor, there is a more shift in the spectrum. As the molecule starts binding, earlier you can able to see if I go back this is your wave okay.

Due to the binding, we can able to see there is a shift in the spectrum. We are going to measure this shift as well as the optical response. The intensity is the function of the shift in the spectrum as well as the optical thickness. This is what the principle behind here.

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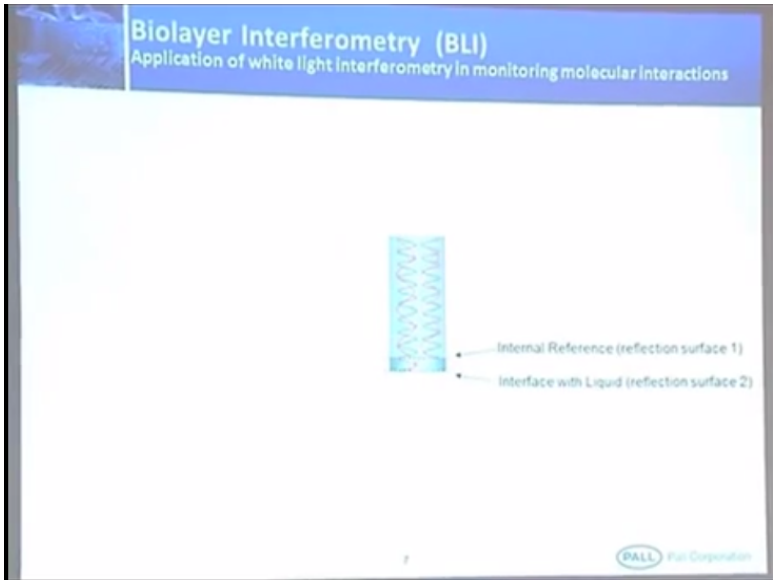

I am passing a white light okay, light get reflected back, one I said from the internal optical layer due to the matrix, another from your ligand end, so you can see that all these is constructive as well as the destructive interference.

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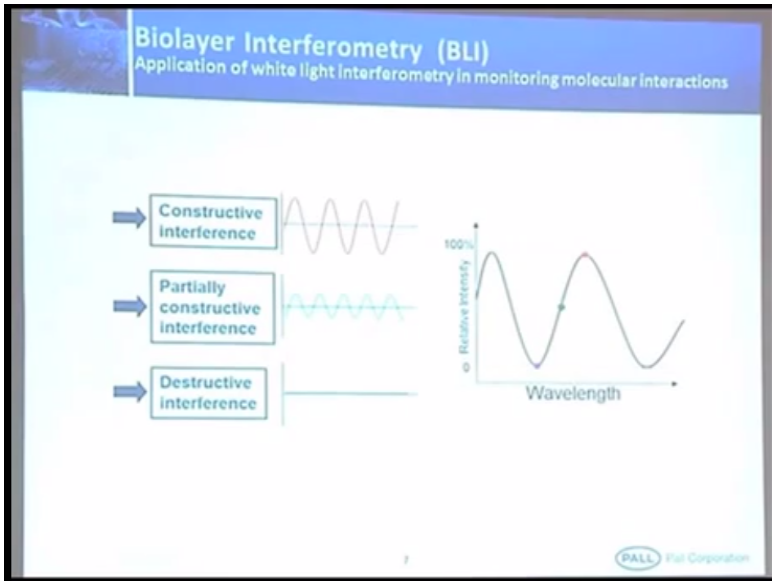

Constructive interference makes the signal rise; destructive interference makes the signal drop. The same sensor when you dip into the say suppose I gave example last time like that protein A immobilized on to the sensor, then I am dipping into the corresponding antibody as a analyte.

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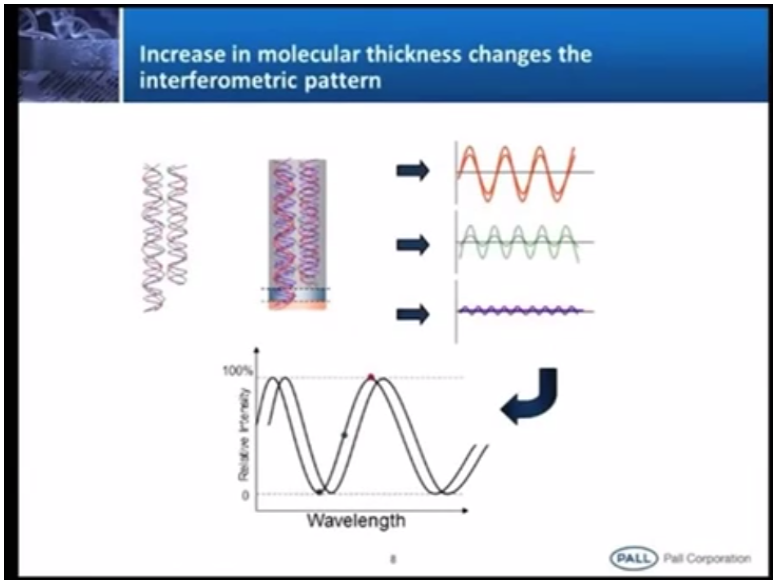

Say suppose sensor is already coated with a protein A, now I dipped into the corresponding antibody solution. Now you can see, there is orange thickened layer right, this is due to the interaction between the protein A and the antibody. Now you can see light get reflected back from here, earlier was from here, now from here. Due to this, you can able to see there is shift in the spectrum.

The path length get increases right, that is the path length is also dependent on the concentration. More the concentration, more the molecules starts, your optical thickness gets increases. If it is a less molecule, then the shift will be less. Due to the interaction, we can see that there is a shift in the waves. Can you able to see these patterns? This is due to the interactions.

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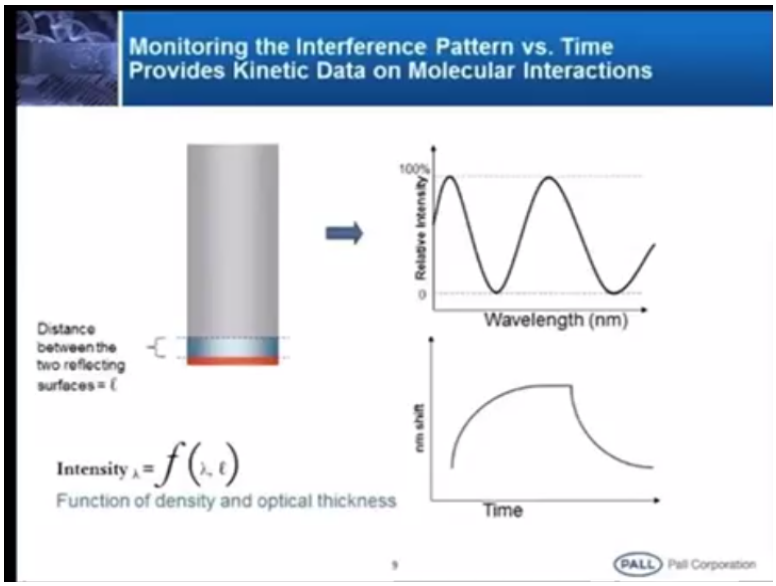

Now as the molecule starts binds, the real time you can see the molecule starts pressing there is a curve like this. This is what the background, this is what the read out okay. See this is what we call it as an association phase as the molecule starts binding to the binding partner for that we can see there is a curve rises, this is we call it as an association. Once if we like same sensor we bring back to the buffer then the bound molecule to the receptor get washed off okay.

Now you can see this phase we call it as a disassociation phase. So disassociation by the association we call it as affinity. So when we say kinetics, say most of the people do the label based platforms, kinetic studies like ITC the people do, the people do the ELISA based methods, but all the other methods only give affinity constants. Say suppose I can plot like sigma like this at the midpoint in the IC50 what we call it as affinity.

There are the equation to calculate the affinity, but label free platforms are the only instruments, which gives the on rate and the off rate value. None of the any other instruments gives the on rate and the off rate. What is on rate? On rate is nothing but the association rate, off rate is nothing but the disassociation rate. So disassociation rate by the association rate gives the affinity.

So we have to determine these affinity constants. These affinity constants if says protein-protein are interacting strongly, this affinity we are in terms of call it as a picomolar affinity. If there is a moderate interaction say suppose when antigen binds to antibody or a protein binds to a receptor, you will see the association phase. Now it starts dropping when in the disassociation phase is if it is like this we call it as a moderate interaction.

If it is like a strong interaction like straight line from here, this is we call it as a picomolar affinity. What exactly this picomolar affinity, nanomolar affinity or a micromolar affinity? Say suppose in case of the drug discovery or anything, you required a small amount of a drug to elicit a response, just like a picogram material or autogram level that much concentration is required to elicit a response or efficacy of the drug even I mean to say.

So such kind of things comes into picture here. Less concentration, more response, longer time in the body that is we call it as a strong affinity towards the receptor. If say suppose giving an example, doctor says that you have to take a paracetamol 3 times a day, it indicates

what means drug is not much have like affinity towards the receptor. Otherwise some tablets people say that you have to take it one tablet per, even some injections are there once in a month.

These are what? These are how strong how weak are these are binding, based on that we can decide the dose okay.

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So I will go through this. We have a (()) (10:59) we have like different platforms for designing the experiments and to perform in the discovery. From some single channel, it is a very small, in one hand you can hold, looks like our NanoDrop machine. Single channel, manually operated, one sample at a time okay. This is 8-channel instruments what I brought this one here.

At a time, you can go for a 8 sample, you can finish off the kinetics or the quantitation study. Say suppose quantitation, you are something like expressing the proteins which have **"Professor - student conversation starts."** These are the instruments like this is the 8-channel, 8 sample that we can measure at a time. We can perform like different dilutions in the kinetics, higher to lower dilutions we can perform and we can conduct kinetic studies here. **"Professor - student conversation ends."**

See here for the red instruments, we can see the dynamic range wise. The maximum we can go from 10 picomolar to 1 millimolar. That is the dynamic range we can go and the size was interactions say suppose small molecule protein interaction, protein-protein interaction,

vaccines, live virus, bacteria and all we can able to perform. Here with respect to the size wise, 150 Dalton is the minimum detection to the megadalton protein interactions.

Megadalton in terms of virus like particles, it is like a very multimer, we are measuring in terms of the nanometer not with respect to KD or size wise. It is such a big we can able to do still quantitation. In case of vaccine industry, people are using this machine for the quantitation purpose and this is the one this is the blue model, it has a similar 8-channel function.

But this instrument not able to useful for the small molecule protein interactions. Only we can go protein to megadaltons, less than small peptides or small molecules are not possible with this. This is the one recently launched, this is the 2-channel especially for the academia instruments. It only takes 2 samples, one at a time. These are the very high throughput instruments.

This one 384-well plate formats, 16-channel instrument, the sensitivity is similar to this red one and we can perform like a quantitation and kinetics at the high throughput level. The blue one once again sensitivity is similar to only for the proteins not for the small molecules and the peptides. This is the high throughput machine. This is also very recently launched. It has 96 channels.

So suppose if I put a 96-well plate, in one stretch 96 sensor it will pick up and go to the 96-well plate. You can read a quantitation in 2 minutes, so such a high throughput level.

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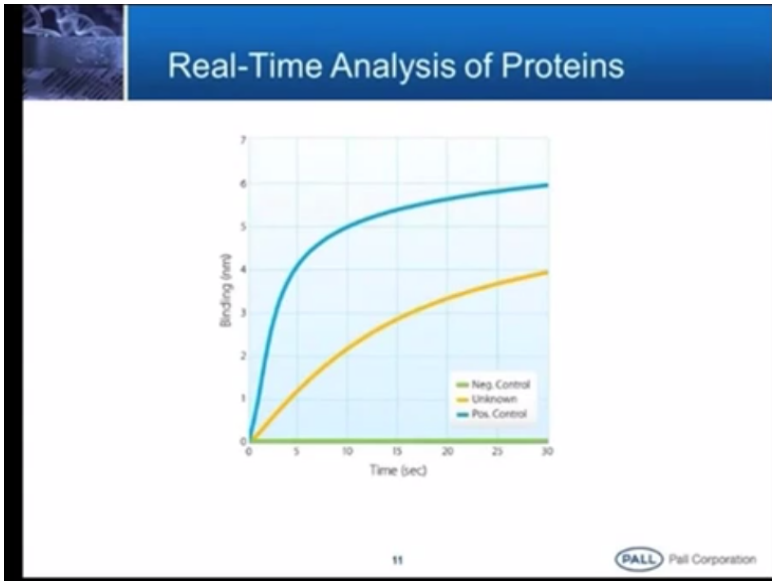

So this is what the read out is, how it comes, the example I had shown.

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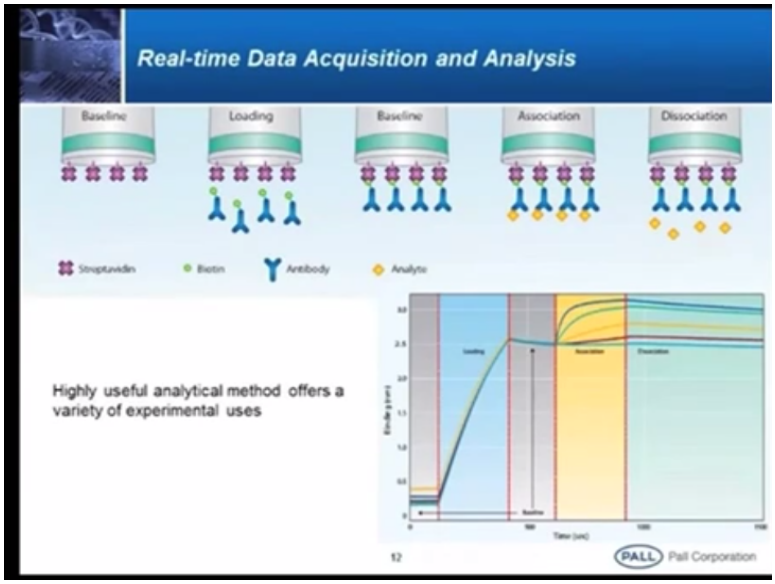

Kinetics actually, how we are performing this kinetics? During the example here one of the sensor it is streptavidin coated sensor, the matrix is streptavidin here and immobilizing antibody which is biotinylated and then this is if you look at, there is a response like this. The sensor just it is a baseline, when I dip the sensor into the corresponding biotinylated antibody you see the gradual rise in that.

The same sensor when I bring back to the buffer, unbound material gets washed off here and the sensor now I depend to the corresponding antigen we see the curves like this, different dilutions 2-fold or 3-fold however you are making. This is what interaction between this

antibody and the antigen. Once again the sensor, I bring back to the buffer you see the disassociation. So this is what completely we call it as kinetics.

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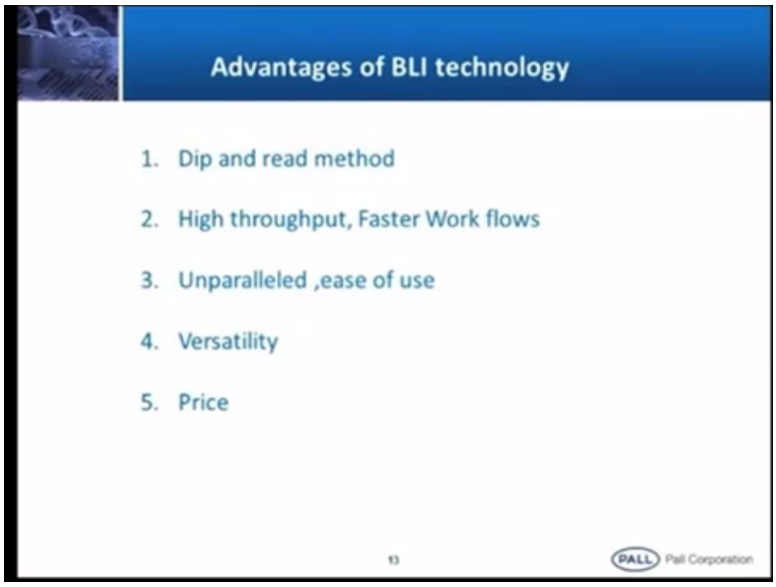

So advantage is like that on the bio-layer interferometry, it works on the dip and read format. So just in the 96-well plate, we have put antigen, antibody and all that, the sensors it will move like this and we can rate and advantage is like that there is no microfluidic devices. In case of the SPR, it works on the principle of the microfluidics. You know in HPLC, there is a peristaltic pump, it will deliver the mobile phases.

In case of the SPR also, there are the buffer systems, it will deliver the mobile phase or the buffers on the sensor surface. So what happens is this in the microfluidic devices, we cannot use a kind of samples like crude sample. Suppose from the serum if you want to do a serum sample analysis or the body fluids or the samples from the bioreactor itself because it contains so many other kind of contaminants and all.

It blocks the flow cell, once if it blocks the flow cell, we have to replace the complete assembly that is what the expense in case of the SPR microfluidic devices, but here it is a dip and read. Something goes wrong also we just we can throw a sensor, but there is no any other kind of such fluidic devices here and the high throughput I said it is like 8-channel, 16-channel depending upon the application we can use, unparalleled and ease of use.

I can show you how the software is so user-friendly, just if I train half an hour for you, you yourself can work on this instrument such user-friendly, anybody can operate. In case of the

SPR, we require a dedicated operator for that. So here that is such an easy system. Versatility, sample I said you can take from the crude or the purified or the unpurified any kind of samples we can go and the pH, we can work from the pH 1.5 to pH 9.

Depending upon your application most of these biomolecular interactions we are working with the physiological pH. The price wise if I compare the sensors SPR chips versus these biosensors, this one sensor cost you something like 5 dollars and you can regenerate this sensor okay. Just I perform the instructions, I can regenerate the surface, I can use next time. At least, these sensors we can use for 10 times okay.

That is what the advantage is like that. In case of the SPR also, people are regenerating. If it is something goes wrong in case of the SPR chips, you have to throw the complete sensor, that sensor cost you something like 150 to 200 dollars depending upon the what chemistry you are buying. Here this is like 96 sensors we are providing, 96 different kinds of experiments you can perform here that is the advantage.

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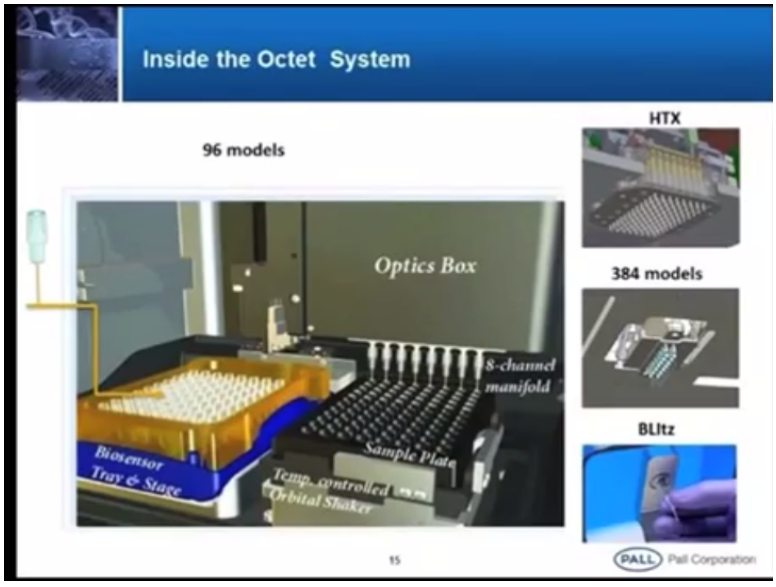

If you look at the inside of the instrument, this is we call it as a sensor rack, this is the optic box consists of a spectrophotometer inside, it is an 8-channel instrument, each channel has a one, one spectrophotometer built inside and this is your 96-well plate sample compartment. It will move in this fashion, parallel okay. It has like a temperature controller as well as the orbital shaker.

So here I said we do not have microfluidic devices, for to assist a binding we required a shaker so shaker enhances the binding. So this shaker we can work from 100 RPM to 1500 RPM depending upon the applications. Say suppose when I can use what kind of RPM? Say suppose if I am working with a high concentration, I can reduce the RPM, 400 or 500 I can work it.

If you are working with a very low concentration, then I have to increase the RPM speed. So shaker will enhance the binding. This is the 8-channel, we have a 384 model, this is what the optical head is. This is the HTX I said high throughput instrument, multiplexing. Here the advantage for this instrument in the HTX is, we can analyze one sample also, we can analyze 96 samples also, it is a multiplexing.

If I have like a 16 sample, I can choose 16 heads, if I have like a 48 samples, you can choose 48 heads depending upon. The last single the Blitz instrument, it is a single channel, manually operated, you have to put the sensors manually and then read, but rest of these high throughput instruments are all are automated.

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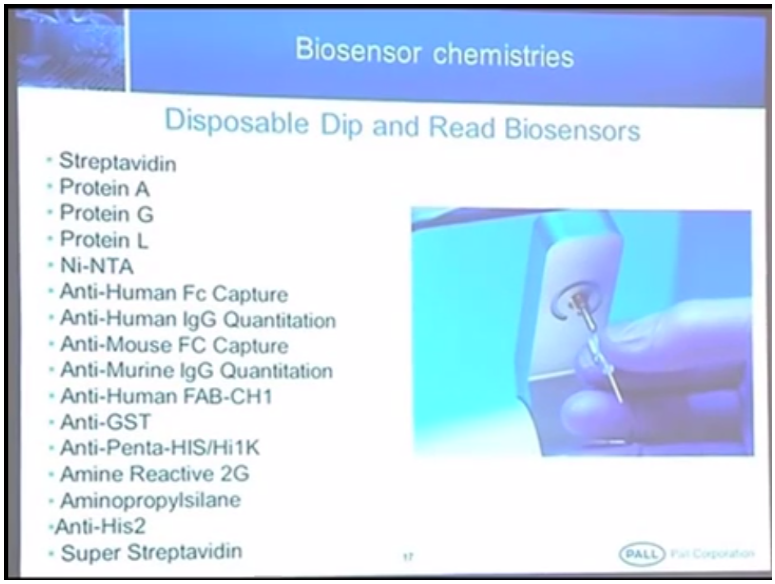

So as I mentioned the biosensors, we have around 16 different chemistries with us and depending upon your application we can choose. Next, streptavidin and the amine coupling, amine reactive, these are useful for the custom protein quantitation as well as the kinetic studies. So streptavidin here one of the protein you have to do a biotinylation, then you have to immobilize.

In case of the amine reactive, there is a kind of chemistry we had to use like it is your end terminal or the lysine get involved covalently coupling to the sensor surface.

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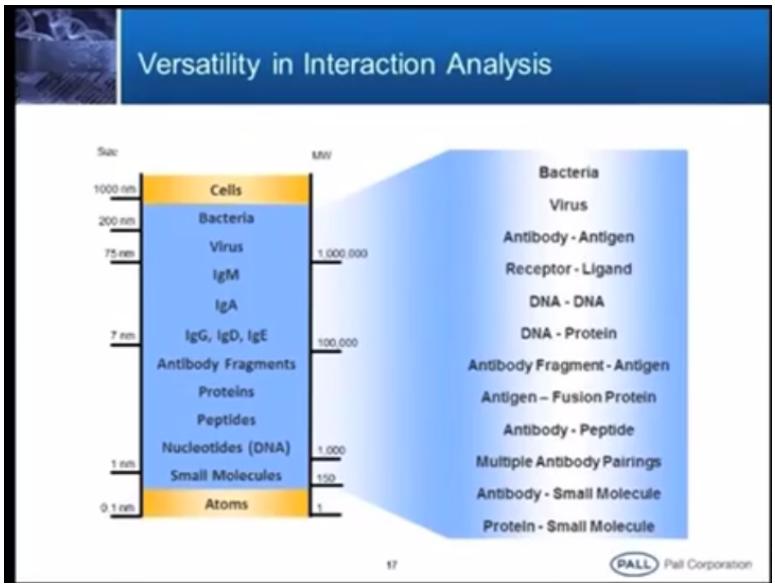

So we can work the versatility of the interaction except cells and atoms, then in the blue whatever is there all things are possible here. Cells is something like in terms of the micron size. If you look at the surface of the sensor, it is just hardly can hold like a 3 or 4 cells on that. This is very difficult to work on the cells. Rest you can even you have like publications on the entire bacteria, virus and all, 0.2 micron such kind of applications we can work.

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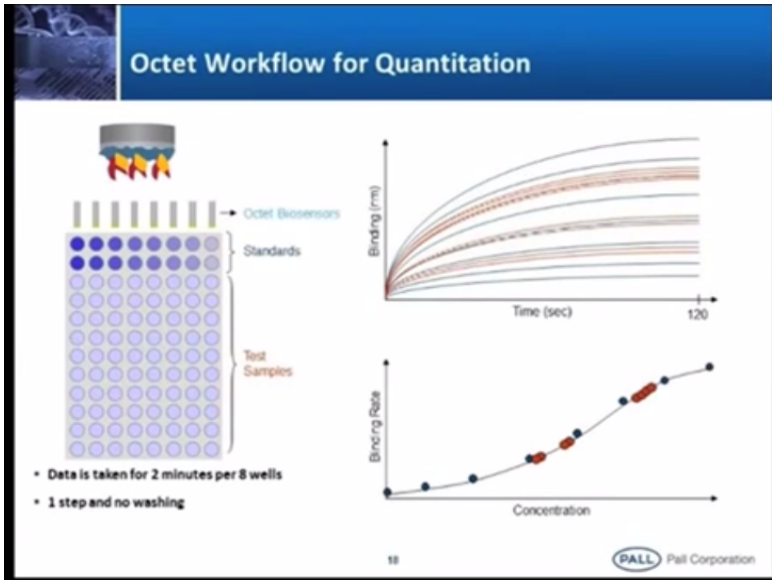

How exactly it works for a quantitation purpose? Eight sensors I am dipping here, you can see, generate different concentrations here say from 100 microgram per ml to 4 dilutions if I go, 8 concentrations we can determine, 8 samples read at a time. Think about if you are

performing even a UV based, one, one sample how much time it will take for a reading? Roughly 8 concentration or a 6 data points half an hour time.

Each time you have to wash the couette and then put next concentration, but here the advantage is like that it is purely an affinity based we will get. In case of the UV, I am giving example, you people are performing the Bradford assays right, it is what you are doing is the purely and total protein quantitation, not exactly the protein of your interest.

Even in case of the HPLC also, it is at a 280 monetary, it is once again resolving but even though you do not know how much pure is your peak. There may be some other proteins which have a same hydrophobicity it will elute at a same time. That is also something like that. Here we can purely an affinity based interaction. If you have a specific antibody or a specific receptor, then you can easily quantitate the how much protein is in your expression.

Now if I dip into the unknown sample, we will see these are the unknown, from this data points we can easily calculate the how much protein is that, very simple.

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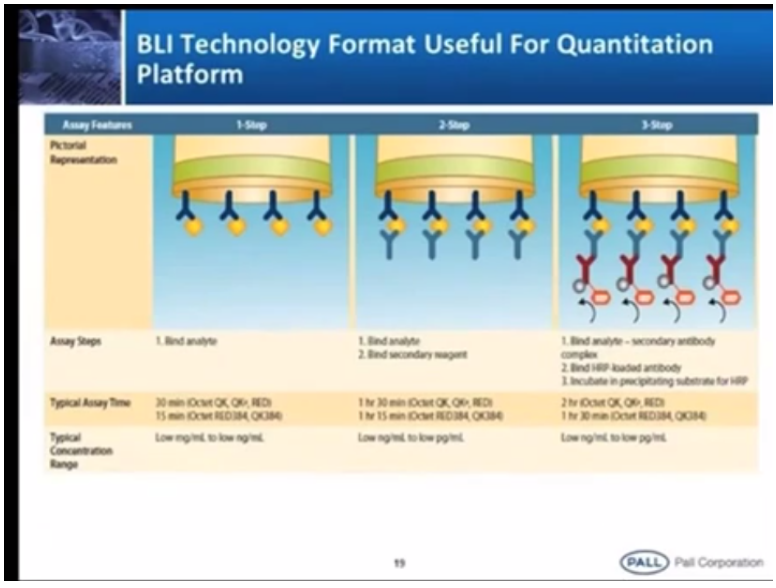

We can do a different kind of experimental designs here, whatever I have shown is called as a direct binding, 1 is to 1, this is our antibody or antigen or a protein-protein. Here we can work from low mg per ml to low nanogram per ml. The instrument dynamic range is 50 nanogram per ml to 2 mg per ml. This is what we called as a direct binding. Say suppose your protein expression is in the nanogram or picogram level.

That time we required a kind of a secondary antibody to amplify the signal. In case of your ELISA, you people use as a secondary antibody right. Similarly, we can use here secondary antibody to amplify the signal. Here the concentration we can go from low nanogram per ml to low pictogram per ml. Say suppose I want to replace the complete ELISA onto this instrument is possible.

That is also there. If you look at the optical thickness right as in the principle, I said more the optical thickness better the amplification. See so many things are here we can amplify the signal.

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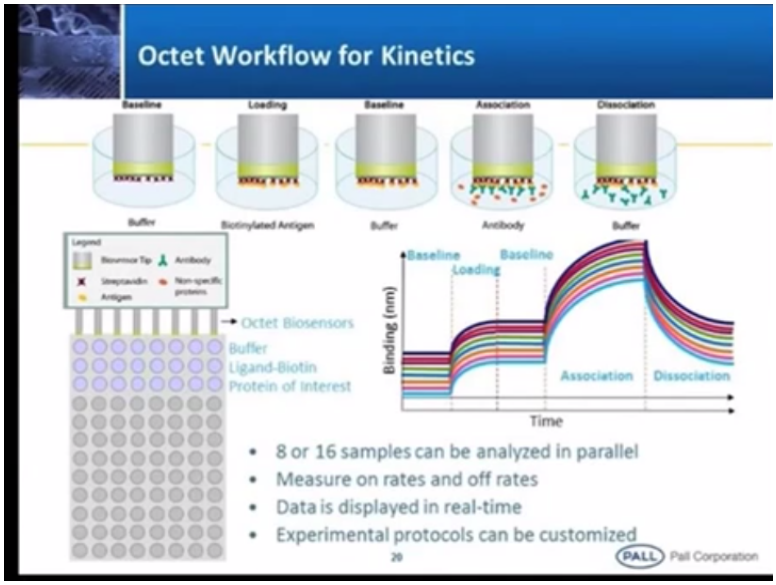

How exactly kinetics works? Just I will put a sensor, take a buffer, dip into the buffer, I will get a baseline and just as a PBS buffer. I dip into the biotinylated antigen; you can see that this step we call it as a loading or the immobilization step okay. Then I will dip the sensor back to a buffer, unbound material gets washed off here, dip into the corresponding binding partner the antibody, we see the association.

When the sensor will bring back to a buffer we call it as disassociation. So in 1 stretch you can do the 8 samples at a time, 8 different concentrations okay.

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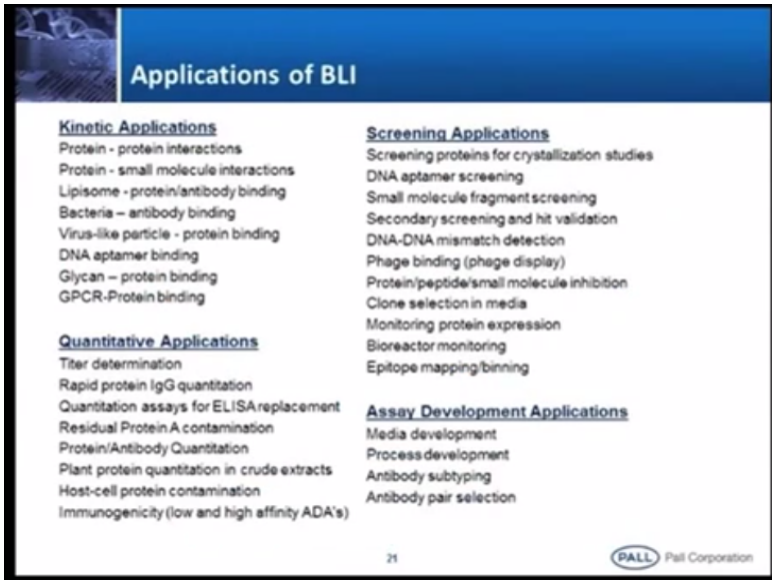

These are some of the applications. We have around more than 1000 right now. This technology started in the year of 2004, ForteBio launched this instrument and later it is picking up and people have a like so many in case of the academia, they come up with this kind of publications like DNA aptamer screenings, liposome antibody like say GPCR, so such kind of things we can immobilize onto a sensor surface and work.

Kinase interactions, yes we have data's on this. So around more than 1000 we have right now.

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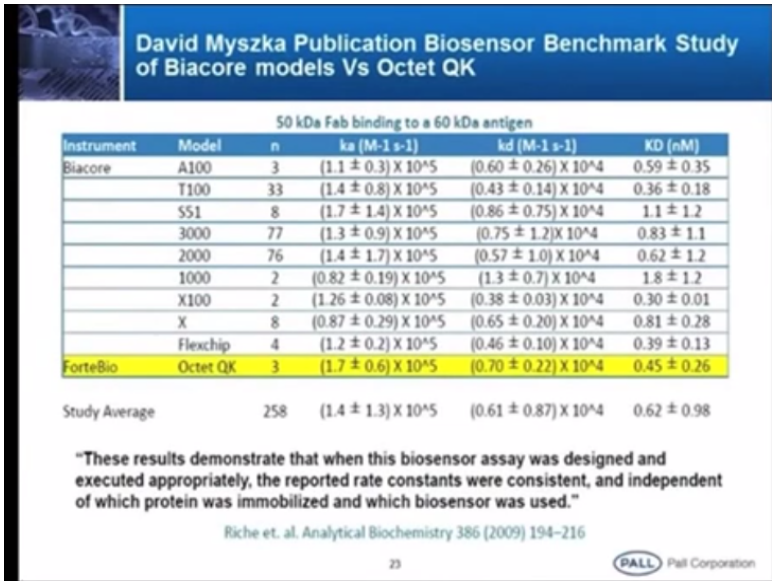

Applications wise this is compared with; these guys are David Myszka from University of Uppsala. He is a pioneer in this area, biomolecular interaction. He has compared the interaction of 50 kilodalton Fab binding to a 60 kilodalton antigen with respect to different SPR models and with respect to ForteBio, it is almost all the data's are same because SPR

technologies are very old almost like a 30 years it is in the market. Now we are the ForteBio almost like 10 years in the market.

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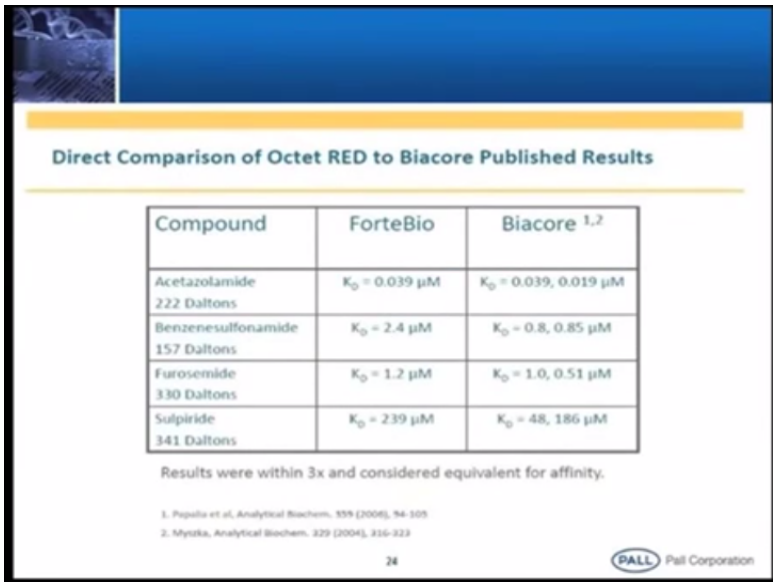

This is the example I am showing a comparison with SPR. This is a small molecule; these are the carbonic anhydrase inhibitors. So carbonic anhydrase was immobilized onto a sensor and then these are the drugs, the inhibitors passed on this. These are (()) (25:16). This is also once again the publications by the Myszka.

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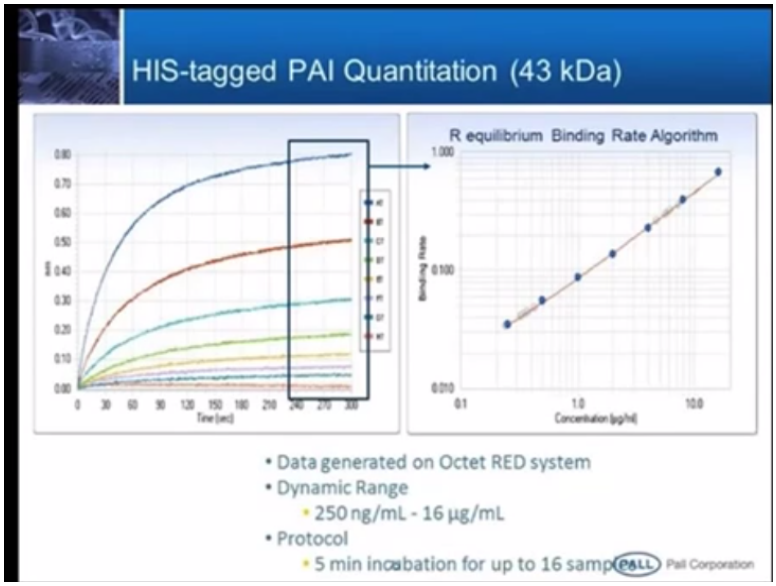

This is the one of the example protein A quantitation, HIS-tagged protein A it is. The dynamic range is 250 nanogram per ml to 16 microgram per ml.

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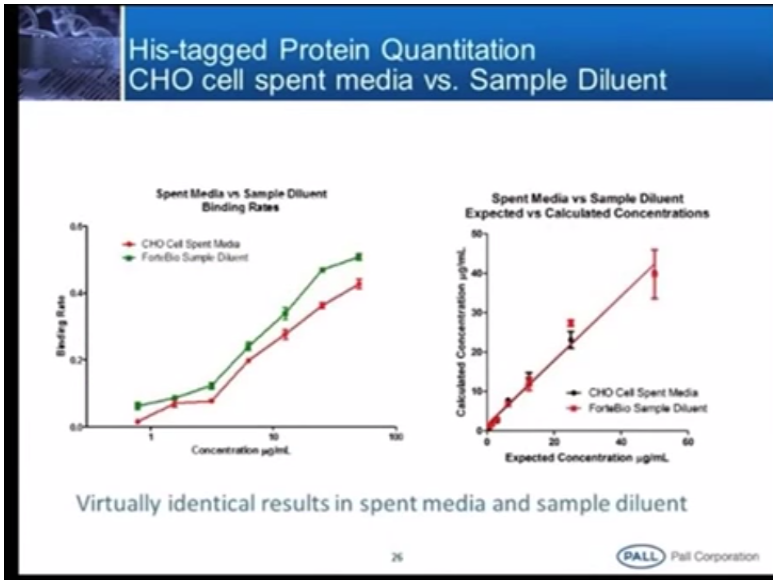

So here they had performed the media versus just only a buffer it is on par the results are. So we can use even to quantify the protein in your culture.

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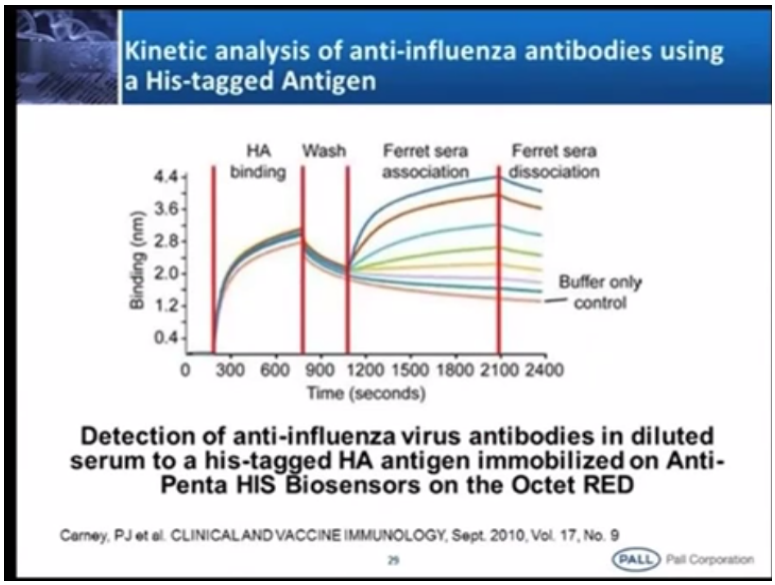

This is one of the example, these are published examples, ant-influenza antibodies using for the HIS-tagged antigen kinetics measurement.

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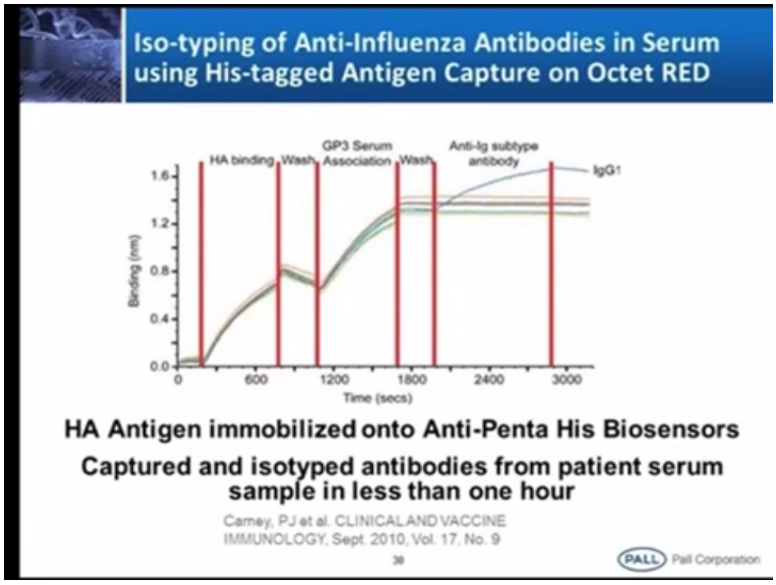

This is the isotyping example, serum using isotyping of the anti-influenza antibodies in the serum using HIS-tagged antigen captured on (()) (26:13). See if you look at this, there is only one which have like affinity towards that we can able to, rest is the baseline.

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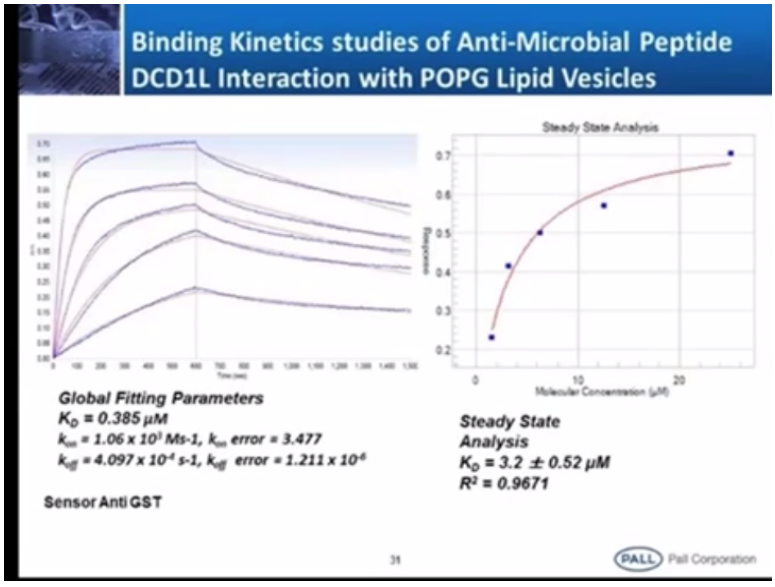

This is the lipid vesicles with respect to peptide.

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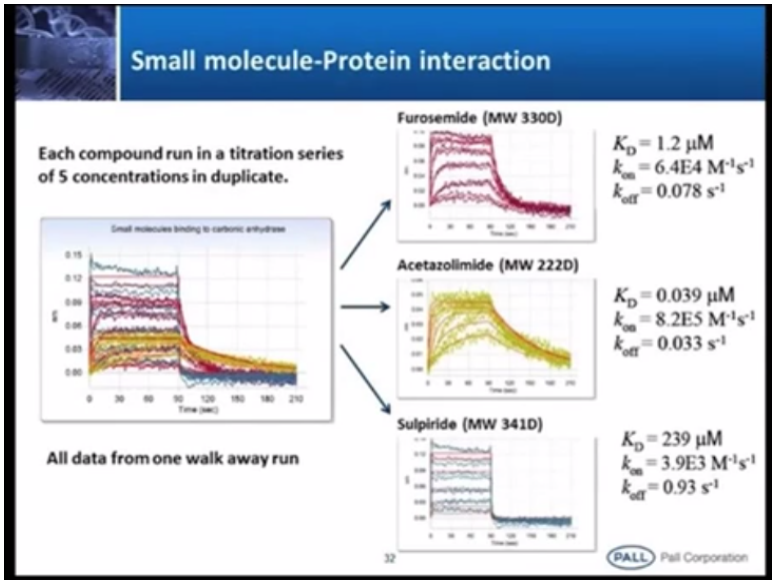

These are the once again small molecule protein interactions.

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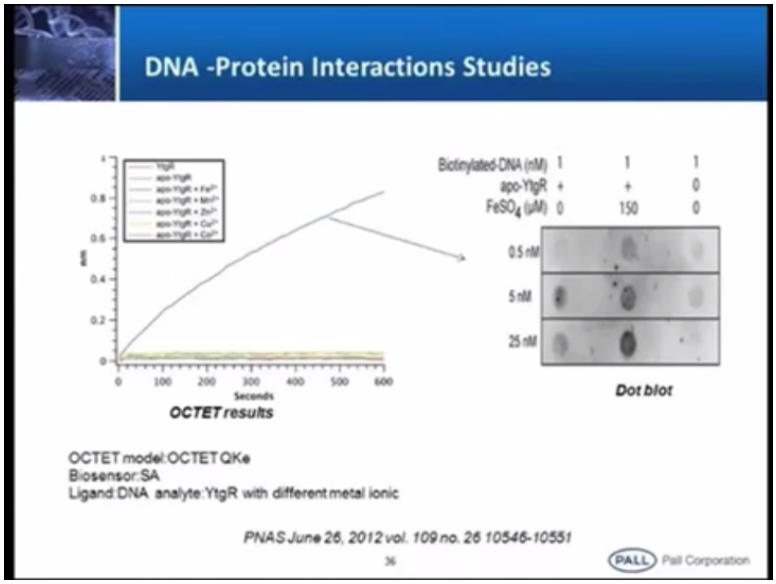

This is the DNA protein interaction studies. Say suppose this is the one of the example that the biotinylated DNA, they immobilized onto the streptavidin sensor, then apo-YtgR protein in presence of the ferrous sulfate, you can able to see there is only binding. They had used different metal ions here, none of the metal ions like used just a baseline, in presence of the ferrous you can able to see this binding.

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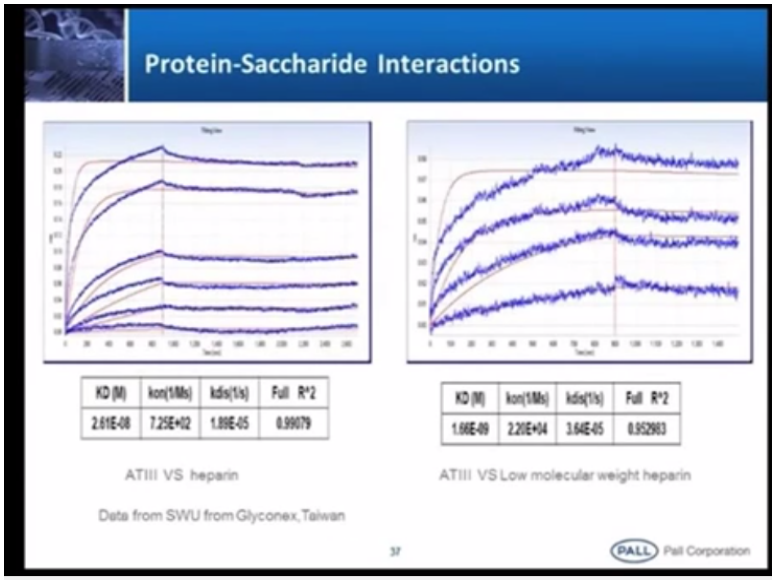

That is what it is a very specific interaction. **"Professor - student conversation starts."** Biotinylated, one of the strand you have to do a biotinylation. **"Professor - student conversation ends."** Protein biotinylation is easy compared to the DNA biotinylation. You have to do using a PCR you have to synthesize that.

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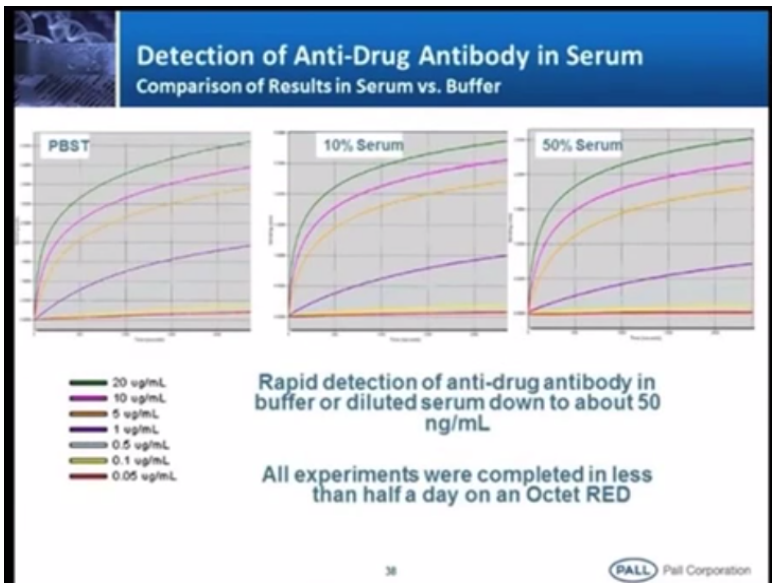

So detection of anti-drug antibody in the serum, this is one of the important for the therapeutic industries because most of these drugs when we are injecting into the body, body itself recognizes that as a foreign particle right. Then once again your immune system get activated, it develops the antibody. So we have to detect that. That is we call it as immunogenicity testing.

It is important for the biotherapeutic industries especially those who do. We have to detect that how much anti-drug is there? Is there any this anti-drug antibody, is the neutralizing your drug of interest.

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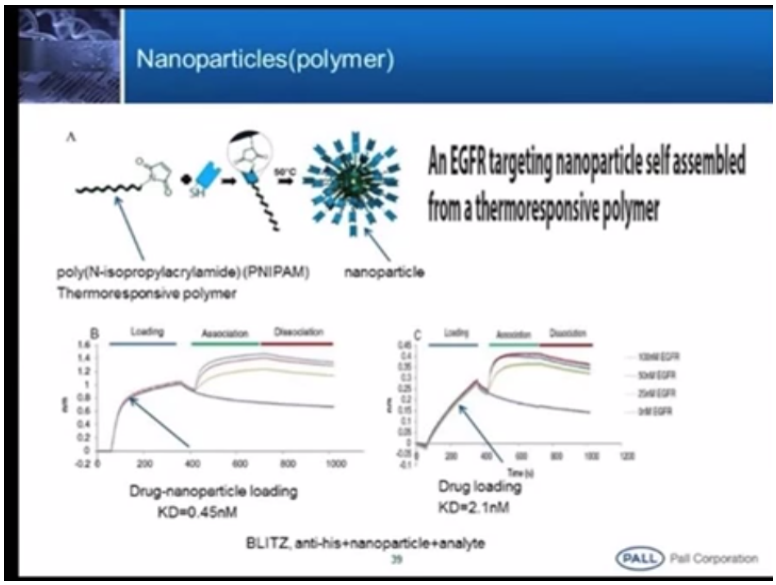

So nano particles, recently this is EGFR targeting nanoparticle self-assembled for the thermoresponsive polymer. This is one of the drug they had make a nanoparticle, then they targeted for the EGFR.

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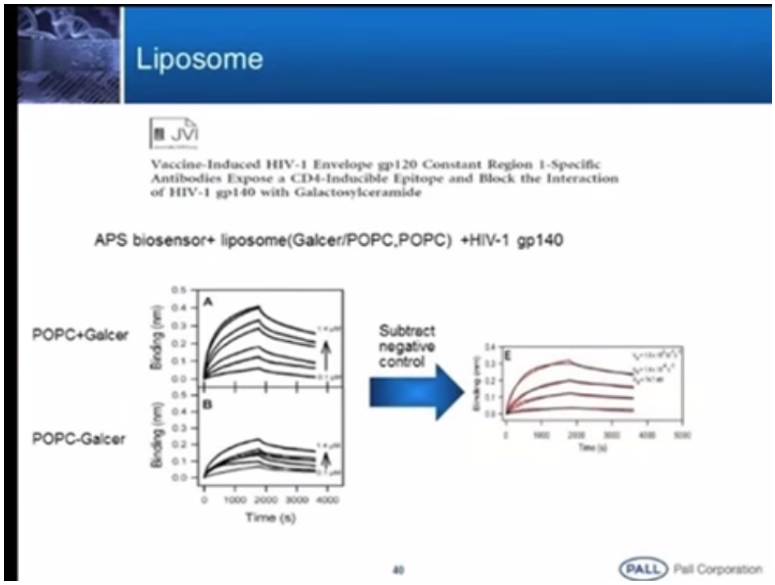

And liposome we have, we can use multiple applications, these are just I am going.

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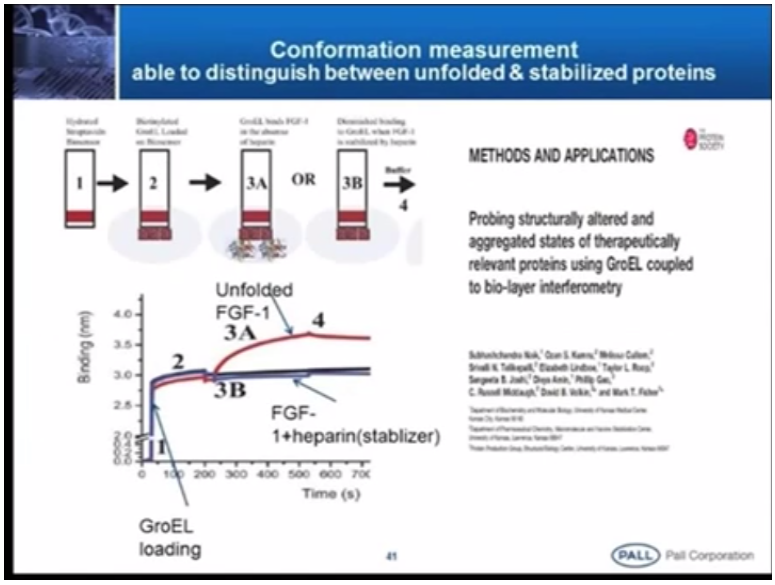

Conformational measurement here, folding, unfoldings and all. This si what right now, this kind of applications in IIT, Delhi Dr. Professor Tapan Chaudhuri is working on this area.

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So in India, we have everywhere industry as well as the academia the installations. Here we have Indian Institute of Technology Delhi, this instrument is there and Indian Institute of Science, IISC we have instruments. We have IISER Mohali, the small Blitz instrument. Madras University, we have a very small single channel instrument. NCBS, we have a small single channel.

CCMB, we have this same instrument in Hyderabad. Rest are other in industries people are using for them.

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Next lecture....
Week-2
Lecture 6: Sample preparation and
pre-analytical factors