Chemical and Biological Thermodynamics: Principles to Applications

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Lecture - 46
Further discussion on Isothermal Titration Calorimetry (ITC)

In the previous lecture we have discussed the principles of isothermal titration calorimetry, which has become a method of choice by many physical chemist, biological chemist, pharmaceutical chemist and many others who are interested in measuring the heats of very small vary. Today we will further discuss how to extract thermodynamic parameters from an isothermal, titration, calorimetric profile and how to interpret those thermodynamics parameters. Let us take a look at this slide.

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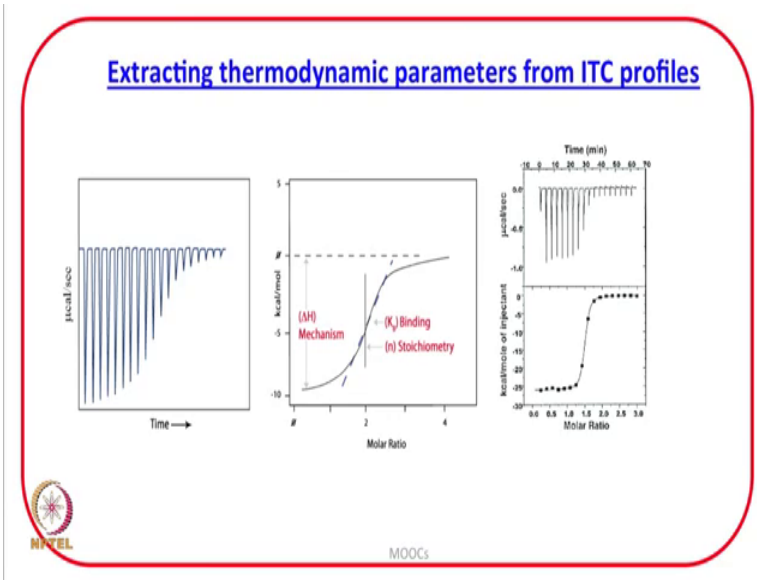

We discussed last time that the raw data from an isothermal titration calorimetric instrument will be in the form of micro calorie per second versus time or micro joules per second versus time. This micro calorie per second or micro joules per second is infect power. So, what this figure actually represents is the differential power, which is supplied or reduced to or from the sample cell to maintain the temperature difference between the sample cell and the reference cell 0. So, let us take a look back again. We also discuss that the area under each peak, will be a point over here which is the amount of heat absorbed or liberated depending upon the reaction. And as you can map both these

slowly and slowly the amount of heat evolved or absorbed goes towards 0 and that is what is seen over here.

And if we take this as a fitted data, now let me talk something about fitting of the experimental data by a suitable model. The experimental points in terms of the amount of heat liberated or amount of heat absorbed, versus the injection numbers or versus the molar ration does not recognize what is the mechanism of the reaction. What you are getting is the actual experimental data. Now it is our smartness to come up with a suitable model which can fit these experimental data points and extract the thermodynamic parameters. Now the commercially available softwares offer variety of models starting from single binding site model. What is a single binding site model? A protein may have binding site for a certain molecule. And it may have one binding site it may have 2 binding site or it is also possible that the 2 protein molecules may be shared by the same the 2 binding sites or individual 2 protein molecules may be shared by the same ligand.

So, therefore, different models are available which are single binding site model; that means, single binding site means one mole of the ligand binds one moles of the macromolecule. Or it may be 2 binding site model in which one protein molecule may bind 2 ligand molecules. Or there may be 3 binding site models or multiple site binding site model. Then there are other binding site models possible for example, sequential binding site model. What is a sequential binding site model? Sequential binding site means there are multiple binding sites available on a protein molecule and the ligands will bind in a sequential manner, means first the ligand will bind to a particular binding site then the ligand will go and bind to another particular site then the ligand will go and bind to another binding site in a sequential manner.

As I just mentioned commercial available softwares are available for variety of binding sites models, and if those are not available one will have to come up with a suitable binding site model which can fit the experimental data points in a nice manner. Now let us go back to the slide and look at this figure. The dark dots are experimental data points and the solid lines is a best fit to the experimental data points. Now as I set as I just said that different binding sites are available and let us assume that one binding site model has fitted nicely to the experimental data points. And if I assume this solid line to be the best fit to the experimental data points, this difference corresponds to delta H of course,

delta H depends upon the mechanism of the reaction and the slope reflects on the value of the binding constant or the magnitude of the binding constant and the stoichiometry n which is the number of binding sites on protein available for the incoming ligand molecule.

So, after we have fit a suitable model to a experimental. So, after we have fit a suitable model to the experimental data points, we have the different thermodynamic parameters. And what are those different thermodynamic parameters let us take a look at the next slide.

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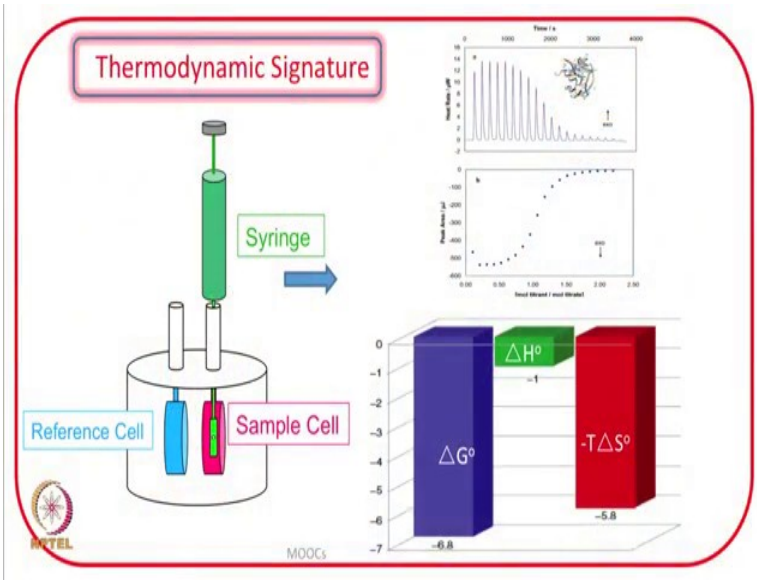

These derived thermodynamic parameters we will call as thermodynamic signature. That is delta G not, delta H not, and minus T delta S not, we will call these as thermodynamic signature accompanying the binding of a ligand with a micro molecule. And the beauty of the thermodynamic technique, which is isothermal titration calorimetry is that all these 3 thermodynamic quantities, that is delta G not delta H not and minus T delta S not or in other words delta S not can be derived from a single experiment. That is we have to do only one ITC experiment.

And what is that ITC experiment? Let us look at take a look at this figure we may choose to add 25 injections of a suitable volume the volume of injection can vary from 2 microliter to 10 microliter or more depending upon how you optimize the experiment. So that means, in one single experiment which may be constituting certain number of

injections. We will get all 3 thermodynamic quantities delta G not delta H not and delta S not. In addition to that we will also get the stoichiometry of binding.

The stoichiometry of binding n, I want to say something about this stoichiometry of binding n, it is determination, n is usually called as the stoichiometry of interaction which is how many ligands, how many moles of ligand bind to how many moles of protein. And as I mentioned in the previous slide it is possible to get the value of stoichiometry. The n value determined from an isothermal titration calorimetric profile will be equal to the stoichiometry of the interaction or stoichiometry of pointing, provided all the concentration in the cell of the isothermal titration calorimeter is in it is active form or concentration is same as it is activity, all the molecules which are present in the cell are in the active form. Otherwise if the concentration and activity are different then one may get a little different number which is relied n may not exactly matched the stoichiometry of interaction. So, this has to be kept in mind.

Now, what we will get from an isothermal titration calorimetric profile is delta G not which one can calculate from the value of k, delta H not and delta S not which will be in terms of some numbers with a magnitude, and also the sign. The sign is very important because it is the sign which helps us in identifying the nature of intermolecular interactions. How to identify those interactions?

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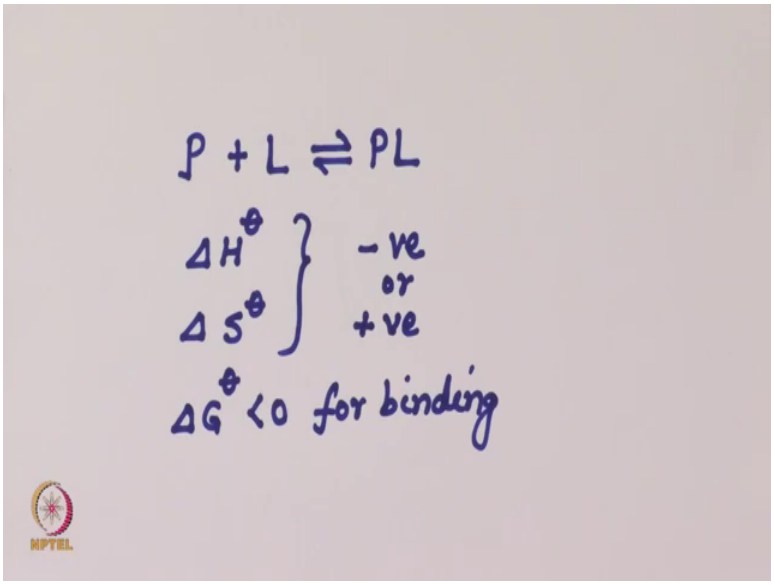

When protein for example, let us see over here when protein is interacting with a ligand. What we are getting is a complex of P with L. Along with this we have delta H not and we have delta S not. Delta G not has to be negative for binding to occur because if delta G not is positive binding will not take place. Now these delta H not and delta S not can be either negative or it can be positive, both possibilities are there. And these sign helps us in identifying interaction.

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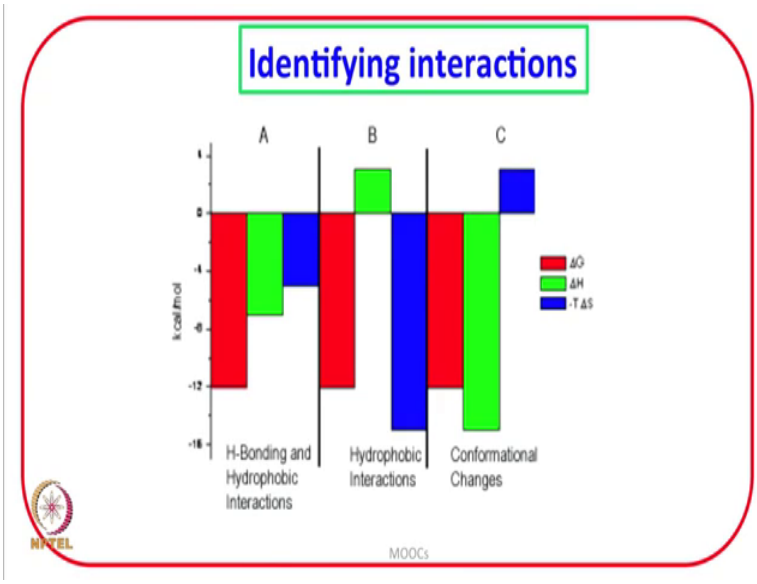

Let us take a look at this figure. The red color bar represents delta G not the green represents delta H not, and the blue represents delta S not, but in the form of minus T delta S. So; that means, this delta H and delta S in the form of minus T delta S are the 2 contribution to delta G. The downward is represented as negative value and the upwards bars will be representing positive values. Let us look at the first panel, when delta G not is negative it has to be negative for binding to occur. And if delta H not is also negative and delta S not is positive.

Remember that we are talking about minus T delta S not. Minus T delta S not is negative means delta S not is positive; that means, if delta H and delta S are both positive it is predominantly indicating occurrence of hydrogen bonding interactions. It is possible that side by side there may be hydrophobic interactions, but a negative value of delta H is suggestive of polar interactions which could be predominantly hydrogen bonding interactions.

And if there is a predominance of hydrophobic interactions, let us take a look at the middle panel. Predominance of hydrophobic interactions are associated with endothermic effects. Delta H is positive and whereas, delta S is also largely positive. Remember that hydrophobic effects, when we talk about hydrophobic moieties this can largely affect the structure of water because hydrophobic groups would tend to be away from water structure. Therefore, wherever there is an occurrence of more hydrophobic interactions the entropy changes will also be significant. Now let us take a look at the last panel, where delta G is negative delta H is negative, but delta S is also negative. Minus T delta S is positive means delta S is negative these trends are representative of conformational changes. Conformational changes can be in the ligand also and conformational changes are also possible in the protein. We will discuss about these conformational changes a bit later, and also we will also discuss how to connect this thermodynamic quantities with the conformational changes a bit later.

This is about how to extract the thermodynamic parameters from an isothermal titration calorimetric profile. And then what kind of information these thermodynamic quantities can give it to us. What we have started now appreciating is that, the thermodynamic parameters which we derived from ITC are not only just telling us about the extent of interaction. Extent of interaction is reflected in the value of binding constant binding constant is equilibrium constant, and if the binding constant is high means delta G not is largely negative. So, we are not only trying to connect with the extent of interaction, we have also started connecting the signs of these thermodynamic quantities with the nature of intermolecular interactions. So, slowly and slowly we have started unrevealing the power of isothermal titration calorimetry in giving the incites of the intermolecular interactions.

Before designing an ITC experiment, an optimization of the experimental conditions is important. By now large amount of data on variety of molecules is available, where isothermal titration calorimetry has been used. And based on that set of experimental data a general equation has been suggested, which should help us in designing an isothermal titration calorimetric experimental experiment suitably.

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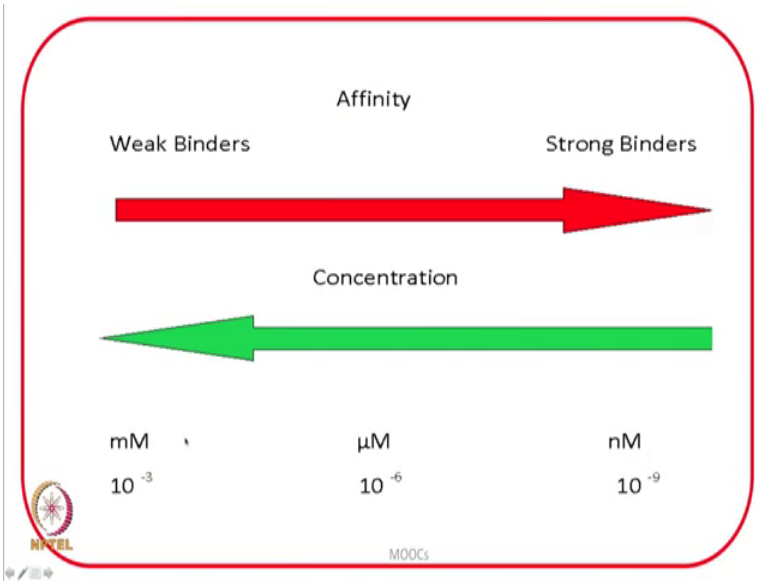

Let us take a look at that. Literature suggests that if the affinity increases, that is if we move from weak binders towards strong binders, the concentration requirement of the macromolecule decreases; that means, if the binding is weak we will require large amount of the macromolecule. If the binding is strong we will require small amount of the macromolecule that is weak binders weak binders, if I say the binding constant of the order of 10 rest to the power 2 10 rest to the power 3.

And strong binders means binding 10 rest the power 8 10 rest the power 9. So, if the binding is weak we will require millimolar quantities of the protein intermediate micromolar quantities of the protein and very strong binders we may require only nano molar quantities of the protein.

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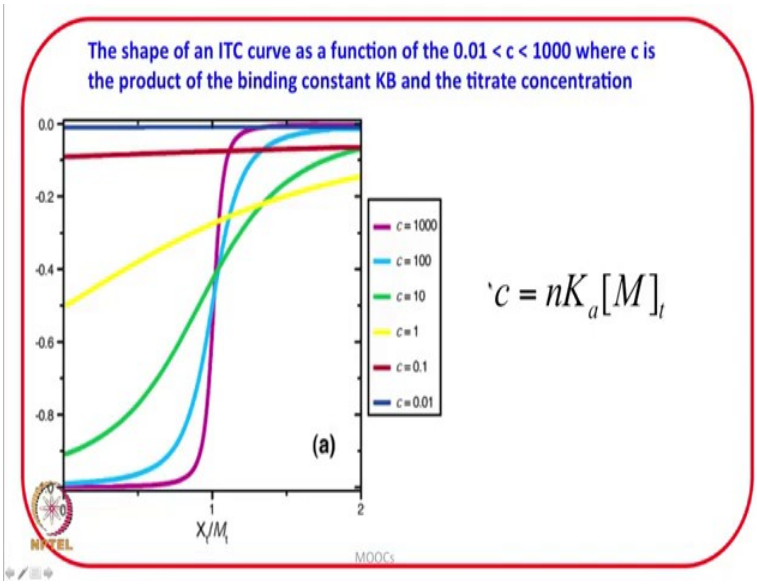

$$c = nK_a[M]_t$$

And the general expression which helps in designing the isothermal titration calorimetric experiment is given in this slide, which is a factor c, c is equal to the stoichiometry of the reaction n which is the stoichiometry of binding into binding constant, or I can also call it as association constant into the total macromolecule concentration. And macromolecule usually is protein in this case. So, c is n times k times the total protein concentration or total macromolecule concentration. And the figure shows how the output or isothermal titration calorimetric profile will look like for different values of c. And this figure represents a range of c value from 0.01 to 1000.

This one let us take a look at this line. This is corresponding to a value of c which is equal to 0.01. This is for 0.1 and for a c value of one yellow line is observed for a c value of 10 green curve, for a c value hundred the blueish curve. And for a c value of 1000, this violate type of curve is observed. So, what is the main observation here? The main observation is that when the c value increases the shape of the isothermal titration calorimetric profile also increases. When c value is 0.01 you see it is just like a straight line and no thermodynamic information can be derived from such an experimental data. Even when the c value is 0.1 it is also like a straight line does not give any information. For a c value of 1 some small curve which are has started appearing, but still this is not sufficient to give an accurate estimation of all the thermodynamic quantities that we would like to derive from an ITC profile.

When c is equal to 10, a reasonably good isothermal titration calorimetric profile can be obtained where you have a good amount of data in the pre region, pre saturation region and toward the saturation and intermediate where the binding sites are getting filled. For a c value of 100 which is blueish line this is also a very good isothermal titration calorimetric profile, but when c value becomes 1000 here we see a large amount of cooperativity kind of profile is seen, but a c value of 1000 has to be avoided for the reason that I will described a bit later, but a general conclusion that can be drawn from this kind of figure is that c value a proper c value has to be maintained if we want to get a good experimental output.

Let us take a look at the slide now. What is the best range recommended in terms of concentration.

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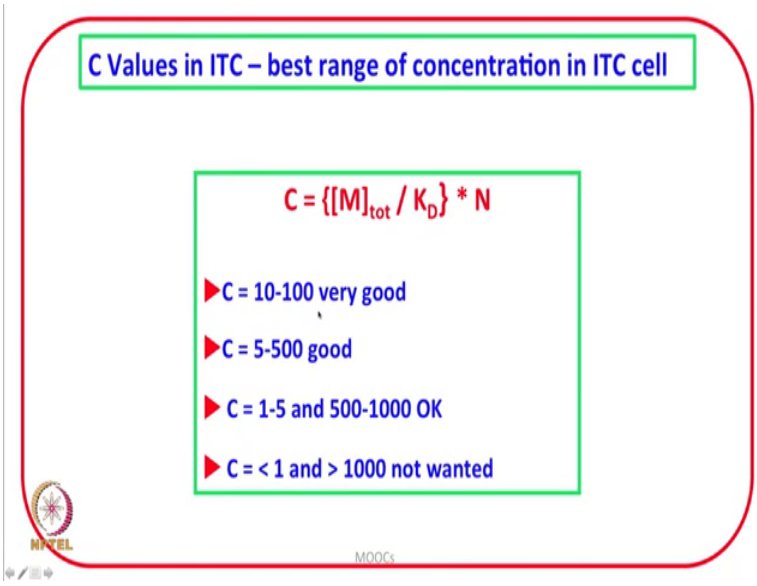

Now, here you see I have defined c as total protein concentration divided by the dissociation constant. And as I earlier mentioned physical chemist would like to talk in terms of association constant and biochemist would like to in terms of dissociation constant. So, therefore, would we should not get confused when we are talking about association constant or when we are talking about dissociation constant, a c value between 10 to 100 is usually considered very good and gives very good isothermal titration calorimetric output profile, a c value between 5 to 500 is good can fall into the

category of good, a c value between one to 5 and 500 to 1000 is type not good not very good, but and a c value less than 1 and greater than 1000 we do not want.

So, therefore, if the c value can be maintained between 10 to 100; that means, in that case under those experimental condition the isothermal titration calorimetric profile will turn out to be very good. And let us try to appreciate form this equation that increasing the value of c means, we will have to increase the concentration of the protein and also as I mentioned earlier that many times we will we may not be able to afford a large amount of protein.

Now these this range of c values for optimizing isothermal titration calorimetric profile is based upon large number of experiments; however, several reports are appearing or have appeared in literature, where different authors are said that it may be possible to obtain good quality thermodynamic data even if the value even if the value of c is low. So, therefore, we must remain in touch with the literature for any updates that is if there are new experiments being designed or if there is a new information on how to get good thermodynamic data when the c value is 1.

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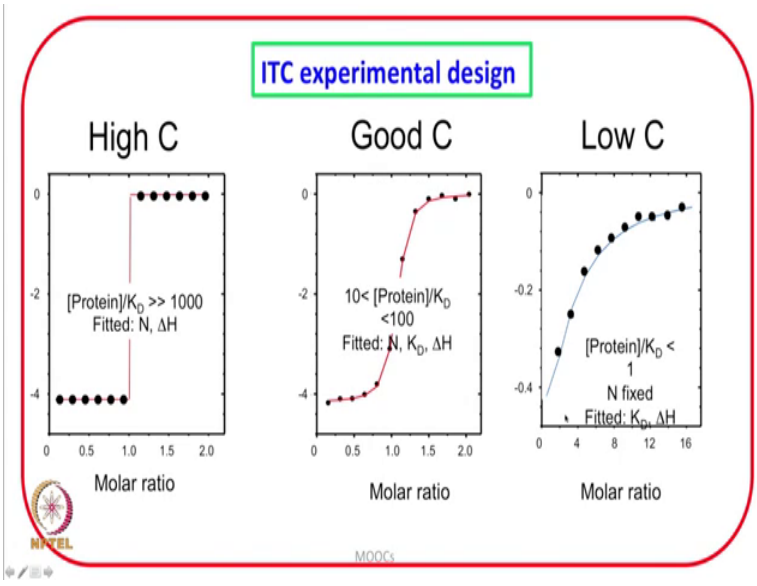

So, in the design of an ITC experiment, a high c value will give an output like this. If c value is more than 1000, we have experimental data points in beginning and we have experimental data points when all the binding sites are occupied and in between we have no experimental data point; that means, all the information which is occurring in this

range where the binding is taking place actually taking place or saturating the binding site that information is lost. Therefore, this kind of experiment is no good to extract the value of binding constant. This kind of experiment is only good to give accurate value of delta H, because delta H is difference between this and this that is accurately known in this kind of experiments, but the information on k is completely loss you cannot draw the value of binding constant.

C value between 10 and 100 you see we have good number of experimental data points and the model also fit is nicely. So, therefore, these experimental conditions are preferred this is a good c value. And if c value is low then we usually will not have the initial data points that is what happens is if you look at this the binding constant is so, large that as soon as you add small amount of ligand, it is completely binding and the next injection also whatever ligand is added that is also completely binding, which is not happening here when you are adding some amount of ligand it is partly binding and partly going into the solution because the value of k is not very large. So, for a weaker binding affinity usually this kind of isothermal titration calorimetric profile is observed.

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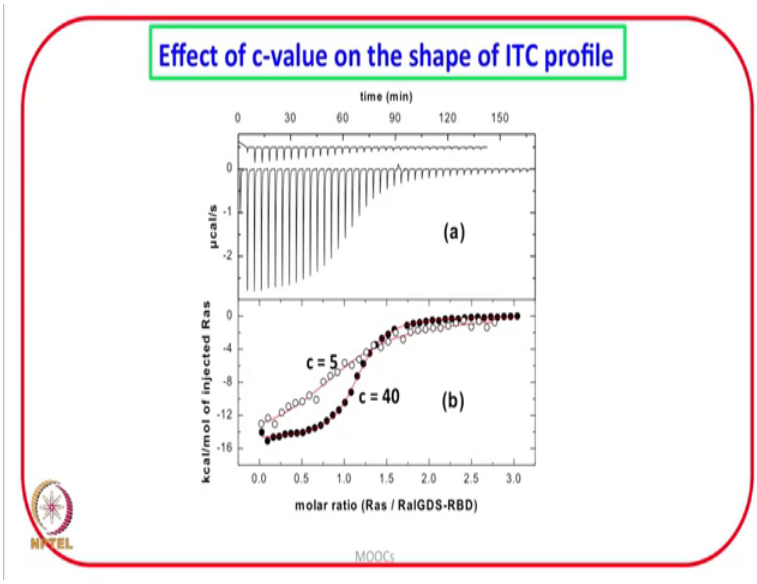

The effect of c value on shape of ITC profile can also be seen in this figure. The upper panel shows the main isothermal titration calorimetric profile and these are the dilution effects. And you see if the value of c is increased from 5 to 40 the shape of the isothermal titration calorimeter calorie calorimetric profile also increases. So, we can clearly see the

effect of increasing the value of c, but increasing the value of c comes at a cost and what is that cost that cost is the concentration of protein. The concentration of protein has to be increased.

So, therefore, now a days there are efforts by the different companies who manufacture the isothermal titration calorimeters to reduce the volume of the sample cell, but let us remember by reducing the volume of the sample cell means we are also compromising with the amount of heat that is either liberated or absorbed, but never the less if the sensitivity improves this factor can also be compensated.

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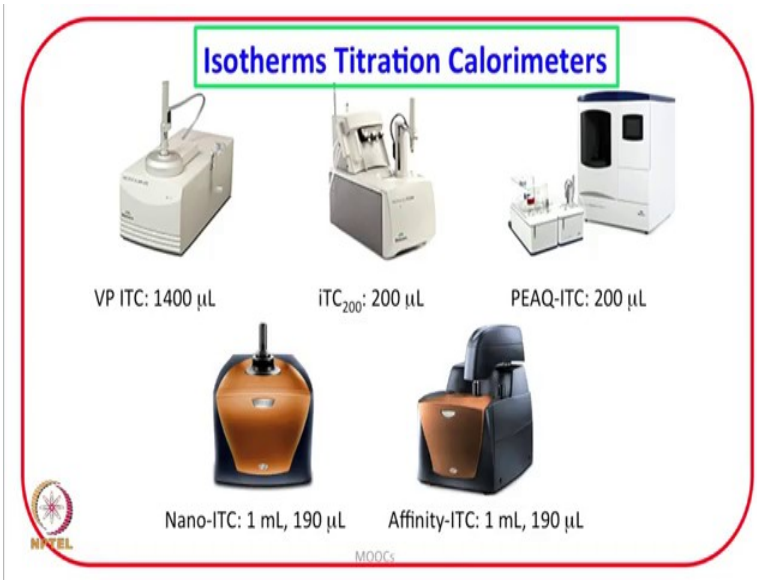

So, what are the different isothermal titration calorimeters available, v P ITC with a cell volume of 1.4 ml ITC 200 with a cell volume of 200 microliter peak ITC, which is an automated isothermal titration calorimeter with a volume of 200 microliter nano ITC with a volume of 1 ml or 190 microliter both are available an automated affinity ITC again with a volume of 1 ml or 190 microliter are available. So, what we discussed in this lecture is how to extract the different thermodynamic parameters from a single isothermal titration calorimetric profile. And that it is not just the number the signs are very important to connect this thermodynamic data with the nature of intermolecular interactions. We also discussed how to optimize the experimental conditions. So, that we get a very good isothermal titration calorimetric profile. We will discuss several applications of the isothermal titration calorimetric data in the lectures ahead.

Thank you very much.