Biomaterials for Bone Tissue Engineering Applications
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Module 7
Lecture No 36

In one of the earlier modules in two modules in this particular course I am illustrating how to develop that antimicrobial biomaterials.

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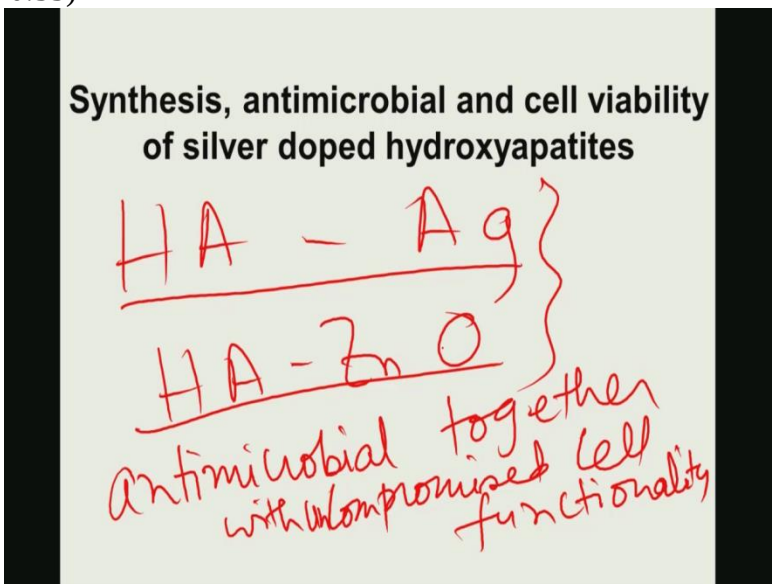

So the two case studies I am presenting; one is the hydroxyapatite silver and one is that hydroxyapatite zinc oxide. The central theme of discussion of these two materials is that while we have to provide antimicrobial properties or induce antimicrobial properties but we cannot compromise on the cell viability. So therefore I am writing antimicrobial properties with together with uncompromised cell viability, uncompromised cell viability or cell functionality properties.

So this demands that very careful and thoughtful design of these two materials that I am illustrating in this particular module I will be discussing on the habits of silver. In another module I am discussing hydroxyapatite zinc oxide composite development.

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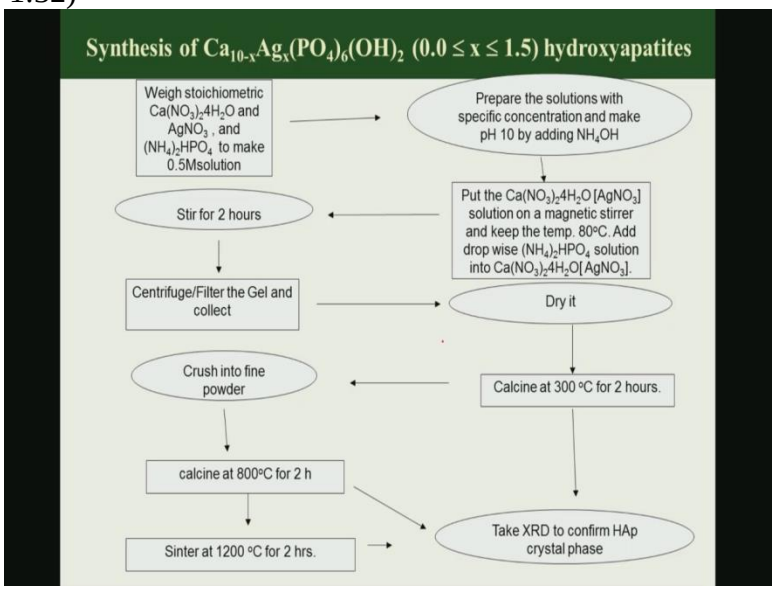

Now hydroxyapatite silver, these materials can be produced by two different approaches. One is called mechanical mixing of silver and then second is that chemical synthesis route.

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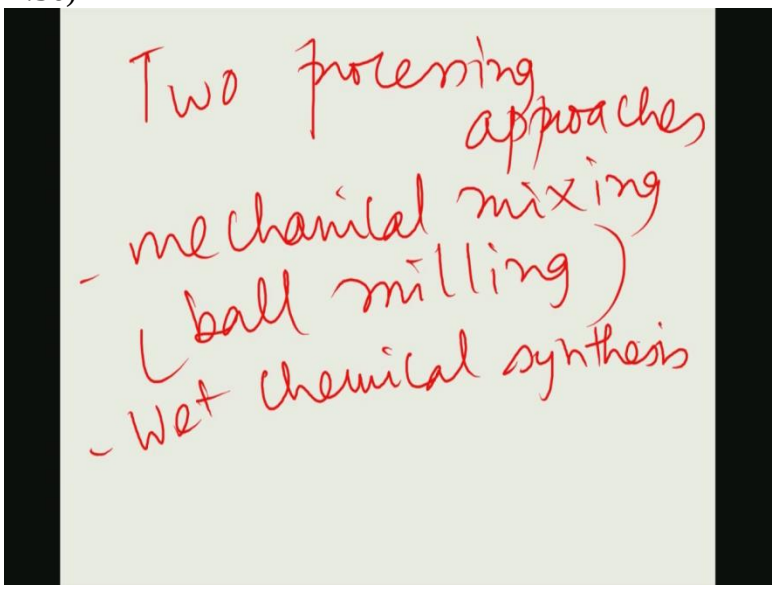

So the mechanical mixing and in both the cases, so two processing approaches. One is that mechanical mixing, so mechanical mixing essentially means that is the ball milling. And then second one is that wet chemical synthesis. Now wet chemical synthesis approach will be emphasised in this module. Now mechanical mixing simply means that you take hydroxyl powder and you take metallic silver powder, you mix it with certain for certain time with the

expectation that silver will be homogenously distributed in hydroxyapatite matrix and that will be followed by sintering.

So this is very much material science based approach, but more chemistry based approach is that wet chemical, wet chemical synthesis.

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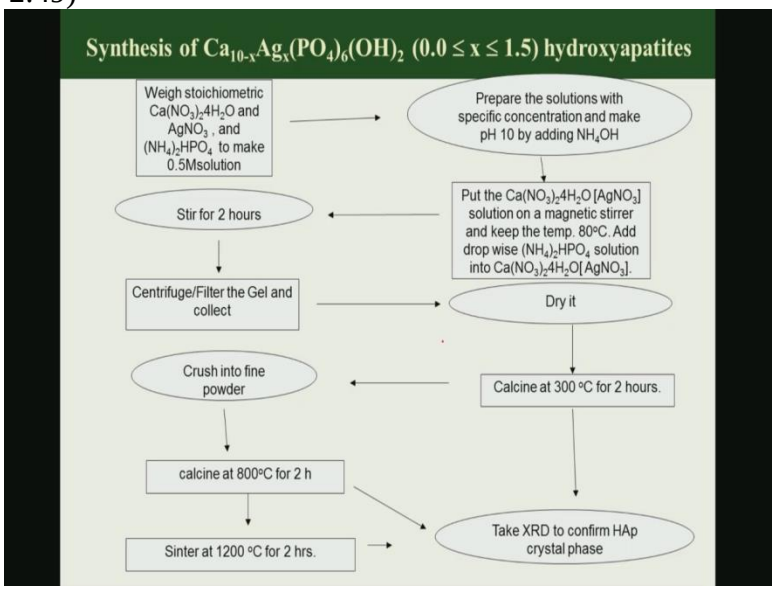

So like any other wet chemical synthesis you start with the precursor solution of this silver as well as the hydroxyapatite, and the precursor solution here is the silver nitrate for silver and then for hydroxyapatite we are using calcium nitrate. 4H2O(Ca(NO3)2.4H2O) and ammonium phosphate that (NH4)2HPO4 and then we make this 0.5 molar solution of this particular precursor, then you stir if for two hours then you centrifuge, crush it and then calcine it at 300 degrees for 2 hours.

After the calcinations you sinter it at 1200 degree celsius for 2 hours, after you sinter it then you can do the XRD test to confirm the hydroxyapatite, whether the silver has doped into hydroxyapatite.

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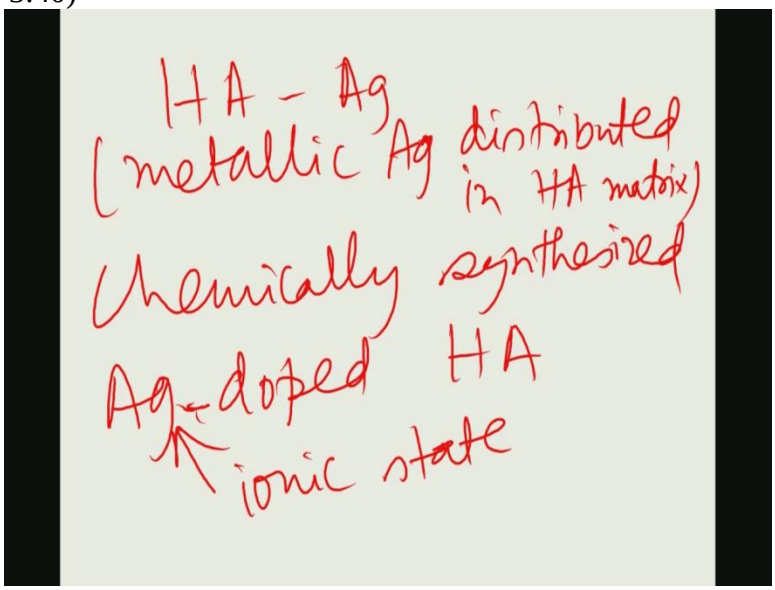

So as I said that this hydroxyapatite silver when it is mechanically mixed then we expect metallic silver to be distributed in HA matrix, and when it is chemically synthesised or to be more precise wet chemically synthesised so what we expect is that silver doped hydroxyapatite and this silver here, it is in ionic state. So depending on whether the silver is present as a metallic particles or metallic nano-particles, or whether silver is present as ions which is incorporated into hydroxyapatite lattice as such, by partially replacing calcium ions and so on, so those kind of things their bio compatibility properties will be entirely different.

Because in one case you get the biocompatibility or antibacterial property because of the silver metal and another case it is because of the silver in ionic state in the hydroxyapatite powders.

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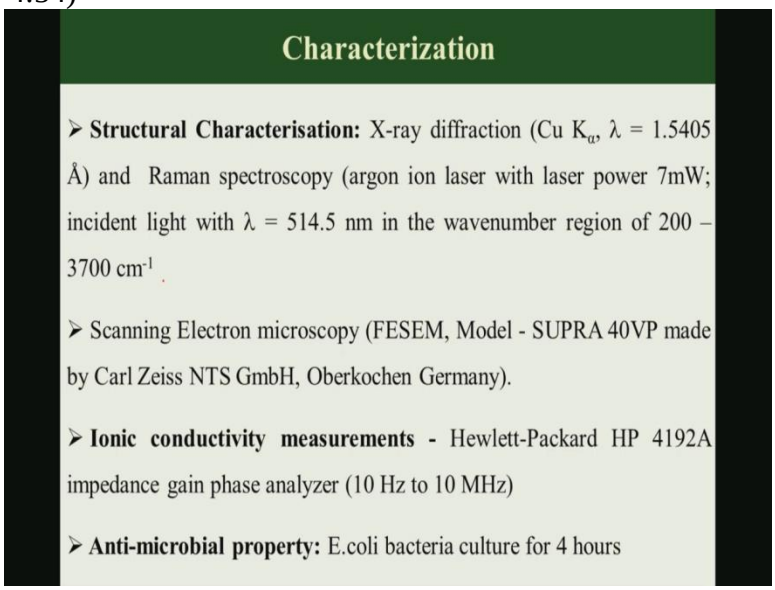

So now how to confirm that whether silver in that ionic state and so on, so you can do specific optical analysis, spectroscopy analysis just to confirm whether silver is in ionic state. Rather than that you can do other standard material science characterisation like Raman spectroscopy X-ray diffraction and so on. Now silver also potentially increases or can also potentially increases the ionable or conductivity of the hydroxyapatite materials, and this has been confirmed using the ionic conductivity measurements.

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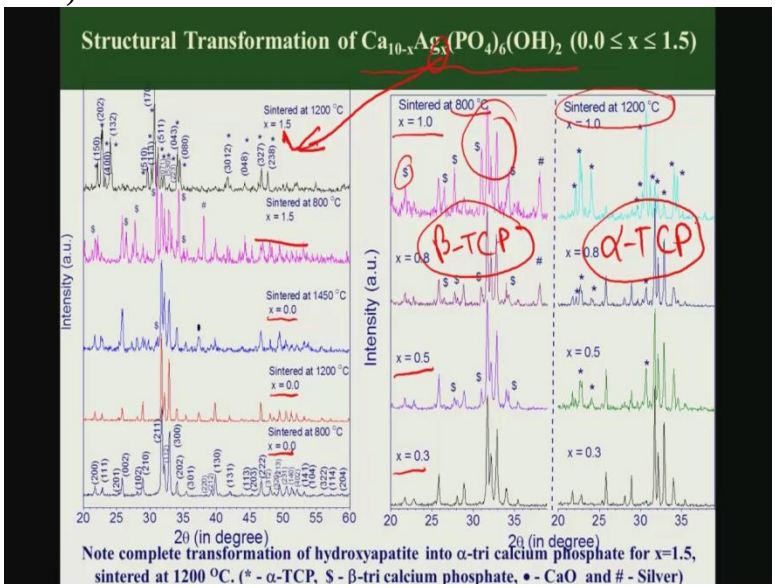

So this is little bit material science based results like you, we have sintered these materials at different temperature from 800 to 40 and 50 degree Celsius. If X is equal to zero means that is pure hydroxyapatite, no silver doping. If X is equal to 1.5 means it is some amount of silver is there, and as you can see here in the title slide of this one, that CA10 xAgx(PO4)6(OH)2. So without silver the structramatic hydroxyapatite has a chemical composition CA10 xAgx(PO4)6(OH)2and as I said silver is incorporated in the hydroxyapatite lattice that is why it is put the silver subscript states, that is X percent is being incorporated and that is replacing the calcium in that hydroxyapatite lattice.

Now when you, when X value is 0.5 here, when X value is 0.5 here then we are seeing that some additional phases that are present tri-calcium phosphate and so on. So now with systematic variation in the composition; like X is equal to 0.3, 0.5 up to 1.0 you can see that when they are sintered at 800 degree Celsius you get a different phase assemblage and when you are getting the same sample sintered at 1200 degree Celsius there is indeed a difference in the terms of the X ray characteristic peak intensity.

Now some of the peaks that are mentioned here, now dollar signed peak is the beta tri-calcium phosphate and then closed symbol it is the calcium oxide and then star is the alpha tri-calcium phosphate. So what you see here this is more alpha tri-calcium phosphate based phase and this is more on the dollar signed is in the beta tri-calcium phosphate. So when you sinter at lower temperature the beta tri-calcium phosphate phase forms, and when you sinter at higher temperature it is more alpha tri-calcium phosphate phase as found.

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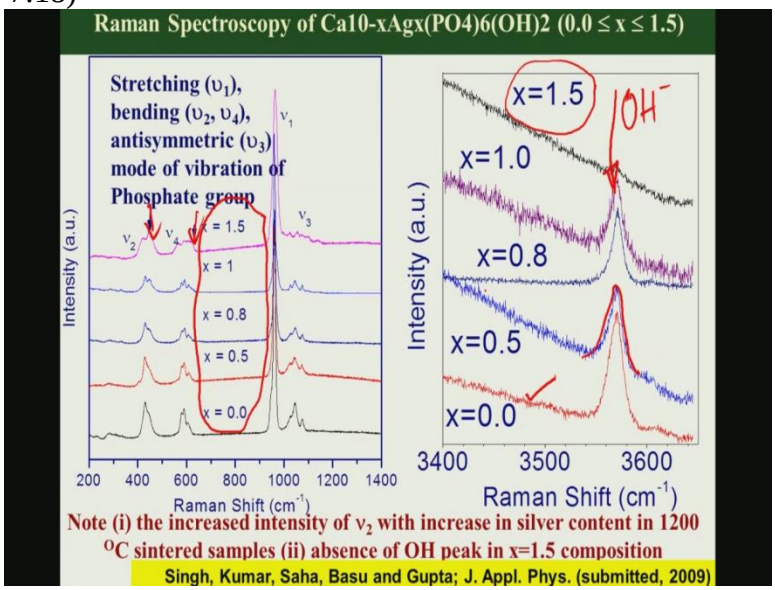

So Raman spectroscopy analysis further confirms that difference in stretching, bending and asymmetric mode of vibration of the phosphate group that has been shown here indicated by different arrow at different places and as you can see that this is also dependant on the different substitution level of the silver into hydroxyapatite.

Now this other things has been mentioned that needs to be clearly mentioned here that this is essentially this particular peak which you see here with this arrow this is essentially indicating or indicates that OH one is a hydroxyl group. Now when you start with the base line hydroxyapatite, you expect very strong OH manner peak simply because CA10 xAgx(PO4)6(OH)2. Now as you keep on substituting calcium by or you are doping that silver into the hydroxyapatite lattice; then this OH manner is intensity that Raman band gets decreased, decreased and subsequently at X equal to 1.5 it is, it completely disappears.

What it means that that X is equal to 1.5 you do not have any presence of hydroxyapatite, instead you will have tri-calcium phosphate and this tri-calcium phosphate is either alpha or beta depending on whether what temperature you are sintering at 800 or 1200 degree Celsius.

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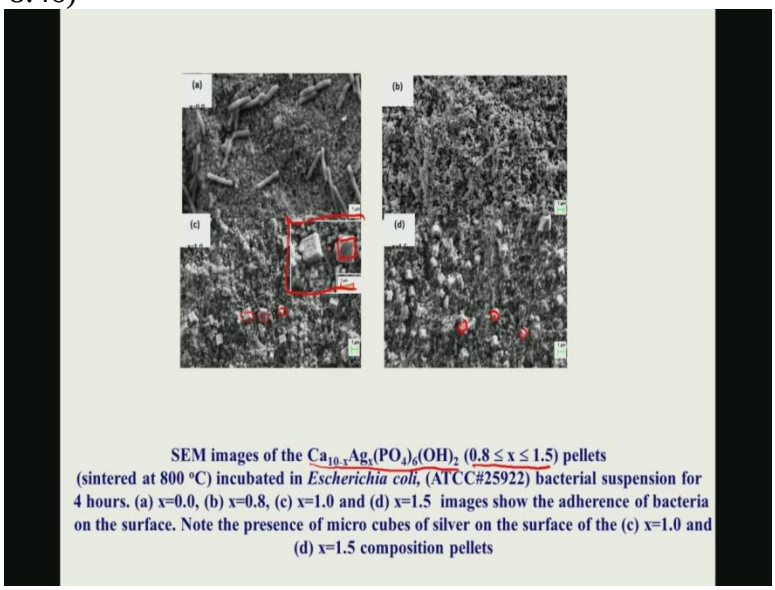

SEM images of this sintered micro structure and at different substitution level and particularly in the window of 0.8 to 1.5. Now what you see, this is like a typical geometrically cubic shaped silver crystal. Also you can see that is present in this micro structure, and this has been clearly shown in the inset. If you look at that micron bar this is one micron, this green line is one micron so therefore individual this crystals is one micron in size. So this one micron n size silver crystals that has been shown to disperse or embedded in this CA10-XAgx(PO4)6(OH)2 ceramic.

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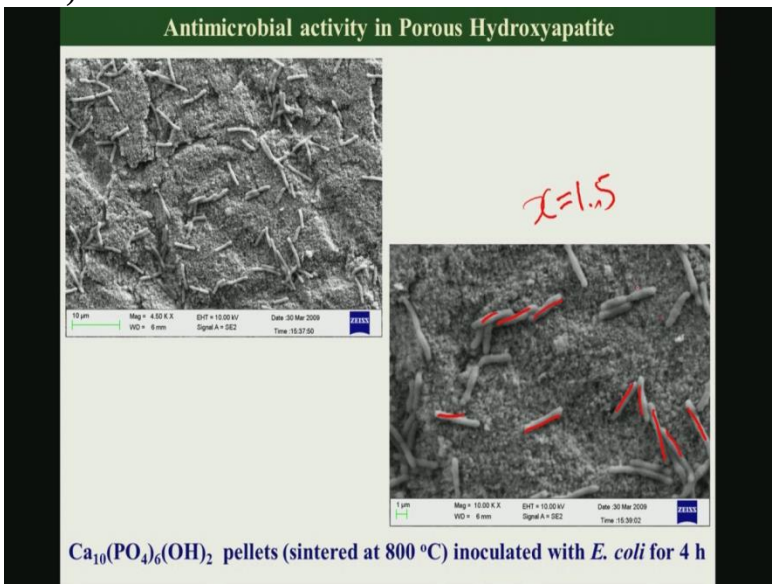

Okay now coming to that antimicrobial property of this materials, so this palettes which are sintered they are inoculated E. coli. Escherichia coli for four hours and after it is sintered, and what we see that when it is, when it is substituted at X at 1.5 much less number if E. coli are found to adhere in the material, on this particular substrate compared to control or compared to hydroxyapatite. It shows certain level of anti microbial action on this material.

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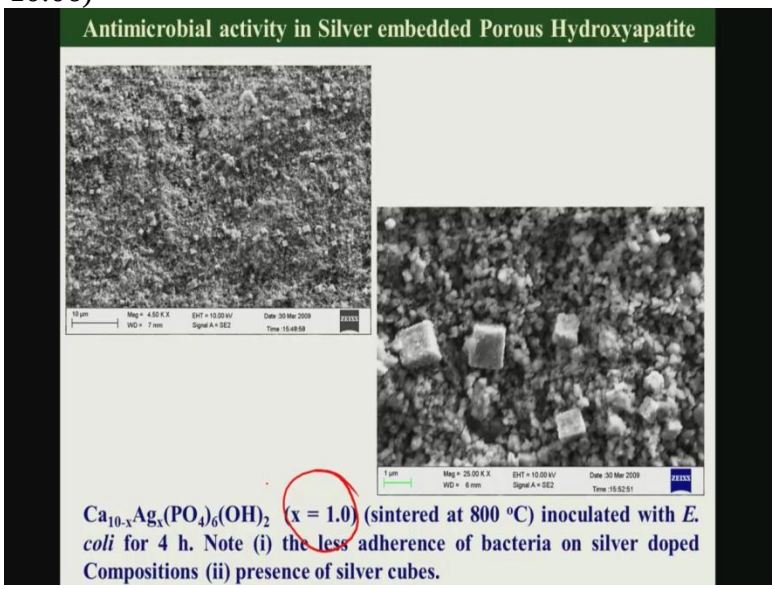

The same is true is true to X is equal to 0.8, it is very hard to find out that one singe E, coli in that, in the entire investigated region that the SEM image after this E. coli was cultured or were grown on this materials for four hours. So at X is equal to 1.0 you do not see any evidence of this E. coli bacteria on this material substrate essentially confirming that from X equal to 1.0 onwards this materials have clearly bactericidal property in vitro. The same is true for X equal to 1 and X equal to 1.5.

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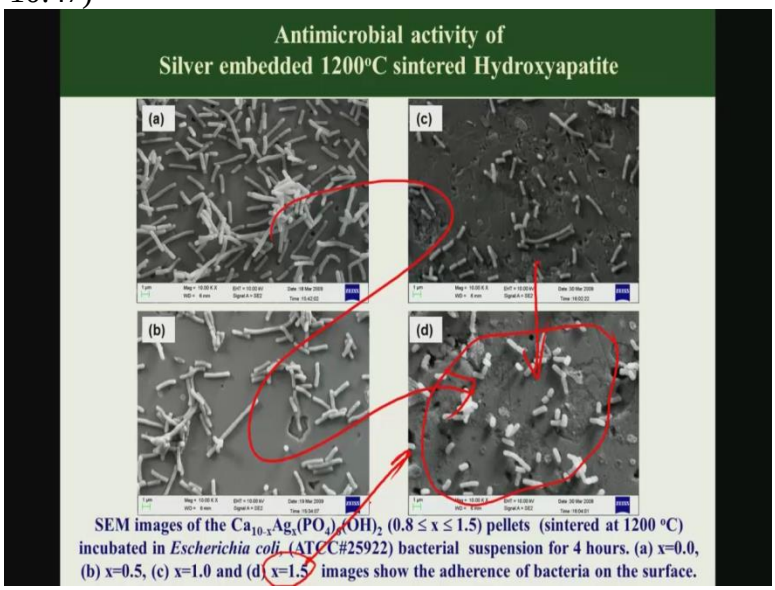

Now when it is, these materials are sintered at 1200 degree Celsius little bit different kind of results are shown and that what what has been found is that although the number of bacterial colony or number of bacteria which is viable on this material substrate, it decreases like this, it decreases in this pattern depending on what is the level of silver substitution on this materials. But certainly you see lot more bacteria what you have seen in the earlier few slides, particularly when that X value or the substitution level is quite high like 1.5.

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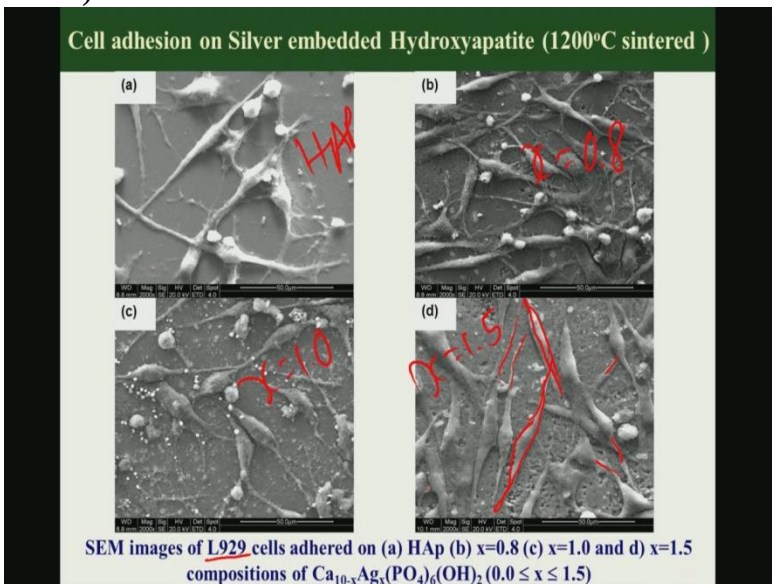

So now in terms of the cell adhesion properties or the cell viability properties of this materials; we certainly got quite an interesting results, because earlier in this hydroxyapatite zinc oxide and so on, so there with depending on the zinc oxide content the cells are not viable particularly at higher zinc oxide adhesion. But here we have grown this L929 that is the mouse fibroblast cells and these fibroblast cells when they are cultured for 24 hours on this materials, now this is your pure hydroxyapatite and this is different level of silver content, and X is equal to 0.8, X is equal to 1 and X is equal to 1.5.

You see that very clear very clear fibroblastic morphology of this material, of this cells and particularly the spindle shaped cells you can see, that expanded and that is grown on this material substrate and also there is some filopodial extension, it certainly indicates the cells are in migratory motion and also there is cell to cell breach formation.

So all these evidences indicate towards that, that this materials does not have any toxicity towards fibroblast cells, not only that; but also this materials supports despite the silver substitution to the level that exceed 1.5, they certainly can be used as a cell growth substrate and this is very encouraging results that this materials while, while inducing that antimicrobial property at the same time it also does not cause any significant reduction or to extent any visible reduction in the cell viability property.

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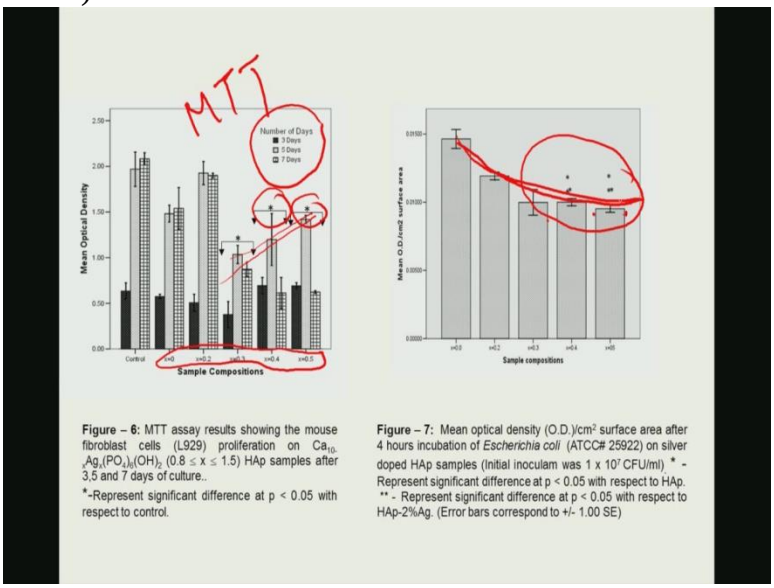

Figure – 6: MTT assay results showing the mouse fibroblast cells (L929) proliferation on $Ca_{10-x}Ag_x(PO_4)_6(OH)_2$ ($0.8 \leq x \leq 1.5$) HAp samples after 3,5 and 7 days of culture..

*-Represent significant difference at $p < 0.05$ with respect to control.

Figure – 7: Mean optical density (O.D.)/cm^2 surface area after 4 hours incubation of *Escherichia coli* (ATCC# 25922) on silver doped HAp samples (Initial inoculam was 1 x 10^7 CFU/ml). * - Represent significant difference at $p < 0.05$ with respect to HAp. ** - Represent significant difference at $p < 0.05$ with respect to HAp-2%Ag. (Error bars correspond to +/- 1.00 SE)

Now the quantification of all this discussion can be found in this particular slide, which this is the results of the MTT assay which has been widely used. Now MTT assay has been done for three different time points, like 3 days, 5 days and 7 days in culture. And then there is different silver substitution level up to £ 0 5 has been shown here and what you see here? That there is little bit reduction in the optical density values.

But this reduction is certainly not extremely significant like what we can see in case of the hydroxyapatite zinc oxide composite. And other interesting thing that what we have found that after 5 days and 7 days that X equal to 0.3 to X equal to 0.5 you see that optical density value has increased essentially meaning that mitochondrial active cells are viable cells that also increases. This is in contrast to the anti microbial results.

In anti microbial results there is a clear reduction in the optical density. Essentially means the number of viable bacteria or number of colony forming bacteria is reduced to quite a significant extent with silver incorporation hydroxyapatite.

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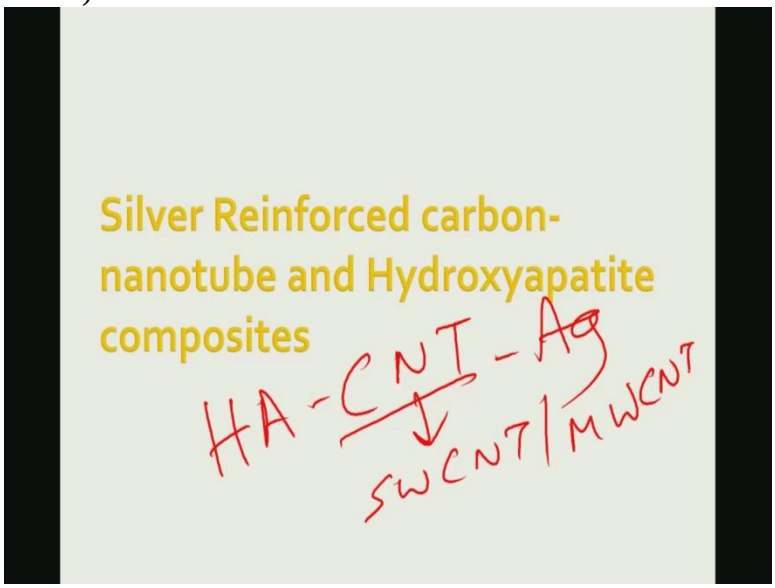

Now next few minutes or so I will give you some I will show you some results related to not hydroxyapatite silver but this materials are essentially hydroxyapatite carbon nanotube and silver composites. Now carbon nanotube as you know that it can be either single valve carbon nanotube

or multi valve carbon nanotube, in both the morphology this carbon nanotube can be present in the matrix.

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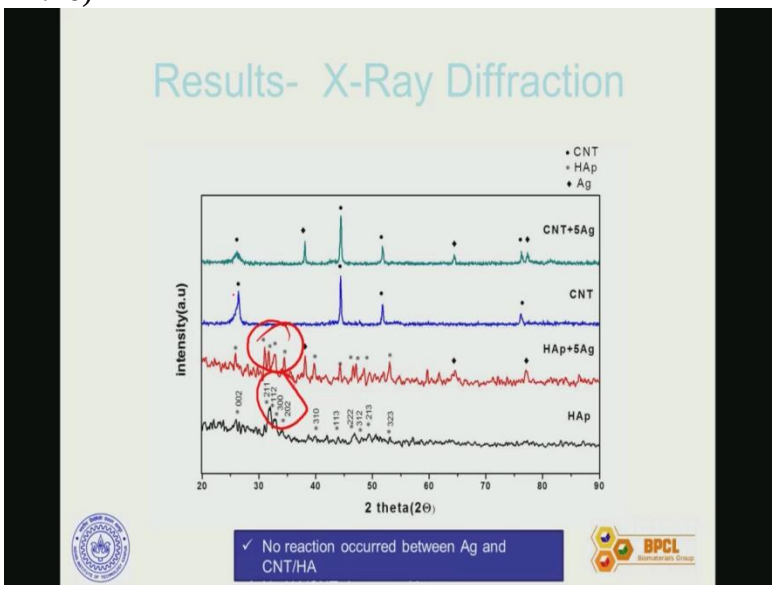

Now when you do this hydroxyl carbon nanotube and silver composites, so we have done classic material science analysis of X-ray diffraction just to confirm that characteristic hydroxyapatite peaks are retained in the spark pulmo sintered composites.

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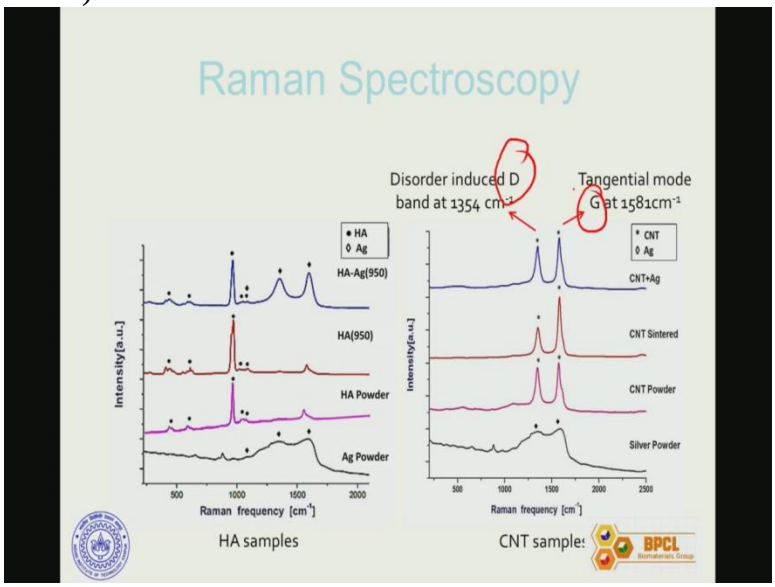

Then second one is the Raman spectroscopy analysis shows that particularly carbon that disorder peak and graphitic peak are clearly present at two distinct Raman bands of 1354 and 1581 centimetres inverse respectively.

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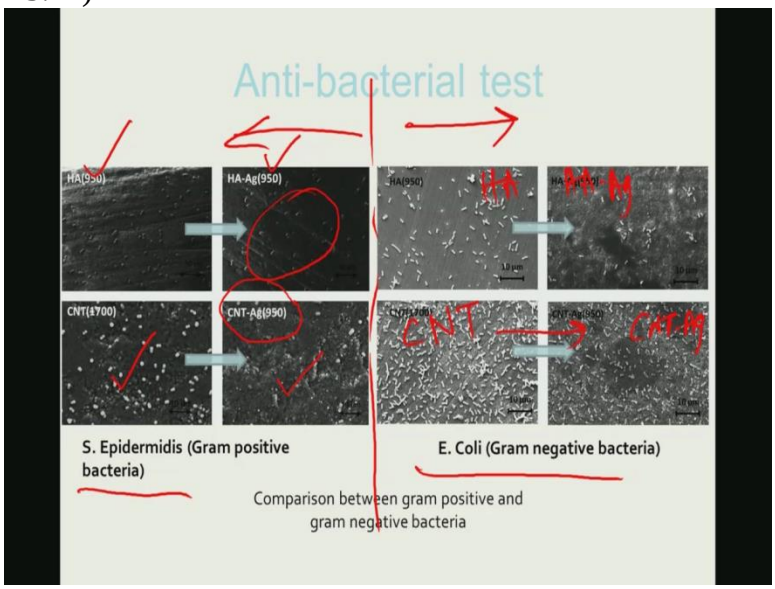

Now in anti microbial tests were conducted using two strains, one is that E. coli strain, that is the gram negative bacteria and one is that is S epidermidis, staphylococcus epidermidis that is the gram positive bacteria .Let me also remind you once more that any time one has to assess that anti microbial property of any synthetic biomaterial it is important to use not only one strain but also more than two strain types, preferably one from gram negative and one from gram positive; so that one can confirm that efficacy of the anti microbial property of these materials.

Now same logic or rationale has been followed here and what you see here that hydroxyapatite thus one hydroxyl silver 950 essentially tells you that that is the SPS temperature at which this materials are sintered. Now when this S epidermidis is there then hydroxyl and hydroxyl silver in silver certainly that number of bacteria which is present that is fairly less.

And what about CNT? CNT as such does not have any anti microbial property, but when we have developed the composite of CNT and silver mix, and then we see that no evidence of that S epidermidis bacteria which is growing as a, or which has a tendency to form any colony on this material. So this is the story for the gram negative; this gram positive this way.

And coming to gram negative E. coli, here again in the hydroxyapatite as such, this is the hydroxyapatite, and this is hydroxyapatite silver. Compared to hydroxyapatite, number of bacteria on this hydroxyl silver is significantly less. This significant reduction is much more evident when you look and compare CNT that was large number of bacteria is growing and compare it with CNT silver, that is the E. coli bacteria; that is the E. coli is significantly reduced.

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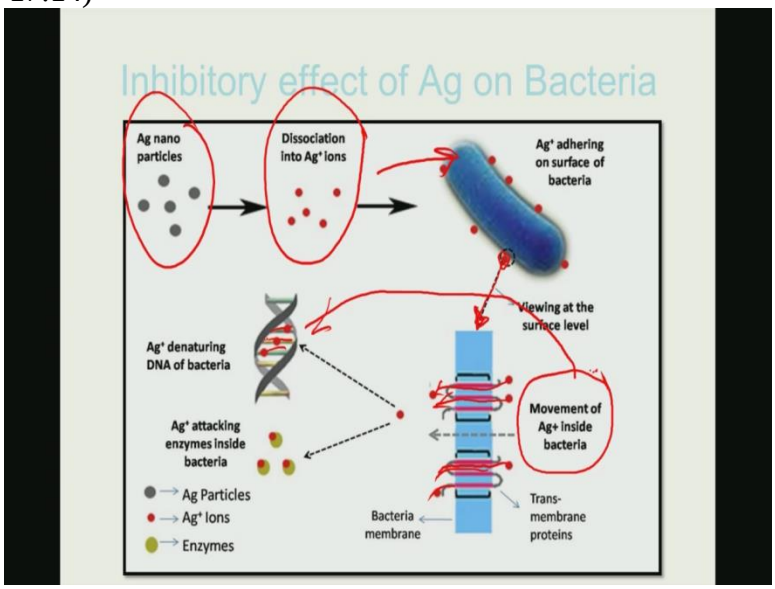

Now the question is that what is the innovatory role or how that silver can cause a reduction or can inactivate bacterial growth. Or why silver is so effective to reduce or to cause inactivation of the bacterial growth. So the theory what is present what is present or the way that we presently understand that bactericidal action of the silver is as follows:

Now silver nano particles become very reactive in the culture medium then it dissociates in the silver, now these silver ions are actually attacking that on the bacterial cell membrane. Now this depending on this how silver ions this is at the surface level, now these silver ions depending on the size of the silver ions like which is less than 100 nanometre, then what you can do, what they can do they can get inside the cytoplasm of this or cytosoul of this bacterial cells.

Now as you know that bacteria does not have a very well developed nucleus like eukaryotic cells instead the DNA is dispersed in this, in this in the in the cytoplasm itself, so these silver ions now these will be there is a possibility that silver also will cause the breakage of this base pairing

of this DNA. Now once the breakage of this base pairing of the DNA takes place; then that will denature this DNA of this bacteria.

And once bacteria is denatured then it cannot grow any further, that entire propagation of the bacteria will be affected. Another thing is that silver through this trans-membrane floating channel it can get inside into the bacterial cytoplasm or cytosoul of the bacteria, this has been schematically shown here. So the two to three things are important here, one is that silver ions getting attached to the bacterial cell membrane. Then they will be internalised into the bacterial cell through the trans-membrane protein channel.

And then they will add, then will get attached to the enzymes which are present in the bacterial cytoplasm. That enzyme consecrated battery of silver ions will be effectively kind of denaturing the DNA in terms of breaking the base pairing of the DNA and thereby that will disrupt the bacterial growth which otherwise can grow very happily in the normal culture conditions without any silver or without any silver containing biomaterials.

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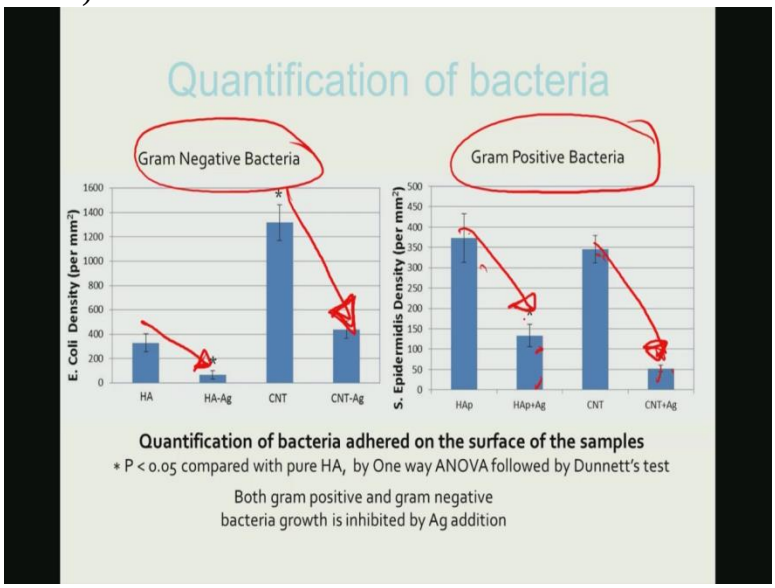

Now this has been shown that whatever earlier discussion or whatever earlier observation of the hydroxyapatite CNT silver and so on. What you see with respect to hydroxyl silver that silver adhesion is certainly effective, but it is much more effective and much more significant when we

compare that CNT with CNT silver. And you can very, it is very clearly shown here in this quantitative results.

So there is gram negative bacteria that is E. coli and gram positive bacteria that is S epdirmidis. Same story is true for hydroxyl hydroxyl silver and CNT with CNT silver. So CNT and CNT silver again, here the reduction is much more significant when you compare with the hydroxyapatite cell. So CNT as you know the carbon nanotube is used as a reinforcement to a multitude of materials for biomedical applications.

So when silver together with CNT can cause so much antimicrobial action, so they can, they this use of silver will now be expanded more to expanded more to incorporate CNT as a potential reinforcement to increase the elastic stiffness property A and B when added with silver that will also cause antimicrobial or bactericidal properties in this materials.

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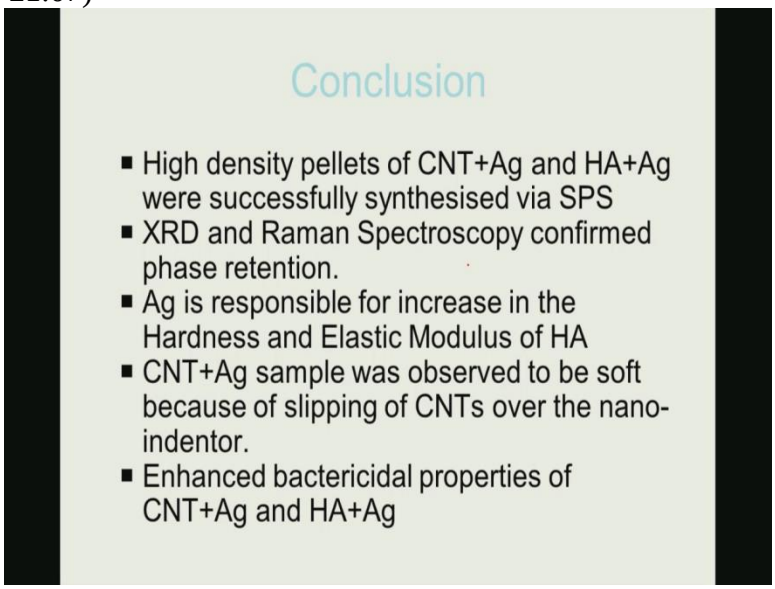

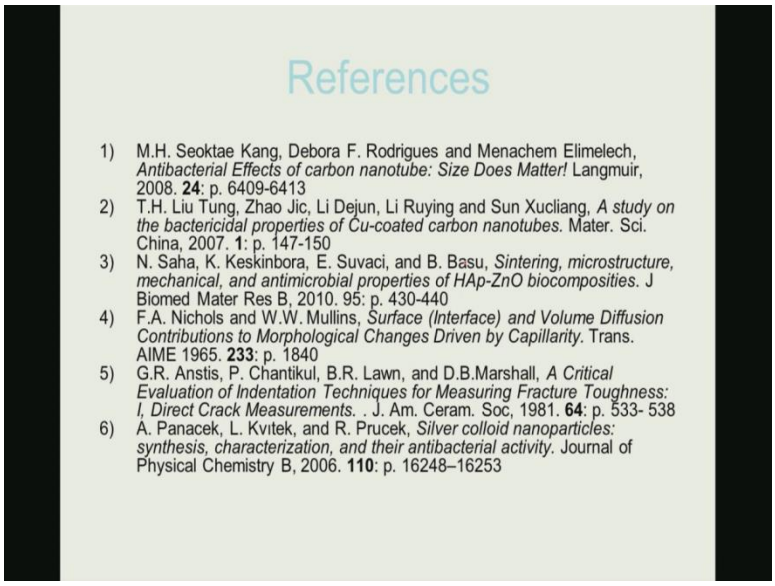

So in closure that you know that we have shown that CNT plus silver samples is extremely good as far as the bactericidal properties are concerned and then more references can be found in some of our some of our published as a results either from our group or from other groups elsewhere in the world.

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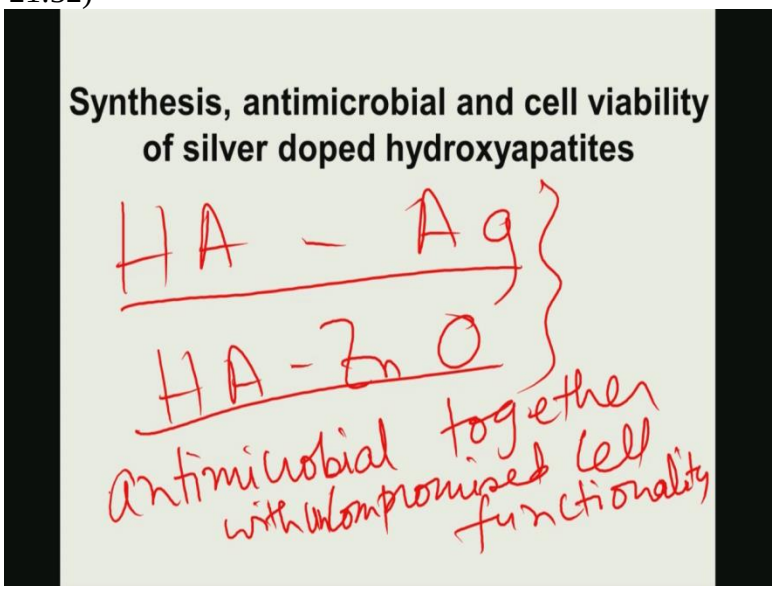

So with this I think I have now completed the the discussion on the antimicrobial properties like hydroxyl silver, hydroxyl zinc oxide and then I have shown this two case studies that how silver and zinc oxide can be effective to cause the bactericidal property in vitro and without

compromising cell functionality only with some specific compositions and this specific compositions can be further tested in the in vivo study or preclinical study further. Thank you.