Structural Biology
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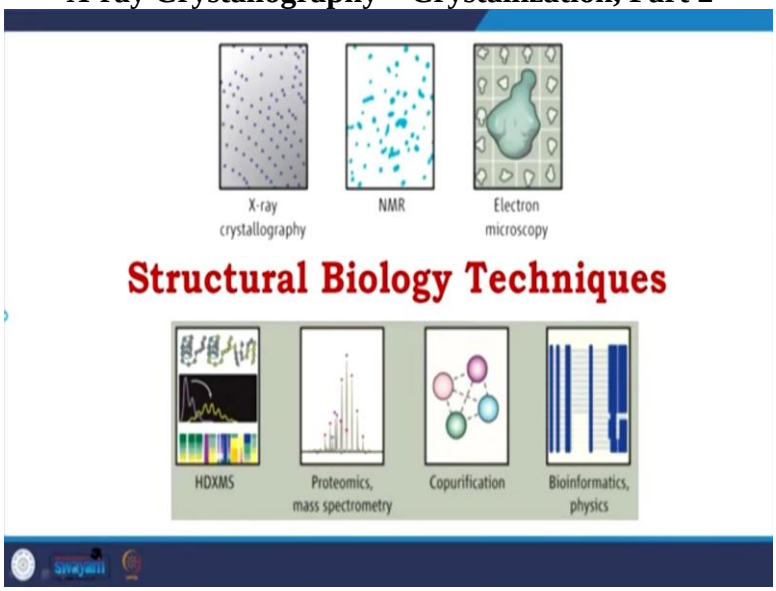
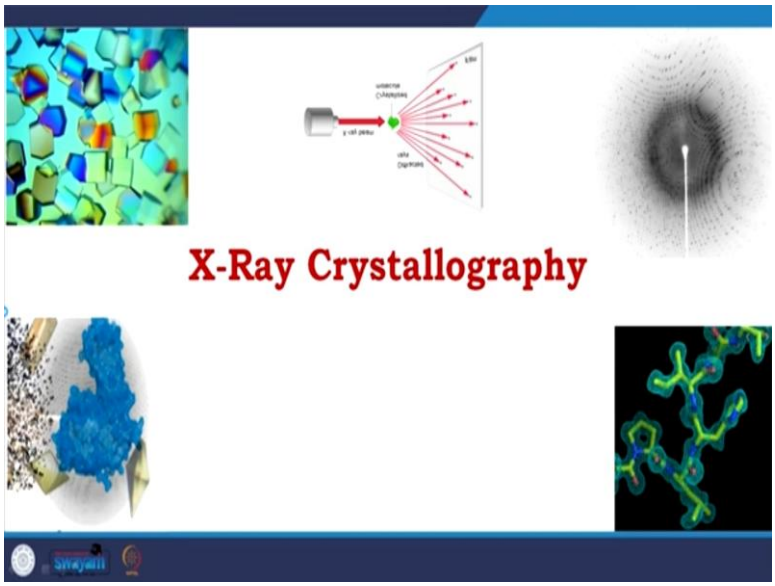
Lecture - 14
X-ray Crystallography – Crystallization, Part 2





We talked about different factors to affect crystallization and strategies to make crystallization more possible. As I told, if you do other things perfectly, only crystallization limits the success of the technique being 8 to 10%. As optimum success, it is always our focus to work on this area and increase the rate determining step.

We are discussing about since last class which is very key to crystallography, that is our crystallization specially, super saturation. So force the monomer out of solution and into the crystal that is called Supersaturate.

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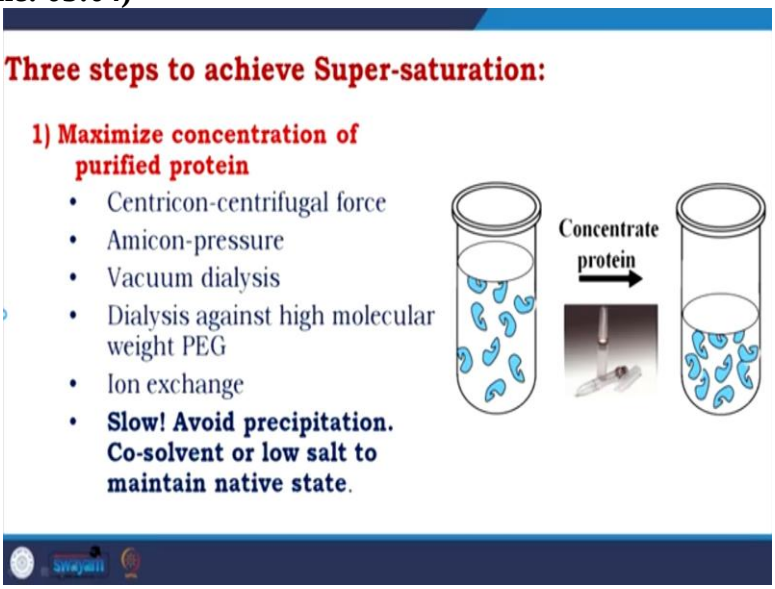

Three steps to achieve super saturation: 1) Maximize concentration of purified protein; Centricon centrifugal force is the common technique used in the laboratory, you have a certain molecular weight cutoff, small molecules will go out, water will go out, and the protein remaining would be higher in concentration. Amicon pressure (it is under centrifugal force). Vacuum dialysis is a easy process, where you have a membrane and the solvent and small molecules will be going out. Dialysis against high molecular weight PEG; PEG is polyethylene glycol ion exchange which is a principle of chromatography and slow avoids precipitation, co solvent or low salt to maintain native state. During concentrating, you are taking out the small molecules, but if you are losing more than enough small molecules like sodium chloride or any other salts, or buffer which are balancing the ionic concentration, you have a high chance that your protein will precipitate.

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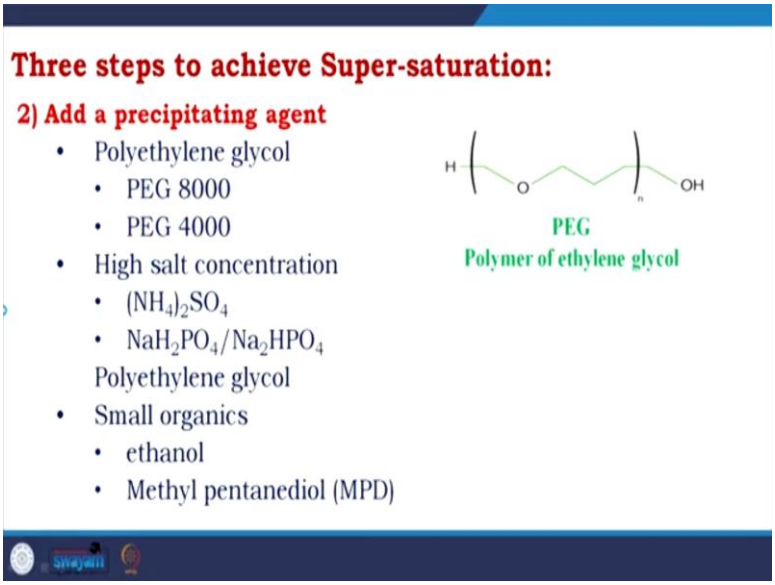

Second step, add a precipitating agent, polyethylene glycol (PEGS) are very useful in crystallization, high salt concentration; ammonium sulfate, sodium phosphate, and small organics like ethanol, MPD (methyl pentanediol), all these are precipitating agents.

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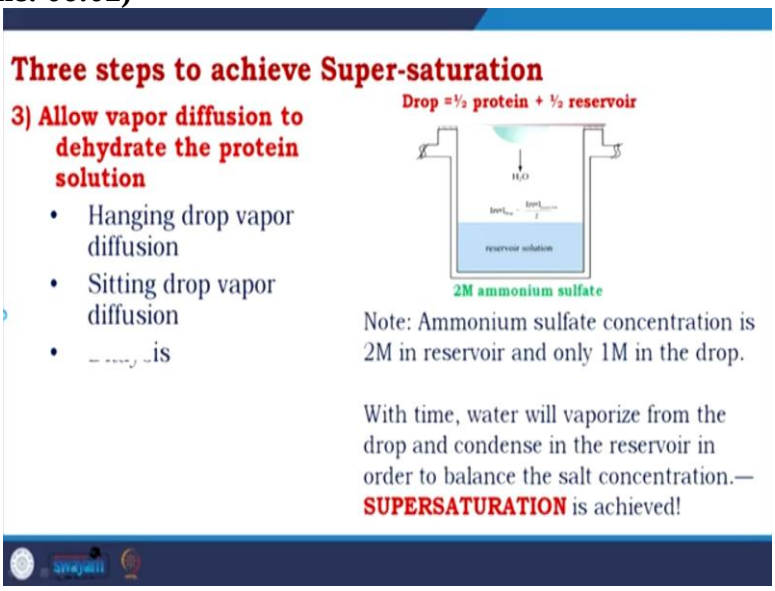

The third step is allows vapor diffusion to dehydrate the protein solution. If you remember when I was talking about the history of crystallization, they have realized that crystallization is a process, where you have a protein in solution and you want it to be crystallized, so in solid state, so there is a phase transition. If you remember, when I describe the process of crystallization, I have shown that you have taken the protein, and you have taken the reservoir solution, because you mix them up, the reservoir solution become half of the concentration. Now you set a drop, water will come from the drop to the reservoir, and in the process, the protein would be more concentrated. It is a process of dehydration, here they are

vapor diffusion to dehydrate the protein solution. So as an example, they have set up a condition, ammonium sulfate concentration is 2 molar in reservoir and only 1 molar in the drop, with time water will vaporize from the drop and condense in the reservoir in order to balance the salt concentration. There are processes like hanging drop vapor diffusion, sitting drop vapor diffusion, dialysis, liquid-liquid interface diffusion and micro batch.

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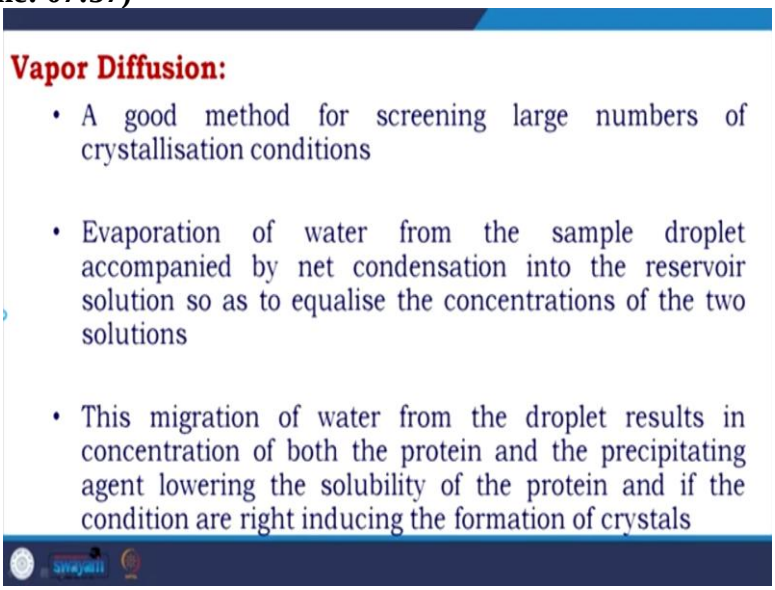

Vapor diffusion, it is a good method of screening large number of crystallizing conditions. Evaporation of water from the sample droplet accompanied by net condensation into the reservoir solution so as to equalize the concentration of the two solutions. This migration of water from the droplet, results in lowering the concentration of both the protein and the precipitating agent, and if the condition are right inducing the formation of crystals.

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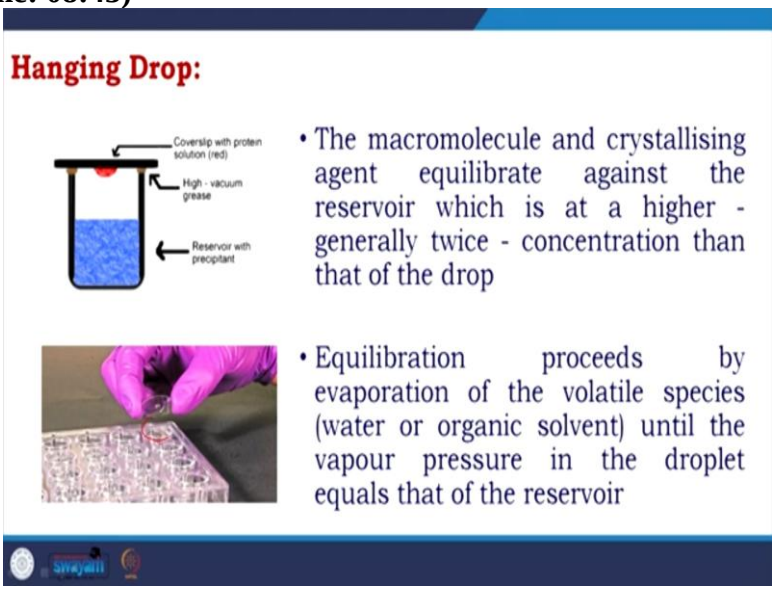

In vapor diffusion, there are different processes like hanging drop, the macro molecule and crystallizing agent equilibrate against the reservoir which is at a higher concentration than that of the drop. Equilibration proceeds by evaporation of the volatile species like water or organic solvent until the vapor pressure is droplet equals to the reservoir.

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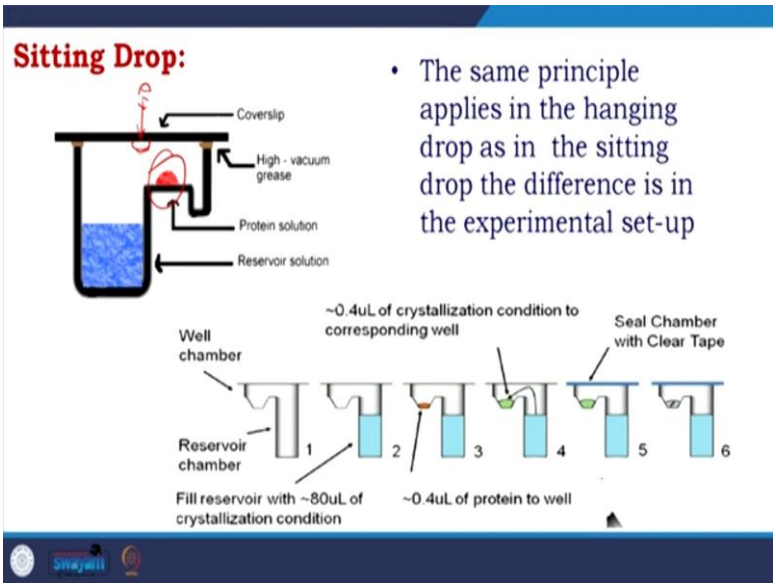

Sitting drop is mostly like hanging drop, but here the drop is not hanging, it is sitting in a place. That is why it is called as sitting drop. How to set it up? You have the reservoir chamber, then you fill up with the reservoir, and you take the protein and then you seal the chamber and you are thermodynamically closed, sitting drop system is ready.

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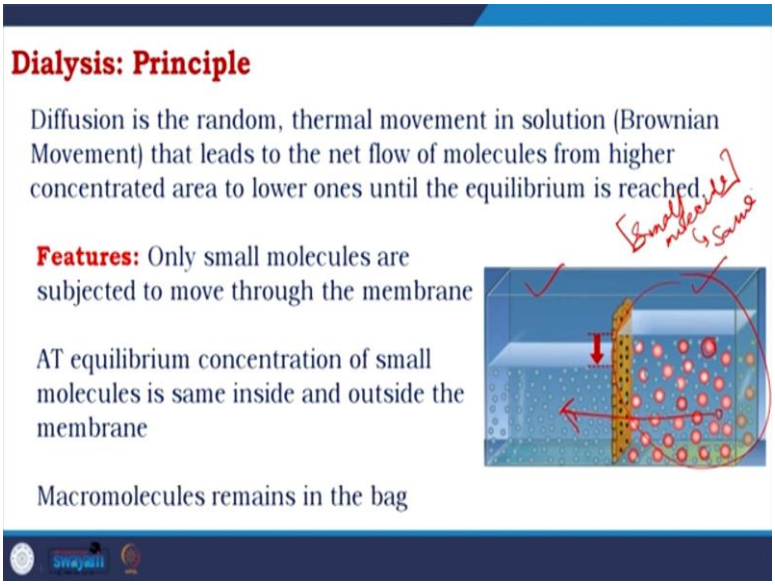

Coming to dialysis, diffusion is the random thermal moment in solution which is generally followed Brownian movement, which leads to the net flow of molecules from higher concentration area to lower ones until the equilibrium is reached. Features: only small molecules are subjected to move through the membrane. If you see here, there is macromolecules and small molecules, so the small molecules only migrate, macromolecules stays there. At equilibrium concentration, small molecule is same in both inside and outside the membrane.

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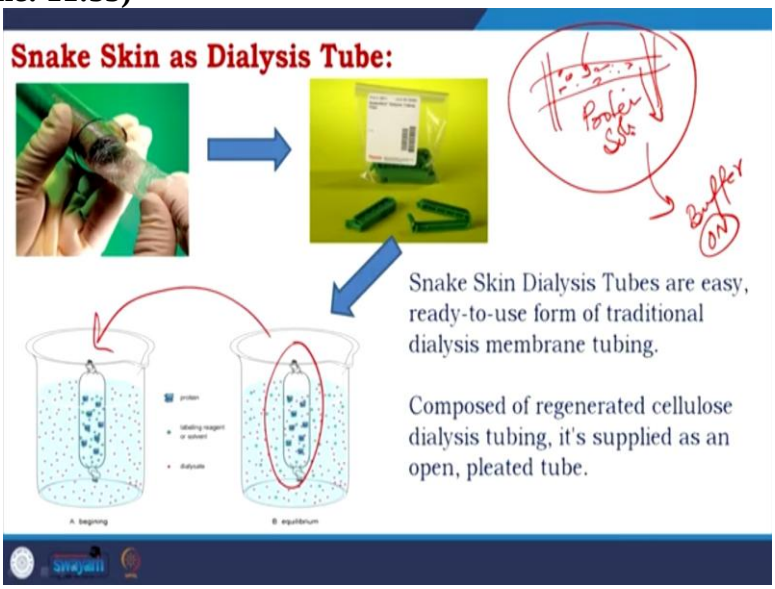

Examples are snake skin, they are very commonly used as dialysis tube, you have the snake skin, it is like parallel pipe, only thing is flexible and you have the clip. So you put the clips in two sides and then put it on the buffer solution overnight, and it would dialyze. The protein

molecules would remain there whereas small molecules are coming out to the solution and concentrate the protein. Snake skin dialysis tubes are easy, and ready to use.

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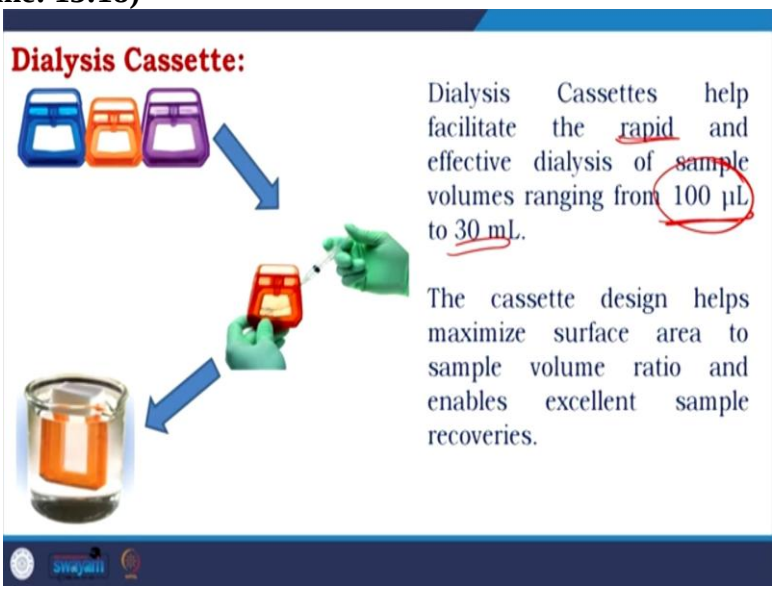

Dialysis cassettes are more advanced form. They are especially good when you have small volume and you do not want to lose your protein. Dialysis cassette help facilitate the rapid and effective dialysis of sample. The cassette design helps maximize surface area to sample volume ratio and enables excellent sample recoveries.

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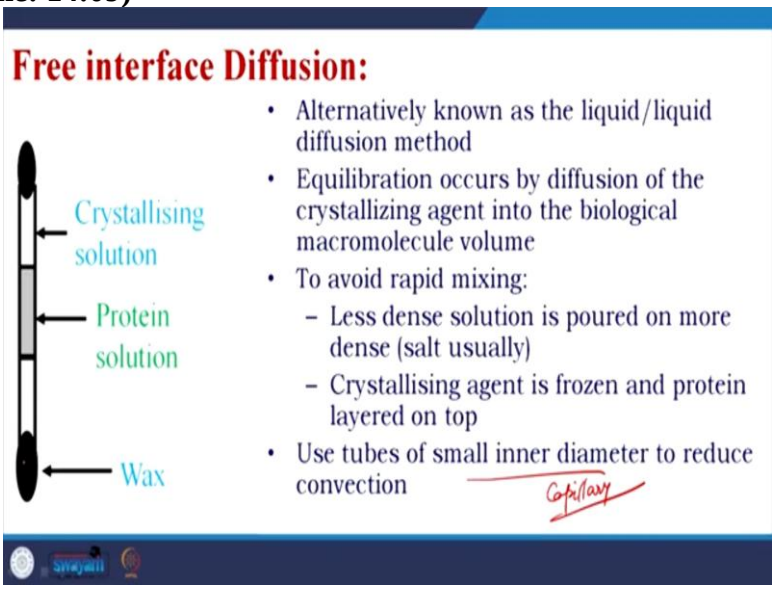

Free interface diffusion. It is alternatively called as the liquid-liquid diffusion method. Equilibration occurs by diffusion of the crystallizing agent into the biological macromolecule volume. To avoid rapid mixing less dense solution is poured on more dense (salt usually), crystallizing agent is frozen and protein layered on top. Use tubes of small inner diameter generally capillary to reduce the convection.

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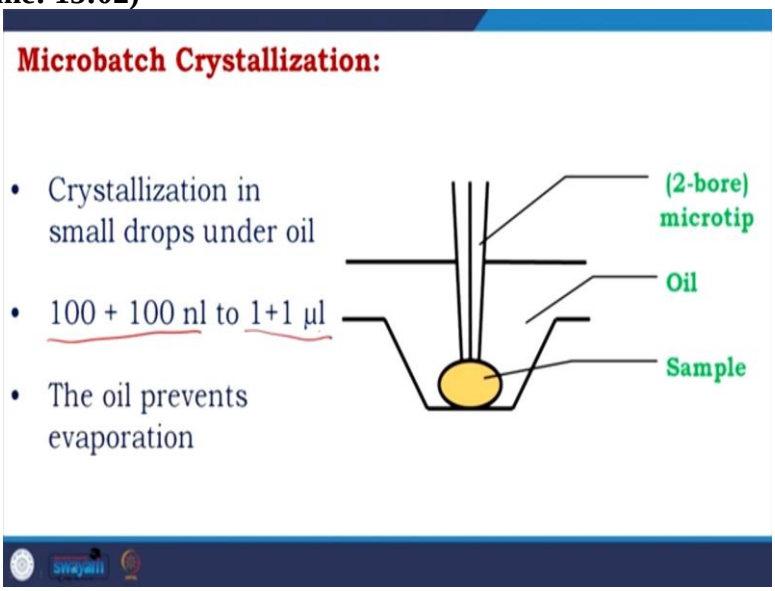

Micro batch crystallization: Crystallization in small drops under oil. It is generally in low volume 100 + 100 nano liter or 1+ 1 micro liter. The oil prevents evaporation. So when I was talking about hanging drop, sitting drop they do evaporation until the equilibrium is reached. And because we have no idea about where the crystal comes, even the crystal comes they keep dehydrating which is not good. That is why you need these conditions, micro batch crystallization, where the oil would make an interface, where slow transition would happen and giving you another dimension to get into variation towards crystallization.

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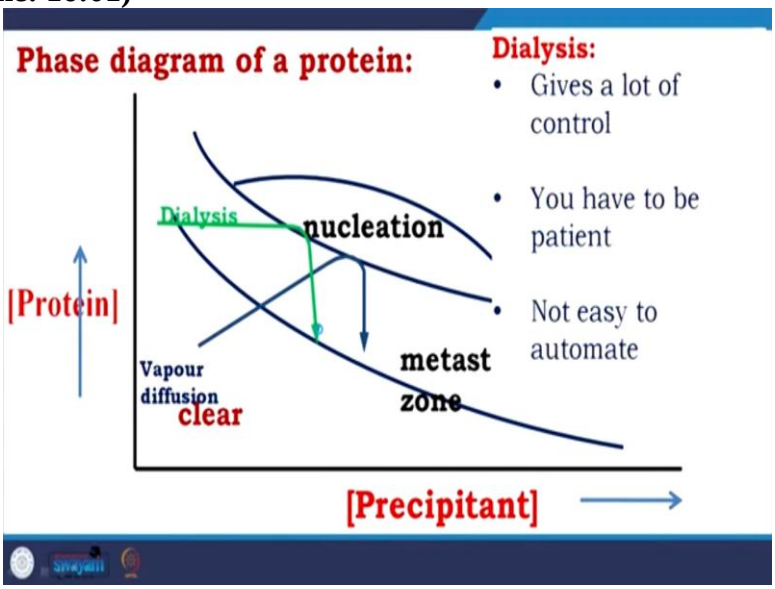

Phase diagram of a protein: we put protein and precipitant, when you see clear, then metastable zone, then nucleation and then precipitation. So for vapor diffusion, it puts you on

the metastable zone. Works well, gentle drop is concentrated after mixing, does not suit all protein. Dialysis gives a lot of control, you have to be patient, not easy to automate.

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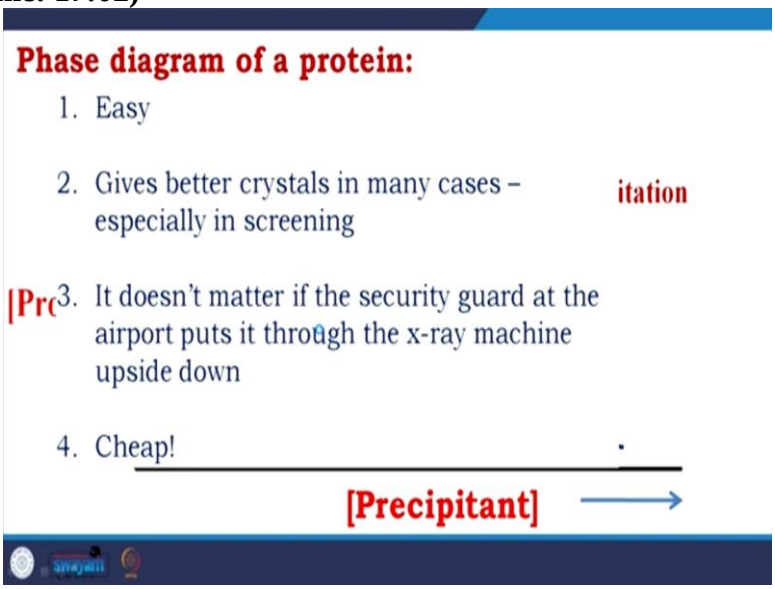

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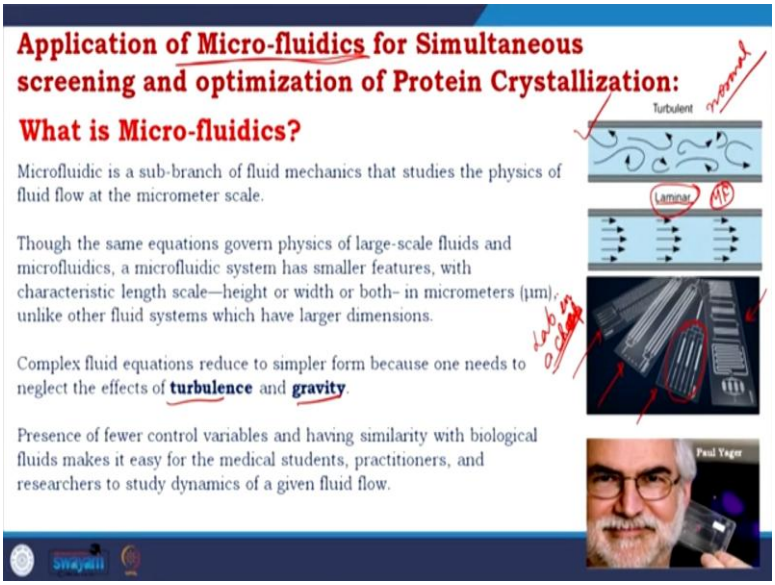

Now I am taking you towards very interesting application, application of micro fluidics for simultaneous screening and optimization of protein crystallization. Why crystallographers are looking for micro fluidics? Micro fluidics is a sub branch of fluid mechanics that studies the physics of fluid flow at the micrometer scale.

Though the same equation governs physics of large scale fluid and micro fluidics, a micro fluidic system has molar features with characteristic length, scale, height or width or both in micrometer, unlike other fluid system which have larger dimensions.

Complex fluid equation reduced to simpler form, because one needs to neglect the effects of turbulence and gravity. Why one needs to neglect the effect of turbulence and gravity? If you imagine you are standing near a river or a sea and the first thing what you will see is the current. The current makes the flow turbulent or turbulence make the current either way. So this is normal fluidics, in micro fluidics this is not there, no turbulence. Now think about your blood flow which is the major control in biology, the bloods are flowing through the vein, arteries and they are in micrometer range. So they are also having laminar flow. So the introduction of micro fluidics have given an advantage to the model, make it more biological.

Presence of pure control variables and having similarity with biological fluid makes it easy for the medical students' practitioners and researchers to study dynamics of a given fluid flow. Until and unless micro fluidics is introduced to us, we love it because the model is simpler, we do not need to remember a lot of equational factors, more importantly, it is more biological fluid like, but there is more, you know even more. Another thing is, when you introduce micro fluidics, because of the volume of micro fluidics, your instrument going into very small size and it introduced something which is called lab in a chip, and it is revolutionary in biological diagnostics.

I will give you a very recent example of Paul Yager, and you see the thing in his hand, this is a beautiful point of care diagnostics with very fast, and is very cheap to do the testing of malaria. So, micro fluidics helps biology to develop diagnostics.

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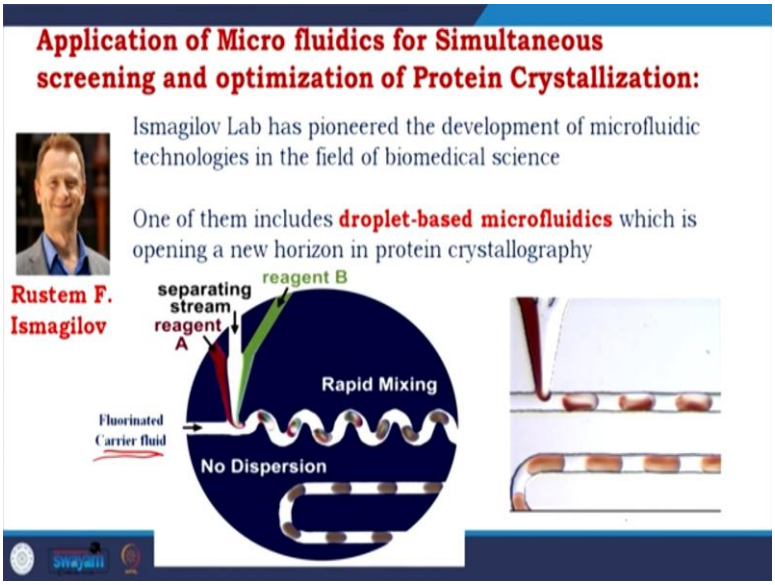

So as I told application of micro fluidics for simultaneous cleaning and optimizing of protein crystallization. Rustem Ismagilov works in University of Chicago that time and he developed fluidics, micro fluidics based crystal screening.

Ismagilov Lab has pioneered the development of micro fluidic technology in the field of biomedical science. One of them includes droplet based micro fluidics, which is opening the new horizon in protein crystallography. This is the system, have different channels, you have reagent A, you have reagent B, you have separating stream, so that they are separated and they are mixing when the fluorinated carrier fluid is mixing there.

So there is three things, reagent A (let us take this as protein), reagent B (let us take this as precipitant), and fluorinated carrier fluid. And now they are making separate droplets (that is why it is a droplet micro fluidics).

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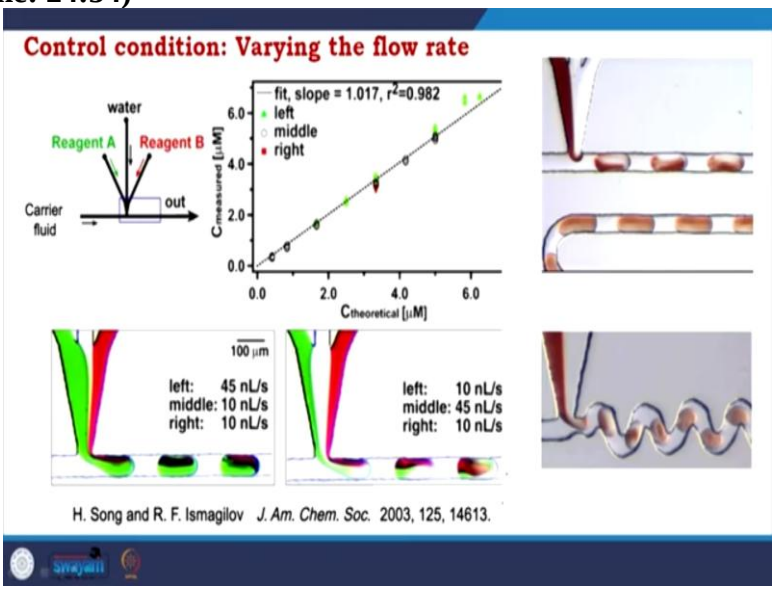

It is more important because, this is slow going. This is a different tube, so it is fast going. So what you could do in this setup, you could control the conditions by varying the flow rate. Here they have different concentration and different speed, and they got the difference here to fit.

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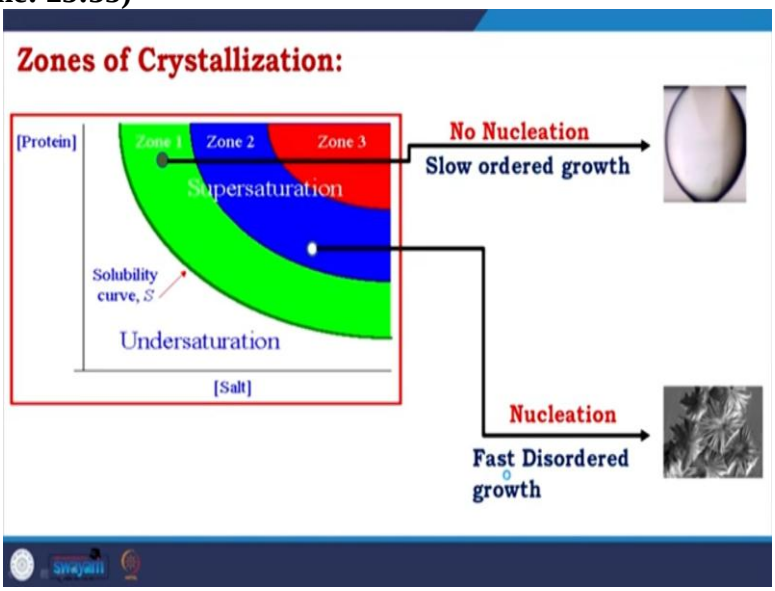

What is the effect of controlling? You remember the zones of crystallization, there are three zones and first zone is mostly clear and third zone is the precipitate in between nucleation happens.

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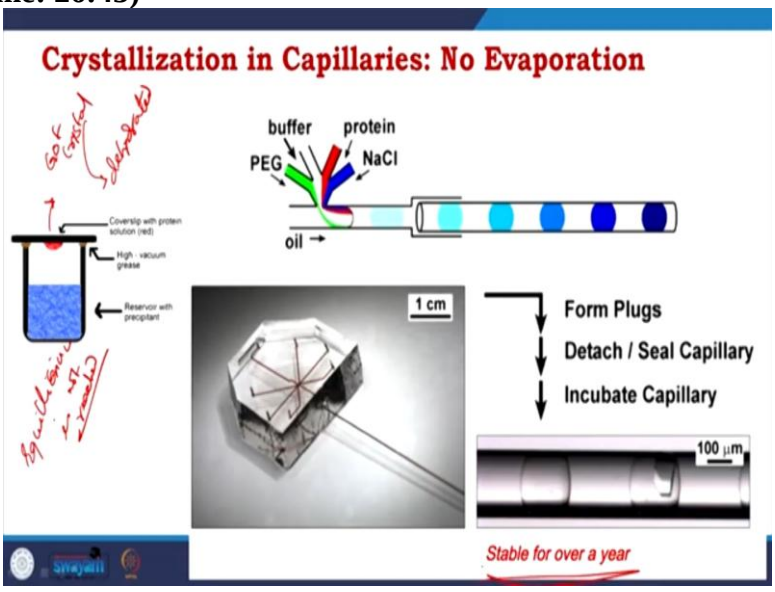

Now when you look at the crystallization capillaries, you get another advantage, remember I talked about when you perform sitting drop or hanging drop, you got crystal, but equilibrium is not reached. So the crystal would be dehydrated that dehydration or any evaporation is not there in capillary giving another advantage. Again remember, I talked about some time

crystals take years to develop. In that time because of the equilibration, the mother liquor might get in a situation where it would spoil the crystallization or the crystal formed but it becomes damaged. Here in capillary, because of the formation of plug, because of seal capillary, it could be stable for a year which is a huge time. So in addition to the advantage of control, you also get no evaporation, so crystals are stable once formed.

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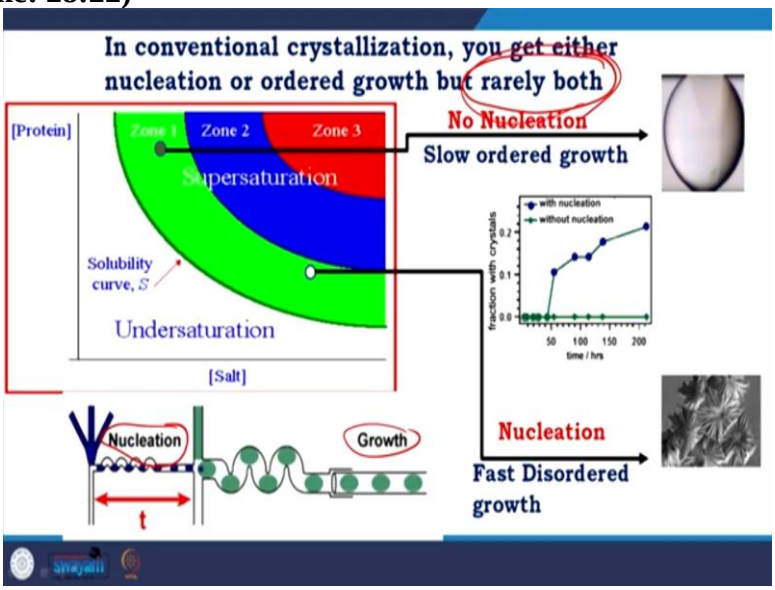

Now as I told in conventional crystallization, if you try to control you get either nucleation or ordered growth, but it is very rare to get both of them. If you get a disordered crystal, you cannot do anything with that. So when I say crystallization, it is an ordered crystallization, ordered growth.

Very interestingly here, you could have separate the nucleation and growth, see there is a time gap. That is the most critical feature which is providing by this micro titer system Also they could see with nucleation and without nucleation. So you could separate them and you could use both of them.

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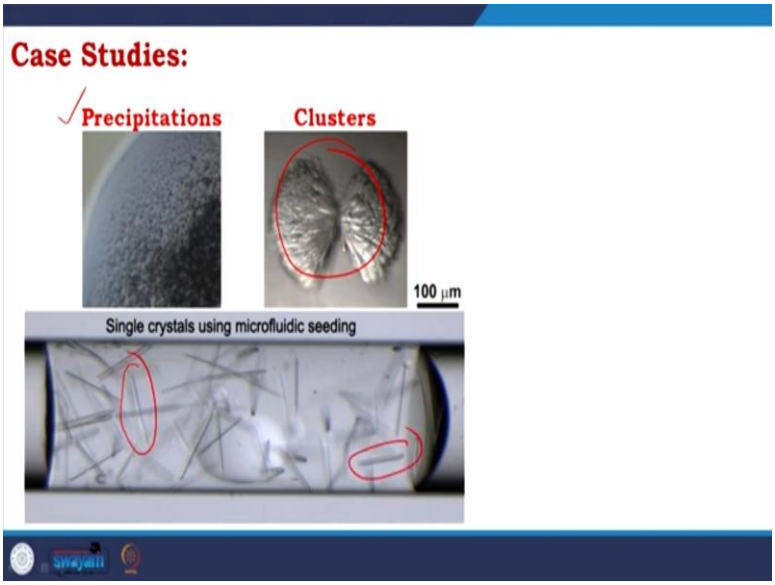

And they also saw a case study of a real crystal, where they got precipitation in normal conventional crystals in most cases. Even if they got clusters, they got very bad clusters, you could see. But in the case of microfluidic seeding, you get single crystals.

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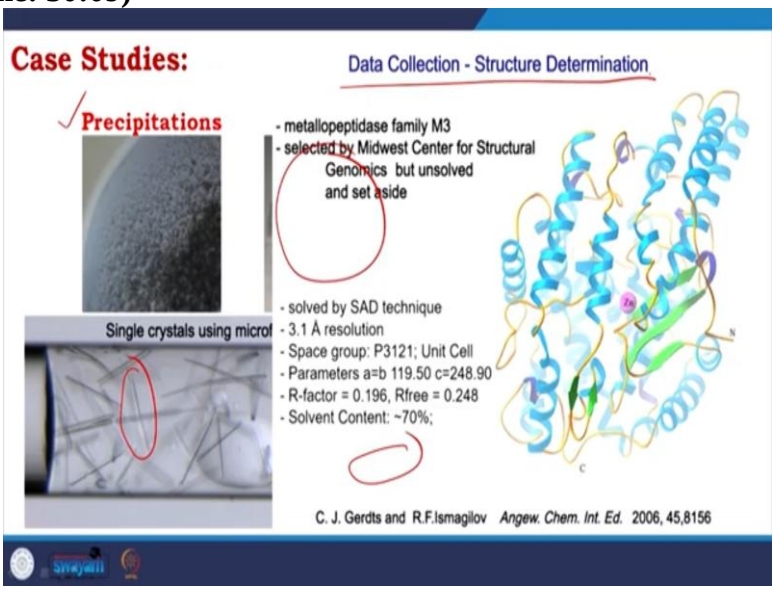

So are they solvable? Yes, they have also shown that the crystal they have used for structure determination is a metallopeptides, this family M 3. This is part of MCSG, which I discussed earlier in structural genomics. They have solved this crystal.

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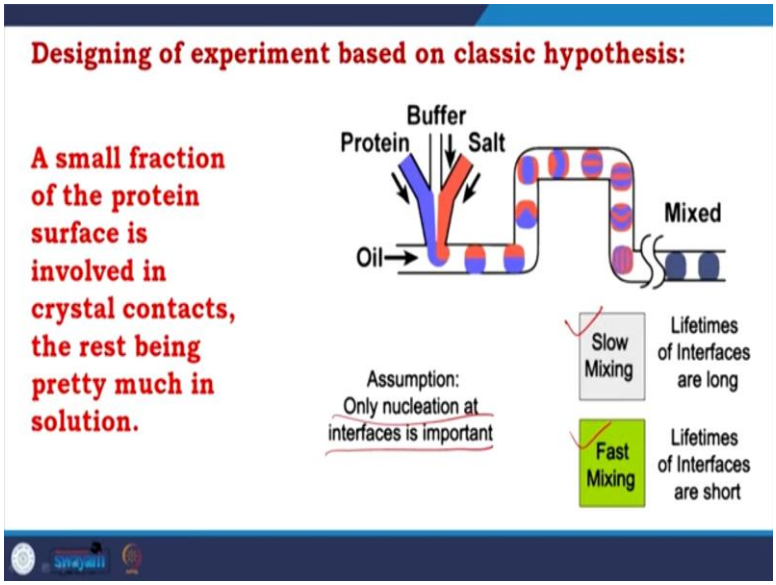

Now they limit themselves to showing the difference, but they also design experiments based on classic hypotheses. A small fraction of the protein surface is involved in crystal contacts, the rest being pretty much in solution, which means for crystallization, it is the protein surface and a small fraction of the protein surface needed.

Ismagilov group has utilized them, they have set up the system so that they are mixing it, and the mixing is either slow mixing or fast mixing, slow mixing means the lifetime of interfaces is long, and fast mixing means the lifetime of interfaces are short. The assumption only nucleation, as I told at interfaces, is important.

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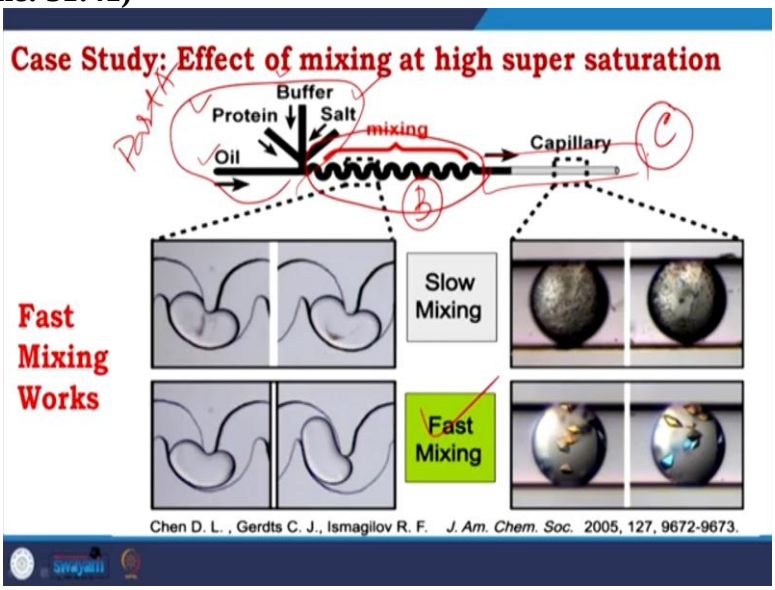

So what they got, they made different case studies of crystallization. In one, they do slow mixing and fast mixing using high supersaturation. And if you see in slow mixing, they got

no crystal or very small crystallites, but in fast mixing, they got the crystal. So, in this case, at high super saturation, first mixing works, and you see what they did? They differentiate the capillary into parts one where everything is mixing, and the oil is coming, the protein is coming, the salt is coming, and the buffer is coming.

Then they go through the turbulent flow where the control is in their hand. They are making it either slow mix or fast mix, and then they again put it on the capillary steadily. The setup has parts A, B, and C, and the result you see fast mixing works.

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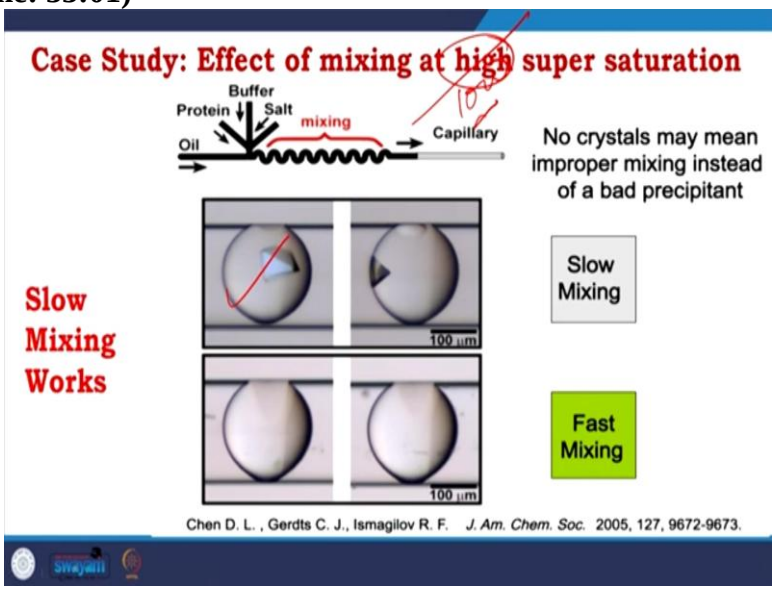

In another case, they do slow mixing and fast mixing, and they got crystal in case of slow mixing, so slow mixing works. So that is how they have shown us that different setup types of micro fluidics work in different conditions.

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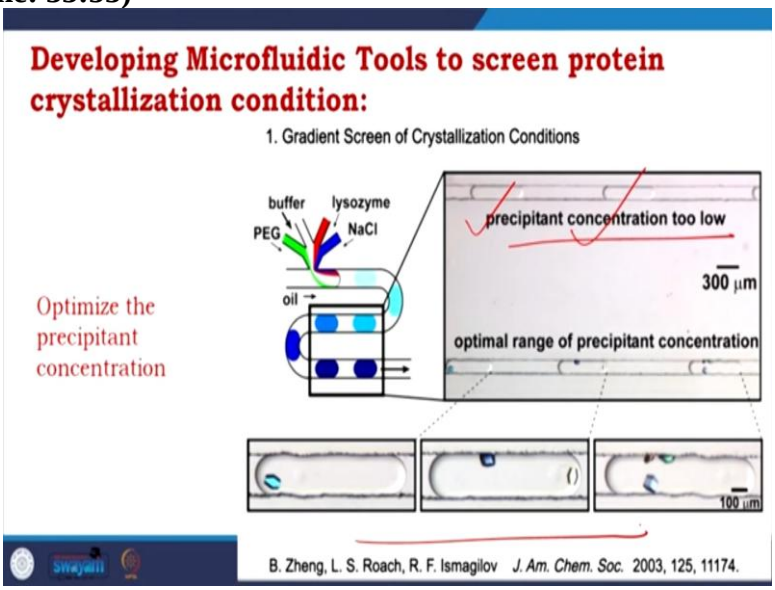

They go for developing for very individualistic conditions, but then they try to develop microfluidic tools to screen protein crystallization conditions. The first thing they do, they use gradient screen up crystallization conditions, which means you have ammonium sulfate as the main precipitant. You use different concentrations of ammonium sulfate in that way.

And suppose here they take lysozyme, buffer, PEG, NaCl, and then develop the differences, optimal range of precipitant concentration.

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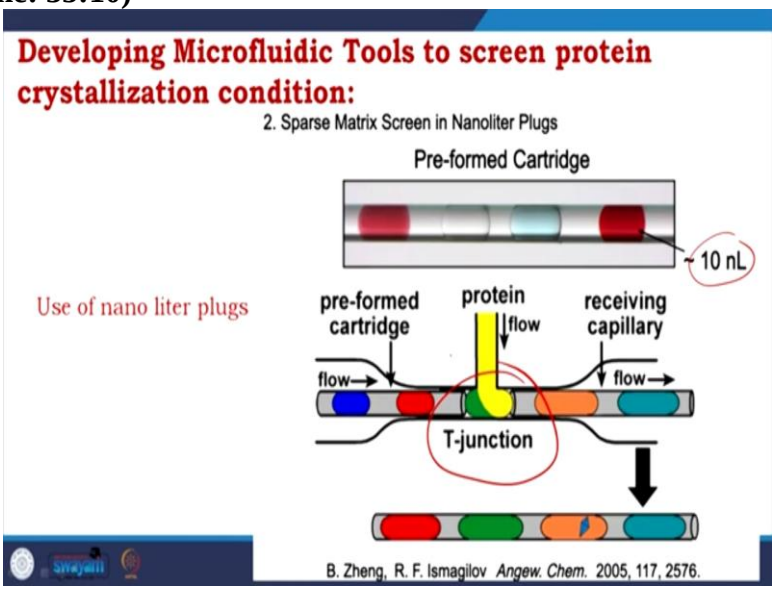

Then the second one is even more interesting. They use sparse matrix training nano liter plugs, so they have developed different plugs of 10 nano liter. So they have the pre formed cartridge and they add the protein and then they have the continuation. So they have developed a T junction, in the T junction, they are mixing the proteins so that every nano litre plugs got the protein and then they could screen it according to different conditions.

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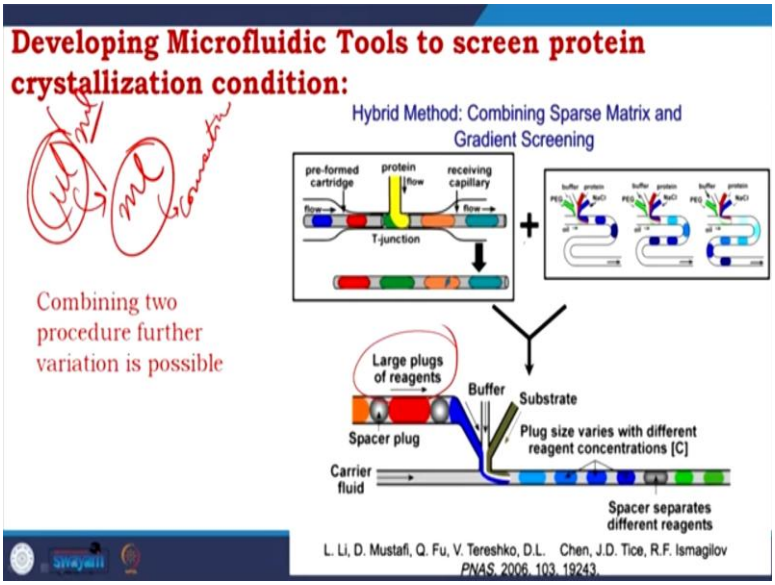

And they also have developed a hybrid method where they combine the sparse matrix and the gradient screening. So they develop the preformed cartridge, they have the protein flow, then they have the receiver capillary, but then they merge it with the different concentrations. So they have these spacer plugs which differentiate the large plug of these reagents and the plug size varies with different reagent concentration. So they are using plugs and each of the plugs are differentiated with different concentration of the precipitant.

So they optimize the nano liters, they optimize the precipitant concentration. So combining 2 procedure further variation. So as I told throughout the course of this crystallization more, you come up with variation more you have chance of getting a crystal, here they are developing the tool to give us the potential to make a number of huge number of variations. And remember, they are doing it in micro and nanometer nano liter, another big advantage.

Remember when I talk about this process of crystallization needs a lot of protein, but actually you need one concentration. So you have to produce a lot of protein and more protein you have more screening you could do but here you are doing in, generally you do in like milli liter range in the conventional one here you are doing micro liter to nano liter. So you could do more screening because you need little amount of protein per go.

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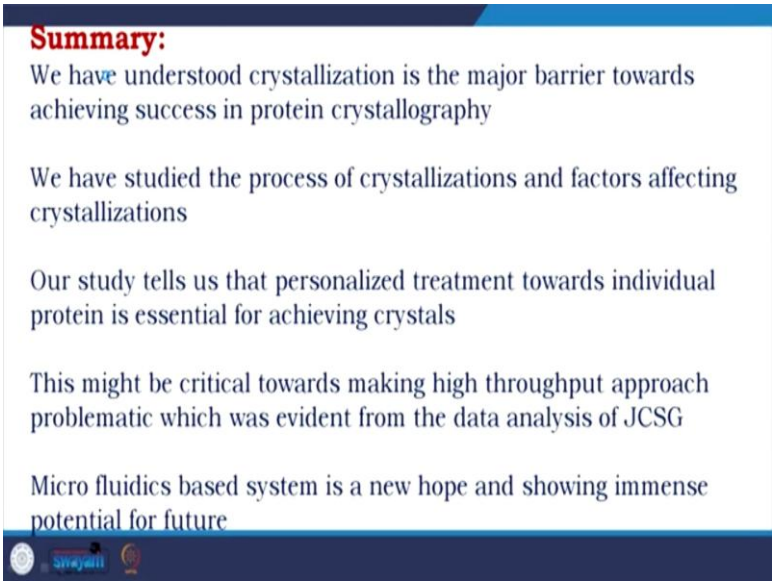

So here I will make summary of today's class, we have understood crystallization is the major barrier towards achieving the success in protein crystallography. We have studied the process of crystallization, different techniques and all and factors affecting crystallization the process also we have studied. Our study tells us that personalized treatment towards individual protein is essential for achieving crystal.

We have shown you that the protein you do everything and not getting crystal with some protein engineering strategies, you could have get crystal with some other strategies of ligand addition and all, domain you could get crystals, so personalized starts are required and when we talk about this personalized treatment that might be critical towards making high throughput approach problematic.

So when people are doing high throughput you like if I say you work on a protein and do your PhD, you have a chance to do personalized treatment, think about the protein think about study about the protein surface proteins property, how to compare with other proteins which have crystallized homologous proteins and all these things you could do. But when I tell you, you have to work with 5000 protein or 100 proteins you could not get those chance.

So that tells us that high throughput is good for genomics. But for structure, it is probably not the optimized approach. And then we look at micro fluidics, the micro fluidics based systems it is definitely a new hope. And I sincerely hope that would so it is already showing immense potential, but it would come out as a device and we could have all access to that because at the end, it is not crystallization.

It is crystallography to solve the structure and relate it to function. That is the goal. Our goal is to go from sequence to function. That is where the structural biology is standing. You all are amazing audience, please talk to us contact us. If you have any question regarding this. I will be very happy to receive them and I will try my best to answer according to my accessibility. Thank you very much.