LECTURE-37

SURFACE PLASMON RESONANCE: DISCUSSION ON Biacore SPR AND DATA ANALYSIS

TRANSCRIPT

Welcome to the proteomics course. Today, we will talk about SPR. We will have a discussion Biacore SPR and data analysis. The biological systems depend upon molecular interactions of two or more biomolecules so that they can form the stable complexes. For biomolecular interactions the principles of thermodynamics, biomolecular structure and recognition play very crucial role. The identification of interacting protein and protein partners of known function with those having the uncharacterized role by performing these types of experiments; it is possible to understand the biological process of uncharacterized protein. The ability to screen large number of proteins rapidly and simultaneously for biochemical activity, interactions of protein and protein, protein-lipid, protein-nucleic acid and small molecules require various high throughput instrumentations.

So 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 that protein microarray, they are one of the very robust techniques for studying protein-protein interactions but these microarrays require label-based detection system often these are fluorescence based detection. However label-based detection techniques have certain limitations such as tags; fluorescent tags may interfere with the function including binding to the interactors and adding them to queries is always not so straight forward. So there is need for label-free biosensors which can avoid and allow for the real time measurement.

So in today's lecture we will have a discussion on SPR and I have invited a guest to discuss BIAcore technology as well as how to perform data analysis using commercial software.

So now let's discuss about SPR this is one of the very emerging label-free technique 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, the 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. Now let's move on to BIAcore SPR technology.

So 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 need label biomolecules. This is one of very versatile platform 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. So let's now discuss the BIAcore technology with Lalit Kishor, the business leader research products in GE healthcare life sciences who handle the BIAcore system.

Here in discussion-

Prof. Sanjeeva Srivastava = A Lalit Kishor = B

A- So how long have been associated with BIAcore SPR technology, GE healthcare?

B- I have been with GE healthcare for last twelve years and I have associated with BIAcore for last four and half years, have been associated with proteomics for slightly longer about seven years.

A- Great. So can you just brief us about your experience of using the BIAcore technology from last several years?

B- My educational background is that I am not a biologist. Though BIAcore is largely biological tool. My background is in chemical engineering so I am a chemical engineer with an MS in management. However I find very varied uses for SPR technology these days. I find it's used in biopharmaceutical; I find it's used in basic research, in nanotechnology, in pharmaceutical industry, in QC labs. So basically I think it's my chemistry and chemical engineering background that helps me to work with BIAcore. I see quite lot applications of BIAcore technology across the country.

A- Alright. So you are utilizing your varied background to apply on the different biological problems.

B- Yes. That is what I think SPR technology also does that it uses very simple technology and applies it to different things in biology and gives out results.

A- So Lalit, can you please tell us how you got interested into working SPR technology?

B- Yes. GE healthcare has been associated with BIAcore as a company for very long time. In year 2006, we actually acquired BIAcore and up until that point BIA was only in some places in India. So I got into BIAcore in 2006, out of interest in chemical interactions and interaction analysis. And ever since I have been just working on BIAcore and label-free interaction analysis technologies.

A- So can you mention currently what are the major applications of SPR in the area of proteomics?

B- Actually, very wide ranging application of SPR-starting from a simple binding analysis or kinetic analysis or analysis of affinity of interaction whether it is protein-protein interactions, protein-DNA interactions, protein-RNA interactions, protein-small molecules interaction and drug discovery, quality control varied applications of SPR. Actually, we support a very large variety of customers from different backgrounds who want to do SPR in their labs.

A- So definitely you can see probably that SPR applications will be broad in almost all the proteomics laboratories depending upon their experiments and questions they want to ask. So BIA-core is one of pioneer in the field of studying the label-free interactions and studying about biomolecular interactions. Can you tell about some of the latest advancement? What are the major applications using BIAcore technology?

B- 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.

So what I show here is basic SPR phenomenon. This is the SPR chip, which you see here, on the top of SPR chip is a gold layer and the top of it flow cell and at the bottom you see a hemispherical prism. So SPR phenomenon is pretty simple that when you actually have a prism and you shine the light through the prism, the light reflects at a angle of total internal reflection and evanescent energy waves are created on the top of the chip and these evanescent energy waves are also called surface plasmons and these surface plasmons are the ones which are used to study biological interactions. When I go to video two you will see 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 one of the interactants 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 interactant 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 also seen in real time. So essentially what you are doing with BIAcore is actually just measuring the amount of mass on 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 BIAcore is BIA for Biological Interaction Analysis

So let me show in few videos examples of how BIAcore can be used to study some experiments-This is an example where A+B gives AB if you see the curve and if the curve exists, 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 so it is very simple example where you can decide whether interaction is happening or not happening.

I will show you another examples now of kinetics analysis where you will see two examples. The first example will be that of an extremely rapid association so you see the slope of the curve goes up very fast and come down very fast so 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. So just by looking at

curves you are able to actually tell if the interaction is fast or slow. So these are the some of the examples of BIAcore technology at work.

A- So I think 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.

B- Yes. And here I have a slide, a very simple analysis that can be done by BIAcore. So if someone asks me what can be done with BIAcore? These are the six things which can be done by BIAcore. So very shortly put these are the six applications of BIAcore. Whenever A+B forms AB, the first question you ask does the interaction happen or not which means is the molecule AB formed or not? The second question you ask how fast is association or how slow is dissociation? This question you is how strong is the interaction so what is the affinity of the interaction? Fourth question you ask is how much of the analyte is there which means what is the concentration of the analyte? Sometimes in drug industry you ask if this interaction is safe or not? And sometimes if you have a heterogeneous analyte and you see that binding is happening, you want to ask what is it that is binding because there are too many components in this analyte. So what is it that specifically binding? And these are the five different things that you can do with BIAcore technology.

A- I think you rightly mentioned identifying the very specific interactor is most challenging aspect of it because that's where people fail and they discover false interactors. I think that is where SPR has edge over conventional techniques like immune-precipitation or yeast two hybrid assays and some other large screening methods where there is a good chance of identifying false positives.

B- Sure. There is lot of false binders in screening experiments which will be avoided if you have specificity that BIAcore gives you. So we start with each of these applications in detail. The first application which we will talk about is the specificity application. Now some interesting questions that are asked when you are doing specificity applications. Is the drug binding to the receptor or not? Is the MAb indentifying 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 my 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 and here we have 40 of these experiments done most of the compounds are not binding or binding at very base level but if you look at presentation I have highlighted one spot here with one molecule that is shown circled in red this particular molecule is actually binding to the

receptor. So 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. Going on to next application-

A- Sorry to interrupt you here, so basically it just demonstrates even if we are unaware about the components which could be interacting this could be good screening tool. Because if there is a real strong and specific interaction then probably we can see it in output.

B- Absolutely, if you want to do that first level screening to just quickly find binders in some kind of screening experiment like you mentioned then this would be a very good starting point for you to quickly find those binders and then take them to the next level.

A- Many times when we have thrust of identifying some large drug library of small compounds at that time may be to begin with.

B- Absolutely, I would also point out that this is a very small experiment it barely takes about 1 minute to do this experiment. So basically per minute you can do one screen and that way you will have lot compounds screened in a rapid manner. So that is the first step in the specificity experiment. 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 so 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 biosimilar manufacturer want to show that their molecule is similar or equivalent to innovative drug. Then one way of showing is that they are similar in kinetics. So 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.

A- So identifying on the rate and off rate and dissociation constant I think this provide you a very strong tool and information for characterization and that is the big thrust for all the pharmaceutical firms.

B- 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. So 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 so that it perform better than existing drug that is to make drugs better or to discover novel drugs that actually act better.

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. So 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) so 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 so what is measured is not just total protein concentration what is measured is specific active protein concentration.

A- I think that is very strong application of it because many we like to know how accurately you can determine protein concentration.

B- Absolutely especially 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. Now once you have seen concentration,

the next thing is 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 so 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 so that is another major application of BIAcore technology. The last application of BIAcore which if you remember when I talked in first slide about specificity, now if you have a heterogeneous mixture that is flowing over a ligand and something within that mixture is actually bound, you see a curve u know that something has bound but you don't what has actually bound so 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 so now you can actually find out what is it that is bound?

A- Definitely, it is very important because many times you will not know what the unknown target is which is interacting or binding.

B- Yes. So if 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. So it is used in a application like ligand fishing where you are fishing for a ligand so these the kind of applications.

A- But in MS you will definitely need certain protein above some threshold so how do you work on that issue because binding will not be minimal.

B- So one thing is that this is very small interaction is happening so amount of protein you collect may not be sufficient. The only way you can overcome is this by doing multiple times, collecting enough so 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.

A- So in BIAcore you have a way to collect the flow in the flow cell and then you can collect it in multiples run and then concentrate and do further MS.

B- So just to summarize, again I will go to my first slide that I showed here, these are the six things BIAcore can be used for- specificity, kinetics, affinity, concentration, immunogenicity and SPR-MS analysis. So those are six broad applications of BIAcore SPR technology.

A- I think those are very important because many times good decision about these products beforehand about characterizing these proteins can save you lot of money and lot of you effort down the road because if you can do those experiments in the beginning.

B- Most often when we actually talk to pharma industry you know we tell them that BIAcore or SPR technology is not for success but more for failure. Only thing is that we say that it is for early failure and it is for cheap failure. So do not spend too much money on that does not work, might as well fail early so that is the basic reason why SPR should be used.

A- I think that is very important. So can you briefly tell about what are the major instrumentation available from the BIAcore technology currently to do SPR-based experiments.

B- There are basically four different BIAcore instruments available. There is a very small BIAcore which is called the X100 you can see it on my page. It has two flow cells and it can do some beginning analysis and then you have BIAcore 3000 which is an academic favourite, lot of academicians like it, lot of customers in India who are in academics have the BIAcore 3000. Then we have latest which is BIAcore T200 very special again because it has all the things which BIAcore 3000 has but it a regulatory up road so if you are a company that works with FDA, that works with DGCA or other some of these regulatory authority then I think you should be using BIAcore T200. BIAcore 4000 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.

A- So how is it to do these SPR experiment and specially the kinetics analysis by using some software using the BIAcore?

B- The most important thing in SPR experiment is actually the experiment design. It's very easy to analysis. Let me show you example of a typical result and how the analysis works out.

So if you can see my computer, I am going to open a typical BIAcore result. This BIAcore result where 5 samples of different concentrations were run over a fixed ligand which was on the chip and now I am trying to do kinetic analysis so if you look at these 5 results I select them and I show the result and these are the results, now this is what typical BIAcore results looks like and there is nothing to be worried about because its

looks odd. The idea is that your results are all embedded here; these two peaks you see here are regenerations. So the first thing I do when I do analysis is to select the regenerations which I don't need and cut them out so I cut them out here and then the rest of the results show up. So now I have 5 different concentrations in 5 different colours, this is the association phase and this is the dissociation phase. Now I am going to baseline this result here so 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 and I have already done cutting and Y-transformation so I say next and then if I want to I can go and adjust the start and end time so 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 so I don't need to much I say next and I enter the different concentrations of the sample which were run. Now this is very important. Now one of the things that is important here is that you choose the model so preferably it is always better whenever you do characterization the more you know about your system the better characterization results you get so 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 so you choose model that you want to and then you say fit.

What happens is curves get fit and the results are thrown up so your Kon and Koff are displayed here so 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 so you ran 5 samples so 50 minutes with the time taken in between the runs is about 10-20 minute so 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 quickly 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.

A- So it was very useful and informative to see the analysis like how easy it is to perform kinetic analysis. Can you give some specific example of kinetic analysis using BIAcore?

B- Let me show one example, this again goes back to why kinetics is important? Kinetics is extremely important by BIAcore technology. So here in this example there are 3 slide showing kinetic analysis using BIAcore so 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 Kon and Koff 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 so 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 Kon and Koff.

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. So 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. So if you look at these two clones for example which clone they should go for? They should go for the clone which is having kinetically relevant properties. If they using affinity to make their choice there is no choice at all they both the same so this is a live example where kinetics data is being used to capture information regarding Kon and Koff and then make educated and knowledgeable decision on which Mab to go forward with.

A- Right. This is very interesting to see because you will fail if you totally rely on the KD value and if you do not go for individual Kon and Koff values.

B- So distribute that KD value in Kon and Koff value and make a more knowledgeable decision.

A- So it was very interesting example to appreciate the power of kinetic analysis. Can you just brief us about what are the major limitations or shortcomings of various SPR technologies?

B- Absolutely. So like any other technology this technology should not be viewed as a silver bullet. It does have shortcomings. One of the significant shortcomings being that if there is any structural differentiation in the protein this would not be able to capture it.

It is a mass based sensor, so any structural changes will not be captured. There is another problem where you are unable to immobilize protein that you have on the chip so you might have to used some capture technique to do that. There is also this big question about why is binding? And why is it binding is answered by structural studies or thermodynamic studies so that is where BIAcore I think can give you little bit of direction but I think you should do NMR study or you should do microcalorimetry and thats what will give you more answers on why is this interaction happening.

A- So further in depth study will be required on those interactions.

B- Of course. When you have an interaction happening and when it is happening very fast, now why is it happening fast is transition state? You will have to ask questions in thermodynamics to get those answers. So sometimes further studies will be required, BIAcore kind of stops with these applications.

A- Would you have any final advice take home to students?

B- Of course. 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, I am sure that you would agree with me is that it garbage in and garbage out so 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 so 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 so I would say make sure your sample prep is correct and design is perfect, make sure your ligand is pure.

A- So I think you rightly mentioned that a good experimental design, identifying the good ligand, working out the chemistry for immobilization as well as doing the very good sample prep. All of these are very essential components for doing any proteomic experiment and especially SPR type of experiment. So thank you very much Lalit for discussing BIAcore technology with us today and I hope it is informative and useful for my students.

B- Thank you!

So after having a discussion with Lalit Kishor 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.

So 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.

So in summary in today's lecture you have learned about significance of learning about biomolecular interactions and how SPR technique can be used to study biomolecular interactions, a brief introduction of BIAcore technology and data analysis and then certain issues and challenges which are associated with SPR technology. So we will continue our discussion on label-free techniques in the subsequent lectures. Thank you.