HANDOUT

LECTURE-37

SURFACE PLASMON RESONANCE: DISCUSSION ON Biacore SPR AND DATA ANALYSIS

Need for label-free analysis

Biological systems depend upon molecular interactions of two or more biomolecules that they can form the stable complexes. The principles of thermodynamics, biomolecular structure and recognition play very crucial role in determining biomolecular interactions.

The ability to screen large number of proteins rapidly and simultaneously for biochemical activity, study of protein-protein, protein-lipid, protein-nucleic acid and small molecules interactions require various high throughput instrumentations.

currently, most popular methods which are being used for detection of protein-protein interactions include yeast two hybrid assay and protein microarrays. As we have discussed in the previous lectures, the protein microarray is one of the very robust techniques for studying protein-protein interactions but these microarrays require label-based detection systems, often these are fluorescence-based detections. However label-based detection techniques have certain limitations: Fluorescent tags may interfere with the function including binding to the interactors and adding them to queries is always not straight forward.

There is need for label-free biosensors which can avoid these issues and allow for the real time measurement.

This lecture will be a discussion on discussion on SPR, with a summary of a discussion with an Application specialist on BIAcore technology as well as how to perform data analysis using commercial software programs.

Surface Plasmon Resonance (SPR)

- This is one of the very promising label-free techniques for studying biomolecular interactions.
- The SPR biosensors are optical sensors which can exploit the surface plasmon positrons, the surface electromagnetic waves that can propagate parallel to a metal or dielectric surface.
- SPR is used to probe interactions between an analyte in solution and a receptor that is attached to the SPR sensor surface.
- Binding of molecules in the solution to the surface immobilized receptor changes the refractive index of the medium near the surface; the change in the refractive index of the medium can be monitored in real time to measure accurately the amount of bound analyte, its affinity for the receptor and the association and dissociation kinetics of the reaction.
- Over the past decade the SPR biosensor technology has made significant advancement and a large number of SPR sensor platforms, biomolecular recognition elements and measurements formats have been developed.
- The major strengths of SPR biosensors are their versatility and ease of use the SPR allows the analytes of receptor-ligand interaction for wide range of molecular weights, affinities and demonstrates the compatibility for small molecules and other chemicals.
- The SPR biosensors have played a very important role in biological research into biomolecules and their interactions and now they are increasingly being used for detection and identification of chemical and biological interactions.

Biacore SPR

- There has been growing interest in commercialization of SPR biosensors which has lead to number of systems available in market.
- The commercial instrument from Pharmacia and BIAcore became available in 1990.
- BIAcore are optical biosensors which can be used to monitor macromolecular interactions in real time without the need to label biomolecules. This is one of very versatile platforms to determine the kinetic rate constants for a variety of interactions.
- A number of commercial SPR biosensor instruments are currently available but since then biochemical analysis for biomolecular interactions have been still dominated by BIAcore system.
- The BIAcore is a versatile platform to determine the kinetic rate constants for a variety of interactions.
- A number of commercial SPR biosensor instruments are available since then however the BIAcore system still dominates this field.

The following is a summary of the discussion of BIAcore technology with Lalit Kishor, from GE healthcare life sciences who handles the BIAcore system

- In 2006,GE acquired BIAcore, which expanded its usage in India Firstly, the applications of Biacore include SPR is used for a wide range of applications, starting from, a simple binding analysis or kinetic analysis or
  - analysis of affinity of interactions whether it is protein-protein interactions, protein-DNA interactions, protein-RNA interactions, protein-small molecules interaction and
  - drug discovery,

Animation 1:

SPR

- Let me start with a video that shows basically what SPR is and then followed by another video of how biological analysis happens on BIAcore and then just few short videos how BIAcore works and then I will quickly come to the applications of BIAcore. Let me show you the first video-
    - Shown here is the basic SPR phenomenon.
    - This is the SPR chip on the top of the SPR chip is a gold layer and above that a flow cell.
    - At the bottom you see a hemispherical prism.
    - SPR phenomenon is simple, when a light is shined through the prism, the light reflects at an angle of total internal reflection and evanescent energy waves are created on the top of the chip and these evanescent energy waves are alsocalled surface plasmons.
    - These surface plasmons are the ones which are used to study biological interactions.

Animation 2

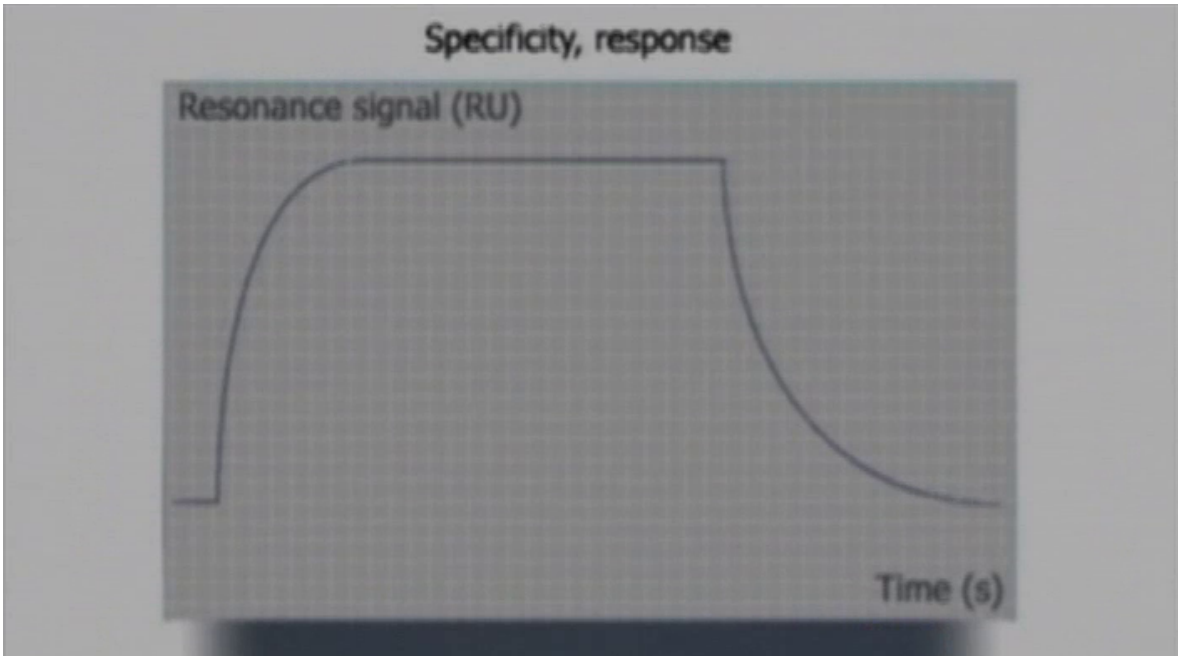
This shows how biological interactions are studied.

- Lets us assume that you have an interaction A+B = AB, What you do in SPR is you take one of the interactants say B, put it on the chip and pass A over it. Let me show you how it happens.
- You take intractants which is B in this case and you actually immobilize it on the chip as you can see molecules getting immobilized right now and when molecules get immobilized there is an increase in mass which changes the refractive index and that is measured in real time. Now, you pass second intractant A over it if binding happens and AB gets formed, you see a further increase in mass which is again measured in real time.

- You stop the flow of A and start flowing a buffer it comes off in dissociation and the dissociation is alsoseen in real time.
- Essentially, what you are doing with BIAcore is actually just measuring the amount of mass on the surface of the chip.
- The mass on the chip increases or decreases and that increase or decrease is measured in real time; this phenomenon is what we apply to study biological interaction. Actually BIA in BIAcore stands for Biological Interaction Analysis

Animation

These explains how BIAcore can be used in certain experimental settings.



- This is an example where A+B gives AB. If you see the curve, it means AB is formed.
- If you pass A over B and there is no response which means a flat line then AB is not formed
- It is thus a very simple indication to determine whether the 2 molecules of interest interact or not

Examples of Kinetic analysis

Example 1

- This will be that of an extremely rapid association, you see the slope of the curve goes up very fast and come down very fast this is a rapid association and dissociation.

- Whereas if you see the slope of this curve that is about to come up, it is very slow association and very slow dissociation. just by looking at curves you are able to actually tell if the interaction is fast or slow. Unlike the microarrays, where we can detect interactions but we cannot tell the nature of the interaction, here the edge is that by looking at this type of kinetics and curves one can tell type of association and dissociation and the overall kinetic
- analysis.
-

Questions about an interaction (wherein A+B =AB) addressable using BIAcore

- Does the interaction happen or not which means is the molecule AB formed or not?
- How fast is association?
- How slow is dissociation?
- How strong is the interaction?
- What is the affinity of the interaction?
- What is the concentration of the analyte?

Relevant to drug discovery,you ask if this interaction is safe or not? We start with each of these applications in detail.

Specificity of the interaction.

- Some interesting questions that are asked when you are doing specificity applications. Is the drug binding to the receptor or not?
- Is the mAb identifying the strain or not?
- Is there any non-specific binding in the interaction that I am studying?

These questions are very easily answered by BIAcore.

Shown here in a slide is actually an example where we are looking at binding and here someone has 40 compounds and they want to see compounds binds to a receptor.

After performing 40 of these experiments, you can see that most of the compounds are not binding or binding at very base level but if you look at presentation, one spot has been highlighted here with one molecule that is shown circled in red this particular molecule is actually binding to the receptor.

At the end of very short experiment of looking at receptor versus candidate binding you are able to determine which of these candidates are able to bind to given receptor, a very simple example of specificity.

Examination of kinetics

- Now let's assume that you found this candidate and you think that it is a specific binder and now you want to look at the kinetics of the interaction and everyone understands that kinetics is very important part in drug discovery.
- In proteomics when you look at the interactions, one of the important things that you need to look at what is the on rate and off rate of the interaction and in that sense how fast is that interaction happens and which candidate is kinetically preferred because if you have two candidates both of them trying to be drugs you should choose the candidate that is kinetically preferred.
- In most recent cases kinetics is being used to show similarity of drugs.

- For example there is lot of biosimilars coming out of India and the biosimilar manufacturer want to show that their molecule is similar or equivalent to an innovative drug.
- Then one way of showing is that they are similar in kinetics.
- That is where kinetics experiments will be helpful to you. Scientists can actually calculate Ka, Kd and KD which is the affinity of the interaction. They can calculate this in a very fast way; actually understand the interaction a little better than they did before. Because in the first instance they only knew that interaction was happening or not whether right now they also know kinetic parameters of interaction and once they know kinetics of interaction they come to affinity.
- Anyone who is pursuing biopharmaceuticals, recombinant proteins, biosimilars or even novel drug discovery people in the small molecule arena want to actually characterize the interactions in terms of the on rate and the off rate and the affinity of the interaction and that is something very well accomplished by the SPR technology.

Affinity of interactions

- When it comes to affinity as you can see here how strong is the bond?
- Is the bonding is strong enough to be physiologically important and this is one very important thing because more and more drug are coming out these days which are one dose a day drug and fast acting drug.
- These kind of discoveries depend a lot on kinetics and affinity of the interaction and that's where BIAcore comes in a very way and help people you know genetically or protein engineer their drug that it perform better than existing drug that is to make drugs better or to discover novel drugs that actually act better.

Concentration of analyte

- Once you know the affinity and concentration and I always think concentration is very less studied but it's very important because if you look at the concentration

analysis across the world for proteins there is no way that someone can measure active protein concentration without having calibrance in their hands.

- Whenever you give a student a protein and say please measure the protein concentration the first thing he will ask you is for calibrants, he will ask you for standards and the problem with the standards is that sometimes standards are not available, sometimes they are very expensive and being proteins they are not very stable.
- There is a great need of having protein analysis technique that does not need calibrants that is where BIAcore comes in again.
- BIAcore does something that is called CFCA (calibrants free concentration analysis) within five minutes if you have a specific binder for a protein you can actually calculate the concentration of protein without the need for calibrants and since you have seen in screen one where we talked about specificity.
- We are talking about specific binding what is measured is not just total protein concentration what is measured is specific active protein concentration.
- Estimation of concentration is especially important in quality control and in filling in biopharmaceuticals again where people need to exactly estimate how much actually they are filling in vial that actually goes to the patient, they need more accurate methods of measuring active protein concentration and that is where BIAcore will play a very measure role in letting people estimate active protein concentration.

Determination of immunogenicity

- Worldwide with the increased biopharmaceuticals, drugs everywhere need to be tested for immunogenicity.
- Immunogenicity is about direct measurement of antidrug antibody which should be measured in serum it's also about bringing a regulatory framework into a system.
- Right now, we talked about technology and science but suddenly when it comes to drugs, regulators come in can we actually accurately, confidently measure

antidrug antibodies in animal sera or human sera at clinical trial levels and BIAcore can actually be used for accurate measurement of antidrug and antibodies and for immunogenicity testing of biopharmaceuticals that is another major application of BIAcore technology.

Identification of binding targets from a complex mixture by SPR-MS

- If you have a heterogeneous mixture that is flowing over a ligand and something within that mixture is actually bound, you see a curve indicating that something has bound but you don't what has actually bound.
- You can use a technique called SPR-MS where you can take the bound analyte separate it into a vial and then take it to a mass spec. and identify the protein now you can actually find out what is it that is bound.
- If you have this unknown target, for example, if you have a receptor and cell lysate or if you have some kind of homogeneous tissue lysate which you are flowing over the ligand and now you can actually find out what is binding.
- It is used in a application like ligand fishing where you are fishing for a ligand these the kind of applications.
- One issue is a limiting amount of protein- the amount bound & collected may not be sufficient.
- The only way you can overcome is this by doing multiple times, collecting enough that you may get a MS response and that is what our most users do is that they run the same binding assay about 10-20 times and collect analyte and then take it to an MS to get their result.

Use in the pharmaceutical industry

It should be noted that during discovery, BIAcore is most useful as a cheap and easy method for eliminating a number of candidates during large-scale screenings. In other words, BIAcore or SPR technology is not for success but more to identify failure. Only thing is that we say that it is for early failure and it is for cheap failure. do not spend too

much money on that does not work, might as well fail early that is the basic reason why SPR should be used.

BIAcore instruments

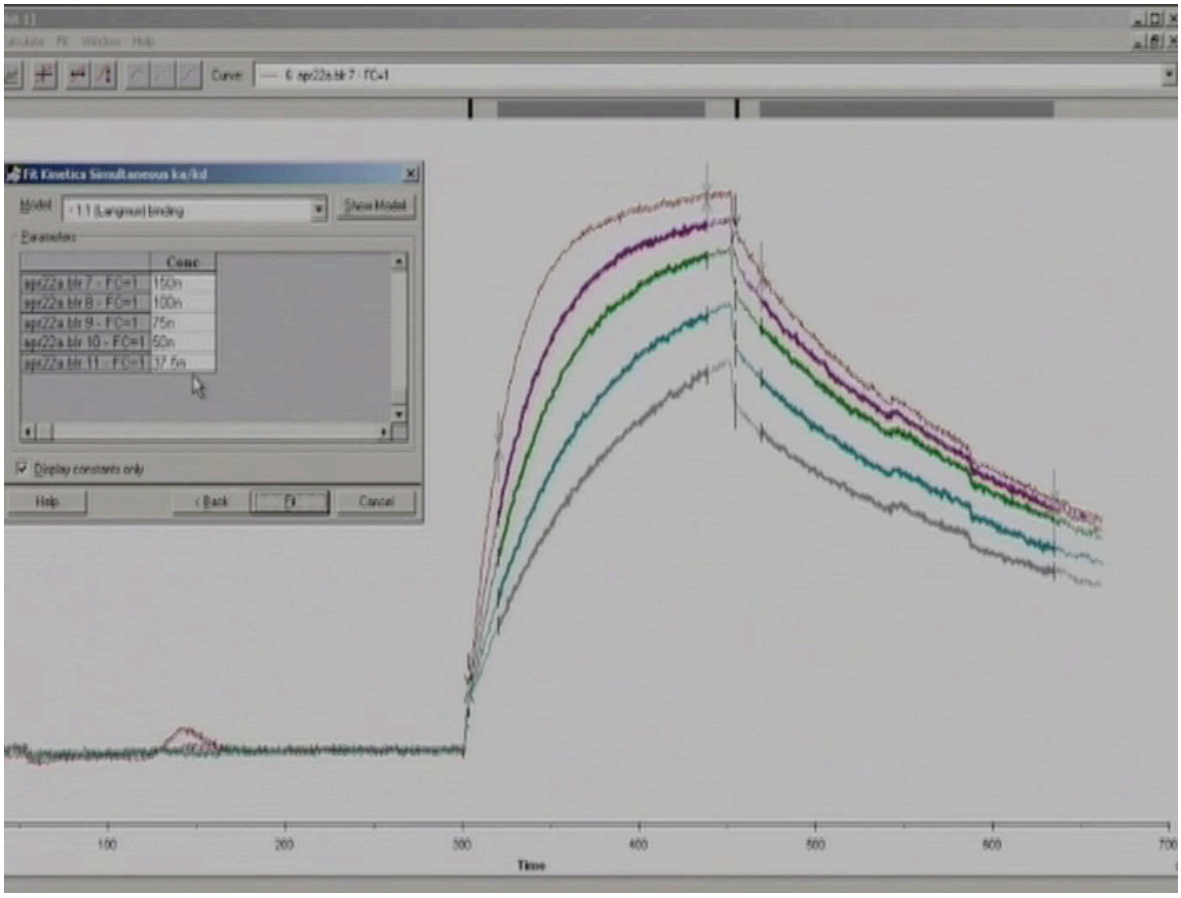
There are basically four different BIAcore instruments available.

- **BIAcore X100:** This is a very small instrument. It has two flow cells and is useful for preliminary analysis.
- **BIAcore 3000**: This is an academic favourite, lot of academicians like it, lot of customers in India who are in academics have the BIAcore 3000.
- **BIAcore T200:** This is new and very special again because it has all the things which BIAcore 3000 has but it a regulatory up road. If you are a company that works with FDA, DGCA or other some of these regulatory authorities then I think you should be using BIAcore T200.
- **BIAcore 4000:** This has 20 different immobilization sites and it can be used if you are a company that has to do extremely high throughput screening, if you are company that does a lot immunogenicity experiments or if you doing batch testing then you should be using BIAcore 4000. I must say that most of the customers in India use either the BIAcore 3000 or use the BIAcore T200.

Example of the results of a typical BIAcore experiment (as narrated by the application specialist)

- This is the BIAcore result of an experiment where 5 samples of different concentrations were run over a fixed ligand on the chip, for which a kinetic analysis has to be performed.
- The idea is that the results are all embedded here; these two peaks you see here are regenerations.
- The first thing during analysis is to select the regenerations not needed and cut them out.

- Now, 5 different concentrations in 5 different colours are seen, with an association phase & the dissociation phase.
- Now I am going to baseline this result here I select baseline here and then I go to adjustment of Y-axis and I say zero at average of selection and then I say add as new.
- Now my all my 5 results are shown here now I am going to do a quick kinetic analysis and it's really extremely simple to do kinetic analysis because all we do is say calculate, I say kinetics simultaneously Kon and Koff.
- I have already done cutting and Y-transformation
- I say next, and then if I want to I can go and adjust the start and end time I can move this to adjust the start time and end time of association and dissociation which I sometimes do but I think this is pretty well picked up by software already.
- I don't need to do much, I say next and I enter the different concentrations of the sample which were run.
- Now one of the things that is important here is that you choose the model preferably it is always better whenever you do characterization.
- The more you know about your system the better characterization results you get in this case suppose you do know that there is one to one binding then you would choose that binding if you want you can to you can actually change the biding model it could be a bivalent analyte, it could be a bivalent ligand, it could be a heterogeneous ligand depending on the model. you choose model that you want to and then you say 'fit'.

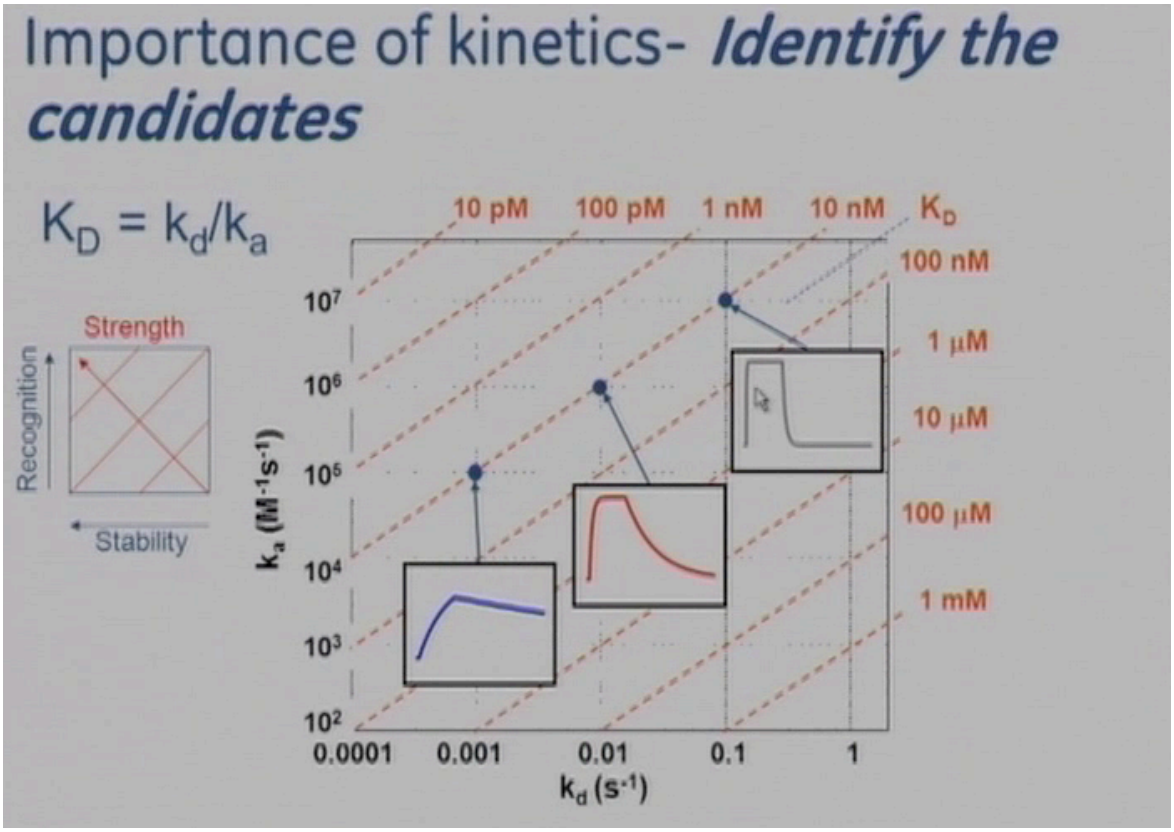


- What happens then is that curves get fit and the results are thrown up, your Kon and Koff are displayed here it is as simple as that.
- All you need to do take a ligand, immobilize it on a chip, run five different concentrations of your analyte over the chip and each of these results if you look at X-axis carefully it is 0-600 that means each one of these runs the entire run was 10 minutes you ran 5 samples 50 minutes with the time taken in between the runs is about 10-20 minute about 1 hour 10 minutes to 1 hour 20 minutes you have your results and you have already characterized your results as you have Kon and Koff calculated and you can also check how good your results are by quickly checking the residuals and you can see residuals here the chi square values are really between –φ to + φ, extremely good fit in reactions and a very a very fast analysis that gives you Kon and Koff.

- It is very easy to use software for analysis.

Example of kinetic analysis using BIAcore

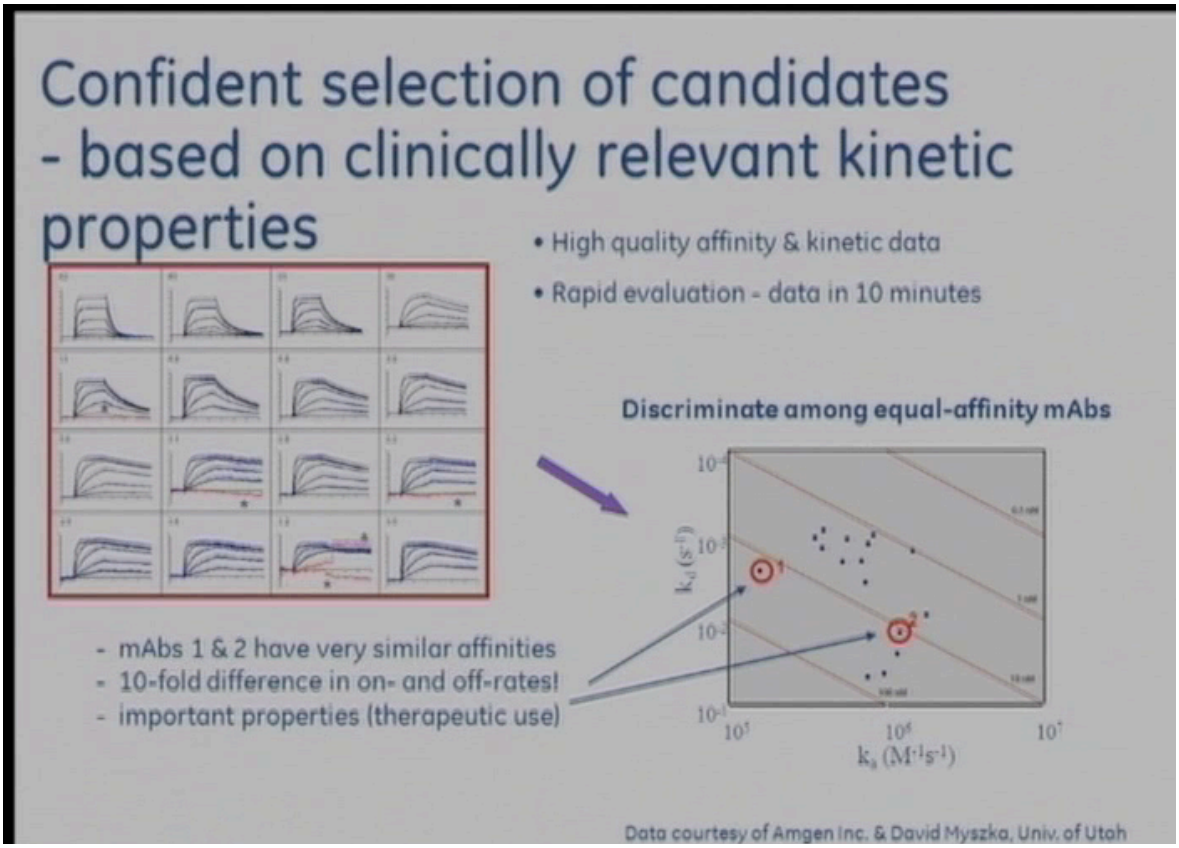
- Analysis of Kinetics is an important application of BIAcore technology.
- Here in this example, there are 3 slide showing kinetic analysis using BIAcore
- look at this slide-



- Now here on this slide, you have three interactions which are captured you have interaction 1 which is captured in blue, interaction 2 which is captured in red and interaction 3 which is captured in black.
- The important thing about all these three interaction is that they have the same affinity. They have varying K_{on} and K_{off} but they have the same affinity and this is

important in drug discovery because let's say for example you are looking for pain relief if you want pain relief you want a drug that acts fast and that stays on for a very long time but if you want a sleeping pill you want a drug that acts slowly and stays on for only a reasonable amount of time and comes off fast enough.

- In other words, kinetics is very important in choosing a drug candidate. This is an example where if someone chose just on the basis of affinity all these three would have been same but since they would make their choice based on kinetics they can actually decide on the basis of K_{on} and K_{off}.



- Now here is real life example which I show you which is a publication from Amjon and from David Mishka of University of Utah where actually Amjon uses this data directly to do their clone selection.

- They are actually doing selection of Mabs and selection of clone of Mabs and if you actually look at this clone 1 and clone 2 have similar affinities but if you look at their on rate and off rate there is 10 fold difference.
- If you look at these two clones , which clone they should go for? They should go for the clone which is having kinetically relevant properties. If they are using affinity to make their choice there is no choice at all they both the same this is a live example where kinetics data is being used to capture information regarding K_{on} and K_{off} and then make educated and knowledgeable decision on which mAb to go forward with.

Major limitations or shortcomings of various SPR technologies

- One of the significant shortcomings is that if there is any structural changes in the protein, this would not be able to capture it.
- It is a mass-based sensor, any structural changes will not be captured.
- There is another problem where you are unable to immobilize protein that you have on the chip, you might have used some capture technique to do that. There is also this big question about what is binding? Why is it binding can be answered by structural studies or thermodynamic studies. That is where BIAcore can give you little bit of direction but I think you should do NMR study or microcalorimetry and that is what will give you more knowledge regarding why this interaction is occurring.

Take home message

Here is basically three rules that we have in BIAcore:

- The first rule in BIAcore experiments is BIAcore technology is extremely easy to understand, it is very easy to analyse. A lot of time must be spent on experiment design. The first thing that I would do if were working with BIAcore, spend lot of time on very carefully considered experimental design.

- The second thing I would do if I were conducting BIAcore experiment is that I will make sure that I have extremely pure ligand that I will put on the chip and that is extremely important.
- The last thing that I will consider and that is true for every experiment: make sure your sample preps are correct and remember that this is an analytical instrument unlike many other techniques in biotech, this is an analytical instrument, it is a mass sensor and end of the day you can call it sophisticated weighing machine. If you put something on it will give you the weight. It is as simple as that only thing is that you have to do it right I would say make sure your sample prep is correct and design is perfect, make sure your ligand is pure.

Summary:

After having a discussion about BIAcore technology, I hope you are very clear about instrumentation, various properties which can be studied by using BIAcore system and how quickly one can perform the data analysis.

The conclusion form this whole discussion is that since introduction of the BIAcore SPR instrument, the SPR spectroscopy has become widely used from chemistry and biochemistry to characterize biological surfaces and monitor the biomolecular binding events. Overall the success of SPR technique is due to following factors-

First, the kinetic measurement in real time that is the major strength of data obtained from SPR. Second, monitoring the adsorption of unlabelled analyte molecules to the surface and third, its ability to monitor weakly bound interactions due to high surface sensitivity of SPR sensors.