Introduction to Biomaterials

Prof. Bikramjit Basu
Prof. Kantesh Balani
Department of Materials and Metallurgical Engineering
Indian Institute of Technology, Kanpur

Module No. # 01

Lecture No. # 24

Alternative Phosphate material HAp-based composites with bactericidal property and glass ceramics for dental restoration

In the last lecture, we have discussed fundamentals of sintering and properties of ceramic, bioceramic materials, particularly that, how this materials can be fabricated using the sintering techniques, and how their mechanical properties are different from that of metals, and what are the different toughening mechanisms, how you can characterize the ceramic materials, in terms of strength, toughness, hardness properties, elastic modulus properties and so on. Now, in this lecture, I will focus on the discussion of the, one of the new alternative Phosphate materials, that is Potassium Magnesium Phosphates. So, this material has been developed as an alternative to hydroxyapatite materials. Now, remember the hydroxyapatite has a typical formula is Ca 10 PO 4 whole 6 OH whole 2. Now, this hydroxyapatite, is one of the important Phosphate materials, which has been widely, now, recognized for biomedical applications. So, in next set of slides, I will show you that, how an alternative Phosphate materials can be developed, based on different inorganic composition, and how these materials can have equally good cytocompatible properties, like that of hydroxyapatite.

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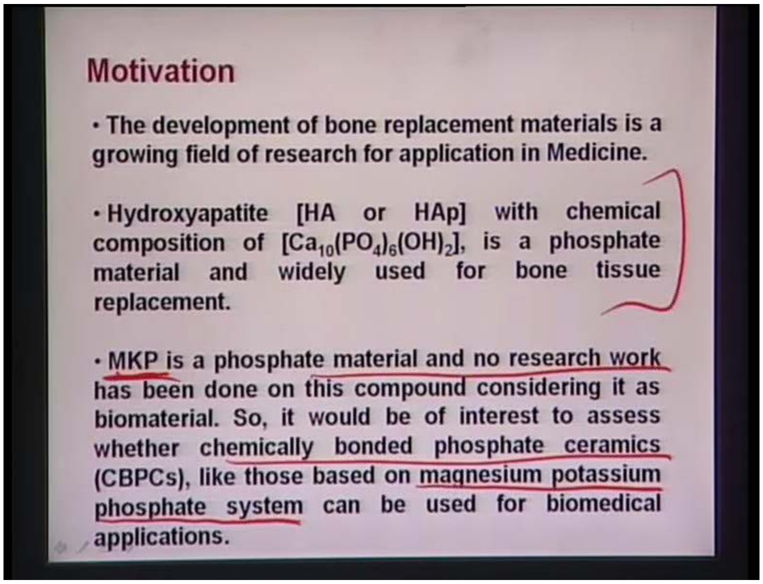

Motivation

- The development of bone replacement materials is a growing field of research for application in Medicine.
- Hydroxyapatite [HA or HAp] with chemical composition of $[Ca_{10}(PO_4)_6(OH)_2]$, is a phosphate material and widely used for bone tissue replacement.
- MKP is a phosphate material and no research work has been done on this compound considering it as biomaterial. So, it would be of interest to assess whether chemically bonded phosphate ceramics (CBPCs), like those based on magnesium potassium phosphate system can be used for biomedical applications.

So, as I said that, hydroxyapatite is an important bio-Phosphate materials. So, MKP, as in Magnesium Potassium Phosphate, is a Phosphate material and then, hardly any research has been carried out on these Phosphate materials for biomedical applications. And therefore, we have investigated this material in our laboratory and this particularly, chemically bonded Phosphate ceramics are essentially based on this Magnesium Potassium Phosphate systems. They have been used, that people have reported their use, particularly in nuclear waste removal applications; nothing to do with the biomedical applications, in the past.

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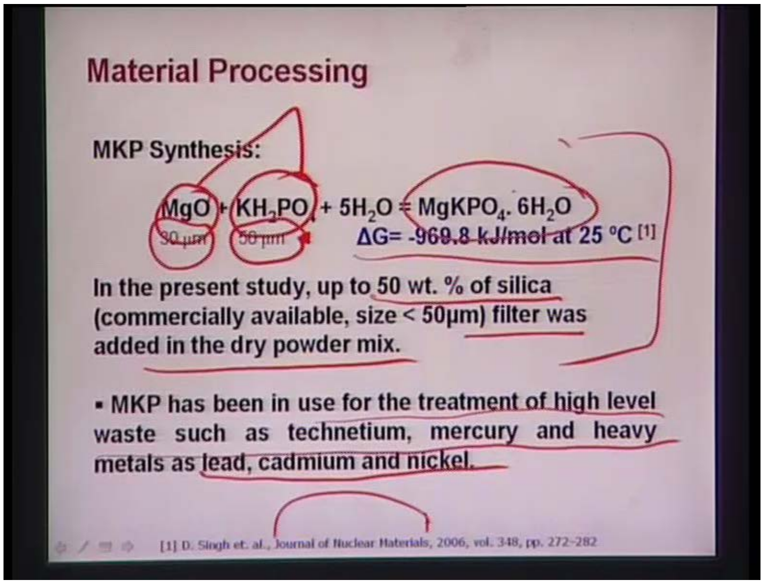

Material Processing

MKP Synthesis:

$MgO + KH_2PO_4 + 5H_2O = MgKPO_4 . 6H_2O$

30 µm 50 µm

$\Delta G = -969.8$ kJ/mol at 25 °C [1]

In the present study, up to 50 wt. % of silica (commercially available, size < 50µm) filter was added in the dry powder mix.

- MKP has been in use for the treatment of high level waste such as technetium, mercury and heavy metals as lead, cadmium and nickel.

[1] D. Singh et. al., Journal of Nuclear Materials, 2006, vol. 348, pp. 272-282

Now, how this material were prepared? You start with precursor powders, Magnesium oxide, then, KH 2 PO 4, and Magnesium oxide, they are mixed together in an aqua solution and then, you get this Magnesium Potassium Phosphate and this Magnesium Potassium Phosphate is an hydrated Magnesium Potassium Phosphate material. Now, delta G values, here, it is largely negative. So, it is almost like, minus 970 kilojoules per mole, at 25 degree Celsius; and now, if delta G value is negative, that means, this reaction is thermodynamically feasible, right and this is number one point. And, number two point is that, you can start with a fairly coarse Magnesium oxide particles and fairly, and you can mix together and this particular study has been taken, where 50 weight percent silica filter has been added in the dry powder mix. Now, why silica has been added to this powder mixer? The silica has been added to, added to this Magnesium oxide and KH 2 PO 4, so that, silica essentially acts as an chemical bonding agent; so that, silica will bond this Magnesium Potassium Phosphate H 2 O in the particular ceramic matrix and this has, as I said that, this has been already used in the treatment of high level nuclear waste like technetium, mercury, or heavy metals like lead cadmium and nickel. So, this has been already published in Journal of Nuclear Materials, by group of researchers from Oregon National Laboratory, United States and therefore, this is the particular, you know, processing technique, that has been developed and which has been used, to make this Magnesium Phosphate; but remember, this has 6 H 2 O. So, it is like a hydrated Magnesium Potassium Phosphate materials.

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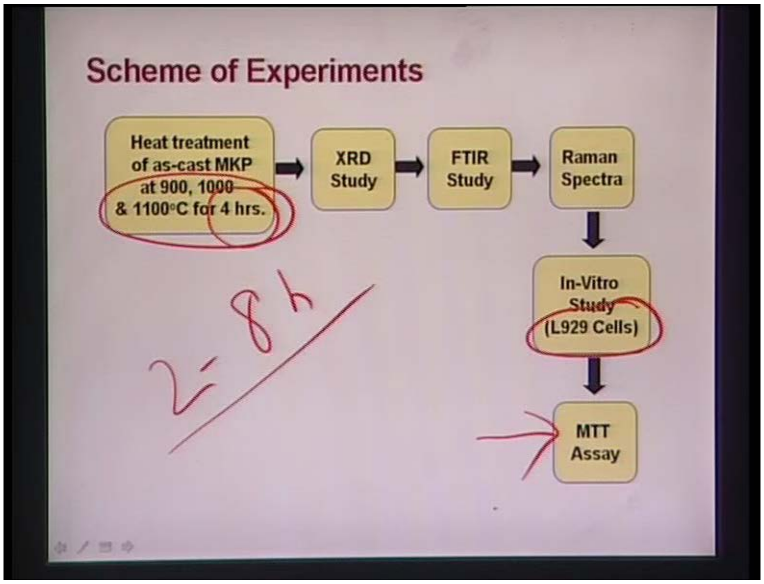

Scheme of Experiments

Heat treatment of as-cast MKP at 900, 1000 & 1100°C for 4 hrs.

XRD Study

FTIR Study

Raman Spectra

In-Vitro Study (L929 Cells)

MTT Assay

2-8h

Now, what we have done, then, in the as-cast, first, we have done this materials, we have processed these materials using slip casting; after the slip casting, we have done the heat treatment of this material at different temperatures and these temperatures were chosen, somewhere around 900 to 1100 degree Celsius, and heat treatment time was initially optimized and then, optimized time was 4 hours. And, how this heat treatment time is optimized? Initially, researchers have carried out the experiments from 2 to 8 hours at regular interval of 2 hours. So, 2 hours, 4 hours, 6 hours, or 8 hours; then, they found that, 4 hours is the optimum heat treatment time and therefore, further experiments were carried out at this temperature range, for 4 hours; XRD was taken, to find out that, what are the different phase assemblage; FTIR was essentially to find out that, whether our Phosphate group is there, or which minus vibration peak is there, or not. Raman spectra has been used, or Raman spectroscopy results were used to provide complimentary information to the XRD result, so that, you know, you can confidently say that presence of certain phases. Cytocompatibility was studied using that L929 cell lines, and also MTT assay was carried out to quantify the cell viability of these materials.

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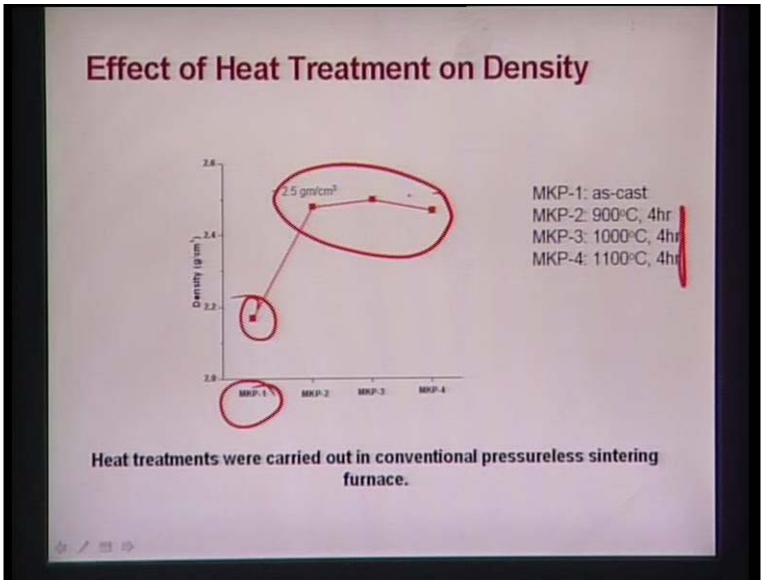

Effect of Heat Treatment on Density

MKP-1: as-cast
MKP-2: 900°C, 4hr
MKP-3: 1000°C, 4hr
MKP-4: 1100°C, 4hr

Heat treatments were carried out in conventional pressureless sintering furnace.

Now, as I said that, these materials were heat retreated at three different temperatures for 4 hours, as you see that, MKP 1 has a very low density and that is because of the high level of porosity in the structure. Now, when you heated them at 900 to 1100 degree Celsius, this material has a largely equal density, almost like identical density, which is close to around 2.5 gram per c c. Now, 2.5 gram per c c density, essentially tells you that, this material has a very low density and this dense, low density materials can be obtained in this Magnesium Phosphate material. As I said in the beginning of the, this course that, typically density is also one of the important factor; if the density is large, then, the patient will feel quite heavy and because of the large weight implants; but if the density is low, then, the patient will find also, you know that, this very light-weight implant materials. So, that is the reason that, you know, density was very low; the density, low density materials is preferred.

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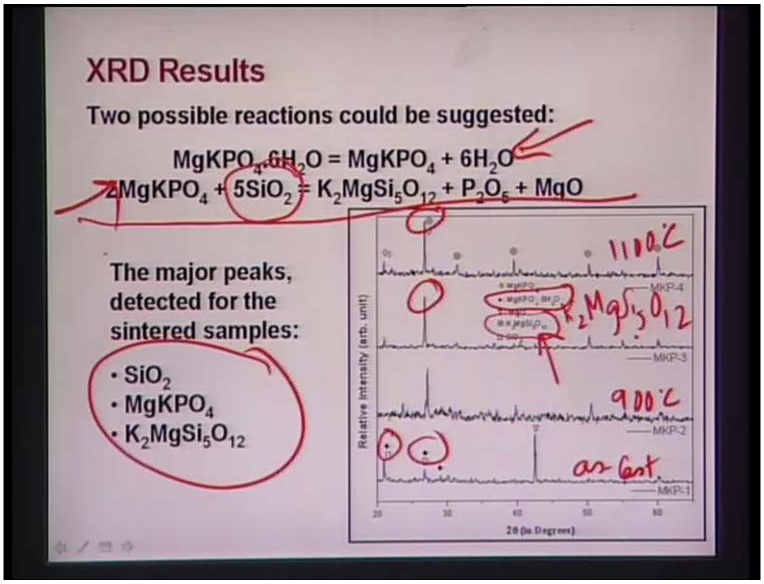

XRD Results

Two possible reactions could be suggested:

$$MgKPO_4.6H_2O = MgKPO_4 + 6H_2O$$
$$2MgKPO_4 + 5SiO_2 = K_2MgSi_5O_{12} + P_2O_5 + MgO$$

The major peaks, detected for the sintered samples:

- SiO_2
- $MgKPO_4$
- $K_2MgSi_5O_{12}$

Now, XRD results, essentially tells you that, there are peaks, which corresponds to K 2 Mg Si 5 O 12. So, this is the K 2 Mg Si 5 O 12. Now, this peaks are the most intense peaks here; that is, the 100 percent intense peaks; also, Mg K P O 4 or Mg K P O 4 6 H 2 O peaks are present in that as-cast conditions. So, this is like, your as-cast phase and this is like 900 degrees Celsius inter, this is 1000, this is 1100 degree Celsius heat treated conditions. So, these are like, three different temperatures these materials were heat treated. And then, what you see essentially that, Mg that, this peaks that Mg K P O 4 6 H 2 O has disappeared in this, when you are sintering at higher temperature, and then, the large silicate peaks that K 2 Mg Si 5 O 12 has been formed. So, that shows that, Mg K P O 4 must have reacted with your silica, which is present in large amount in the starting powder mix, to form this particular silicate phase. Now, this is the reaction we have proposed, that which essentially tells you that, why K 2 Mg Si 5 O 12 phase is formed and then, what should be the reaction and that reaction has also involved Si O 2 phase. The major phase that has been detected for this samples, which are already identified Si O 2, that comes from the precursor powder mix, Mg K P O 4, because that H 2 O has been released because of the heat treatment conditions, at a high temperature and then, K 2 Mg Si 5 O 12, that comes from these particular, this particular reactions, that is between Si O 2 and Mg K P O 4.

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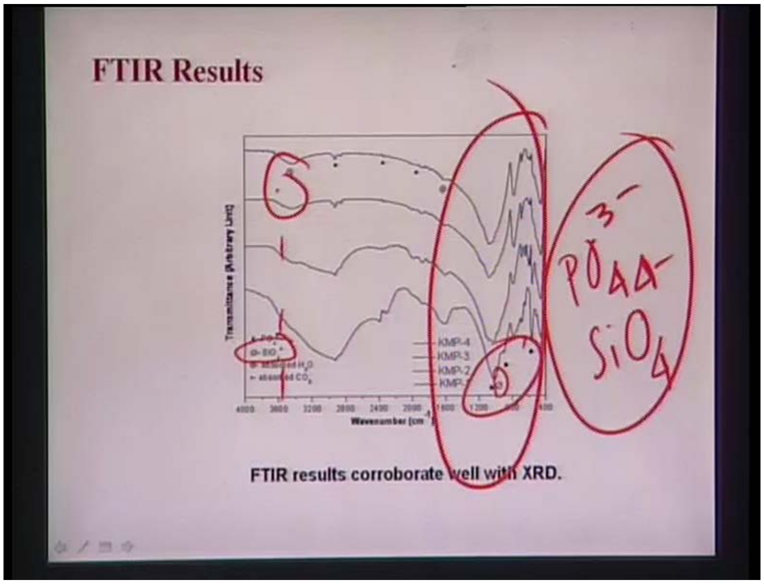

FTIR Results

FTIR results corroborate well with XRD.

Now, FTIR results, essentially tells you that, you have this phosphates peak in this material and also, you have some OH minus vibration peak, which is roughly around here. So, this is your OH minus vibration peak, essentially tells you that, this material has some hydrated phosphate phase and this phosphate phase essentially indicates that, you know, you have that Mg K P O 4 also, because, you have the silicate phase you have also Mg K P O 4 phase also. Silicate peak essentially, is coming from this particular place. So, this is also, that your silicate phase is there; this is phosphate and silicate, they almost like overlaps. So, P O 4 minus 3 and Si O 4 4 minus, these peaks, this FTIR bands overlaps and essentially, this region indicates that, you have a phosphate peak, as well as the silicate peak also. And, if you go back and correlate with your XRD results, then, that correlates well, because that correlation sounds very perfect, because you have this K 2 Mg Si 5 O 12, that is the silicate phase and also, you have Mg K P O 4, that is also the phosphate phase. So, essentially, from FTIR also, you should essentially find that, there the presence of the phosphate peak, as well as the silicate peak, and exactly, that is what you have seeing in this FTIR graph.

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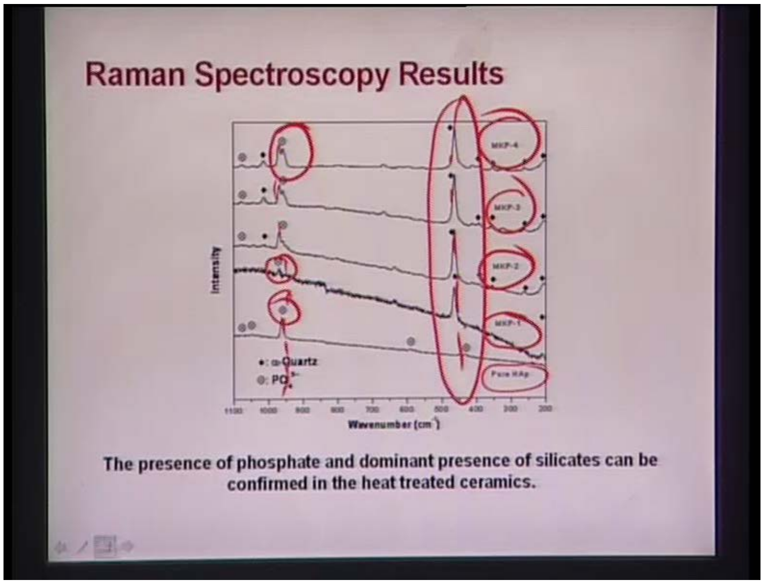

Raman Spectroscopy Results

The presence of phosphate and dominant presence of silicates can be confirmed in the heat treated ceramics.

Now, Raman spectroscopic results, essentially shows you that, pure hydroxyapatite, you have a phosphate peak here, and in this Mg K P O 4 phase also, that in the starting powder itself, or in the, prior to the heat treatment, you have this MKP 1 and then, prior to the heat treatment, your peak intensity is fairly low. But, when you are heat treating at 900, 1000 and 1100 degree Celsius, then your phosphate peaks also becomes more prominent, and therefore, this phosphate peaks are in line with their phosphate peaks of your Calcium Phosphate Calcium hydro[xyapatite]- Ca 10 P O 4 whole 6 OH whole 2, that is your hydroxyapatite phase. So, this, the presence of this phosphate peak, is quite important here. The other important thing is that, alpha quartz; the alpha quartz, it comes from your silica peak, and this alpha quartz phase also present in this MKP 1 to MKP 4 materials, and that is, even after the heat treatment also, you have the significant amount of silica in the materials.

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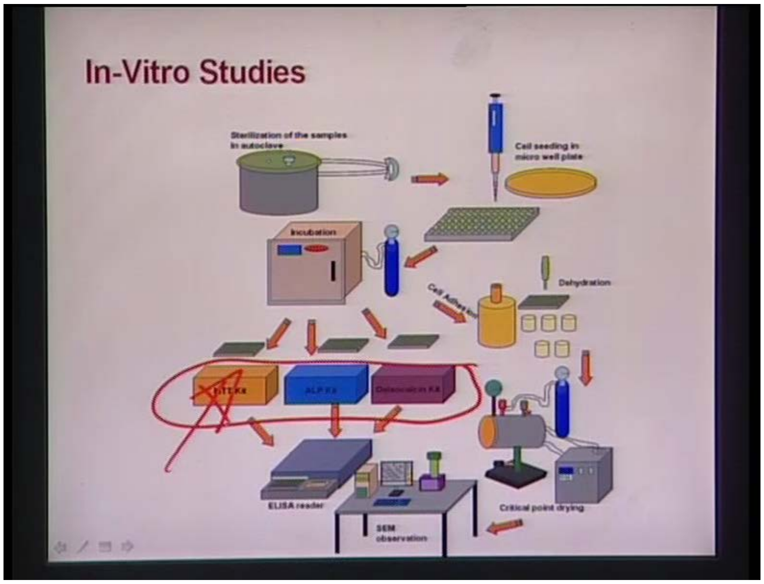

In-Vitro Studies

Now, as I have shown you earlier that, during this in-vitro biocompatibility assessment that, this standard protocol is followed in our laboratories, to find out the cell culture studied, to assess the cell culture behavior, as well as, we have used also standard biochemical access and that is the combination of MTT ALP and osteocalcin. So, this standard biochemical assess has been also used to find out, but in this particular case, we have only used that MTT, just to quantitatively state that, what is the cell (( )) adhesive property of these materials.

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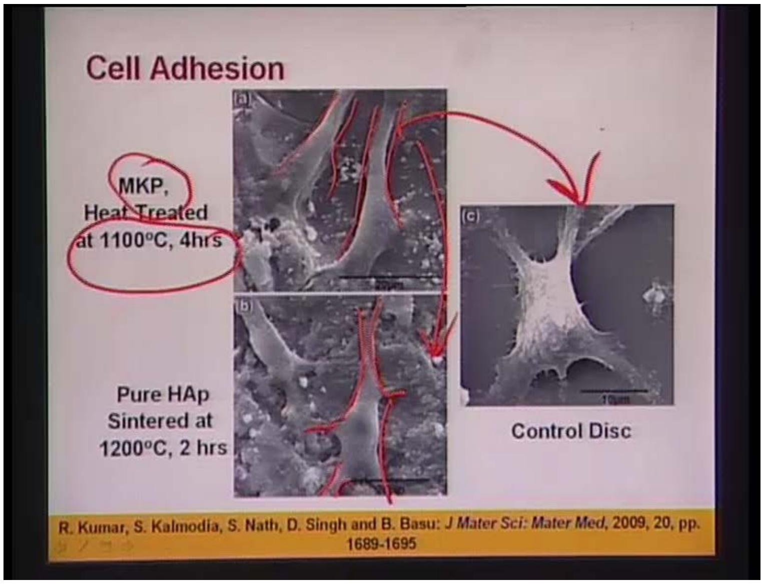

Cell Adhesion

MKP, Heat Treated at 1100°C, 4hrs

Pure HAp Sintered at 1200°C, 2 hrs

Control Disc

R. Kumar, S. Kalmodia, S. Nath, D. Singh and B. Basu: *J Mater Sci: Mater Med*, 2009, 20, pp. 1689-1695

Now, this cell adhesion property, what you are seeing here, here, you can clearly see that, this MKP, that is Magnesium Potassium Phosphate, which is heat treated at 1100 degree Celsius for 4 hours, this is the L929 cells and you can see the filopodia extension very clearly in this material; now, if you compare with the this and these, what you have seen in pure hydroxyapatite also, that your filopodia extension and the cell adhesion property is quite similar, or it is very much comparable and as comparable with the control disc. So, therefore, you can qualitatively say, not quantitatively, that, MKP material which you have, which we have developed, they have comparable cell adhesion property, as like that of the hydroxyapatite.

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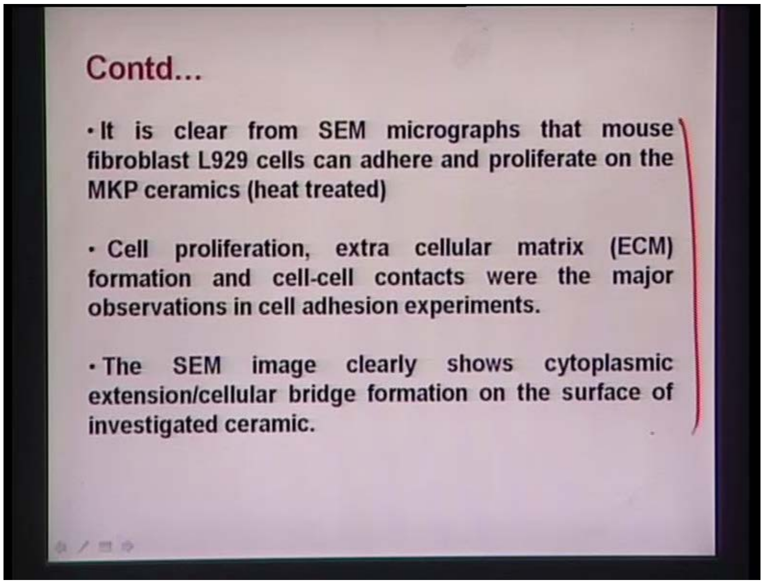

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- It is clear from SEM micrographs that mouse fibroblast L929 cells can adhere and proliferate on the MKP ceramics (heat treated)
- Cell proliferation, extra cellular matrix (ECM) formation and cell-cell contacts were the major observations in cell adhesion experiments.
- The SEM image clearly shows cytoplasmic extension/cellular bridge formation on the surface of investigated ceramic.

So, this is some comments based on the, actually, what I have just told you; we have further carried out that MTT assay, and this MTT assay as you know that, it quantitatively state you that, what is the cell viability property in this materials.

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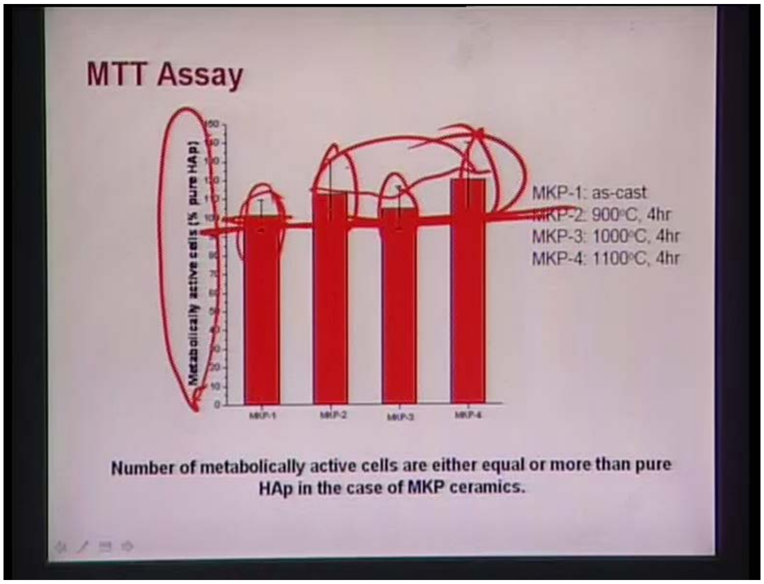

MTT Assay

Number of metabolically active cells are either equal or more than pure HAp in the case of MKP ceramics.

So, this is prior to the heat treatment; this is 900, 1000 and 1100 degrees Celsius; and, this is metabolically active cells; that means, your MTT reagent actually reacts with the mitochondria of the cells and that gives you that, what is the total number of metabolically active cells present on the material; and then, what you see, their error bars actually overlap with each other. Now, if the error bars overlap with each other, that shows that, they have a statistically similar level of MTT values; there is no statistically significant difference among all these materials. And also, it tells you that, since percentage pure HAp is plotted along the y axis, so, it is comparable with that of pure hydroxyapatite, that MTT values, or in some materials, it is little higher than that of the pure hydroxyapatite values. So, that is very good, because you are developing, you are proposing an alternative phosphate material, where the MTT values, after culturing with L929 cells, that shows like, you know, good cytocompatibility property, and as well as good cell viability property, like that of pure hydroxyapatite.

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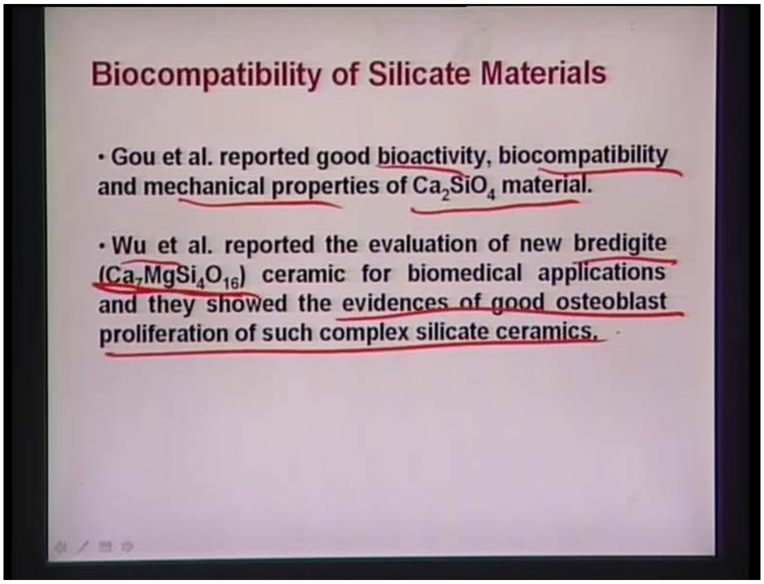

Biocompatibility of Silicate Materials

- Gou et al. reported good bioactivity, biocompatibility and mechanical properties of Ca_2SiO_4 material.
- Wu et al. reported the evaluation of new bredigite ($Ca_7MgSi_4O_{16}$) ceramic for biomedical applications and they showed the evidences of good osteoblast proliferation of such complex silicate ceramics.

Now, why we think that, you know that, these materials have a good biocompatibility property? Now, there are some literature reports, mostly from Chinese researchers and then, what did they have found, that, for the Ca 2 Si O 4, that calcium silicate materials, they have reported good bioactivity and biocompatibility and mechanical properties. Also, another group led by Wu and they have also reported that, there is a one particular complex silicate phase, that Ca 7 Mg Si 4 O 16, and that ceramic for biomedical application, because this ceramic showed evidences of the good osteoblast proliferation; and, from this literature, we believe that, the our materials which has a combination of the Magnesium Phosphate phase and silicate phase. Now, phosphate phase is already well known that, it should have a good biocompatibility property; that you know from your Ca 10 P O 4 whole 6 OH whole 2, because hydroxyapatite has good biocompatibility property. So, we are little bit worried that, because this has additional silicate phase, that K 2 Mg Si 5 O 12, that whether the presence of the additional silicate phase will degrade the MTT values, or will degrade the cytocompatibility property or not; but what our results show that, you know, the additional presence of the silicate, in addition to phosphate, essentially gives you comparable cytocompatibility, comparable MTT values, like that of the pure hydroxyapatite.

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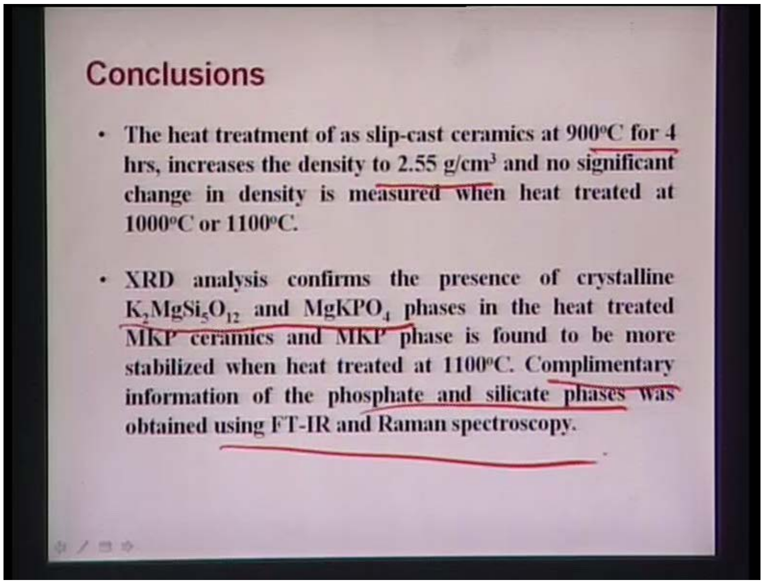

Conclusions

- The heat treatment of as slip-cast ceramics at 900°C for 4 hrs, increases the density to 2.55 g/cm^3 and no significant change in density is measured when heat treated at 1000°C or 1100°C.
- XRD analysis confirms the presence of crystalline $K_2MgSi_5O_{12}$ and $MgKPO_4$ phases in the heat treated MKP ceramics and MKP phase is found to be more stabilized when heat treated at 1100°C. Complimentary information of the phosphate and silicate phases was obtained using FT-IR and Raman spectroscopy.

So, these are the conclusions from this part of the study, that is the heat treatment at 900, 4 hours and little bit above it, gives you a density, low density alternative phosphate materials and this density has around 2.5 gram per c c. XRD analysis confirms the presence of this K 2 Mg Si 5 O 12, as well as the Magnesium Potassium Phosphate phase in these materials; and then, complimentary information on the Phosphate and silicate phases were also obtained by FTIR, that is Fourier Transform Infra Red Spectroscopy, as well as the Raman spectroscopy.

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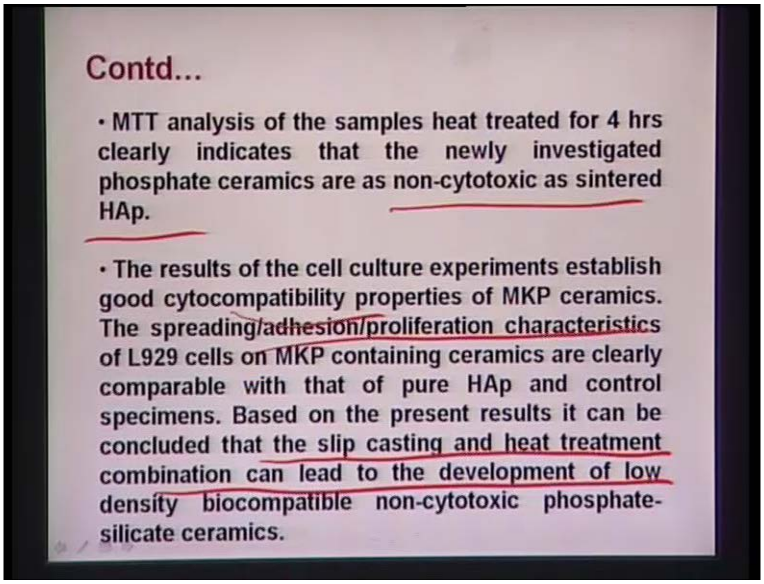

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- MTT analysis of the samples heat treated for 4 hrs clearly indicates that the newly investigated phosphate ceramics are as non-cytotoxic as sintered HAp.
- The results of the cell culture experiments establish good cytocompatibility properties of MKP ceramics. The spreading/adhesion/proliferation characteristics of L929 cells on MKP containing ceramics are clearly comparable with that of pure HAp and control specimens. Based on the present results it can be concluded that the slip casting and heat treatment combination can lead to the development of low density biocompatible non-cytotoxic phosphate-silicate ceramics.

And, the MTT analysis of the samples, that indicates that, you know, this newly investigated phosphate ceramics are non-cytotoxic, as sintered hydroxyapatite. And, on the basis of this good cytocompatibility, spreading and adhesion, and proliferation characteristics, we believe that, the combination of the slip casting and the heat treatment combination, can lead to development of low density bio-composite with non-cytotoxic properties.

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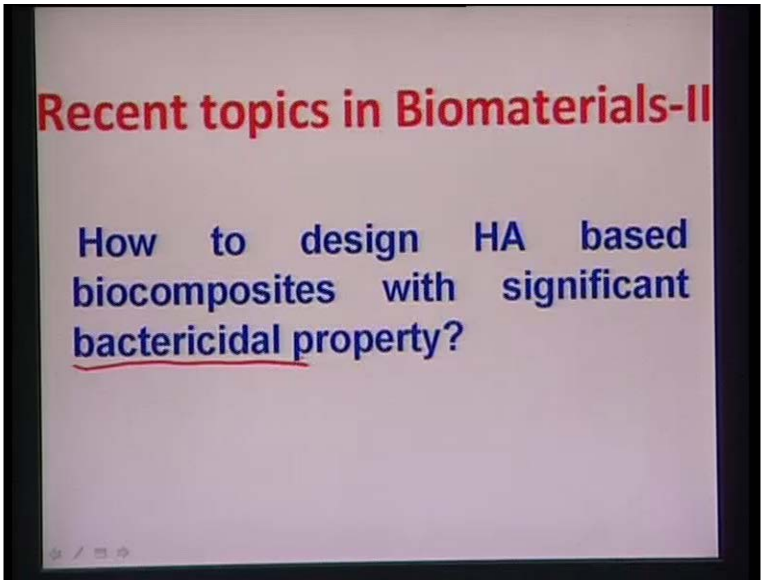

Recent topics in Biomaterials-II

How to design HA based biocomposites with significant bactericidal property?

Today I will be showing you some slides, which will demonstrate, as how to design the hydroxyapatite based bio-composites with significant bactericidal property. If you recall that, hydroxyapatite has two major problems; one is the brittleness, that is the low crack growth resistance, or very low fracture toughness; and, then, second one is that, it has lack of bactericidal properties, or it has lack of antimicrobial property. So, therefore, there is indeed, a need to improve the antimicrobial property, as well as the fracture toughness properties in this materials.

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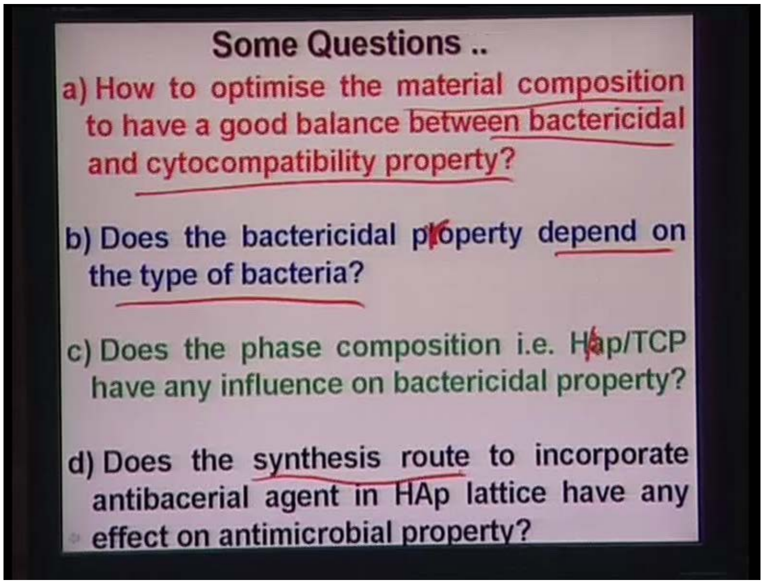

Some Questions ..

a) How to optimise the material composition to have a good balance between bactericidal and cytocompatibility property?

b) Does the bactericidal property depend on the type of bacteria?

c) Does the phase composition i.e. HAp/TCP have any influence on bactericidal property?

d) Does the synthesis route to incorporate antibacerial agent in HAp lattice have any effect on antimicrobial property?

Now, there are some questions that will be answered with the help of our experimental results. The first one is that, how to optimize the material composition to have a good balance between the bactericidal and cytocompatibility property? Bactericidal means, it will kill bacteria, by…Cytocompatibility means that, it will not have any cytotoxicity effect and it will, the cell will proliferate, or cell-fate processes will be enhanced in contact with these materials. Second one, does the bactericidal property also depend on, does the bactericidal property depend on the type of bacteria. Type of bacteria means, there are two types of bacteria; one is that gram negative bacteria; one is gram positive bacteria. Now, the question is that, whether a given material, which has good bactericidal property, or antimicrobial property against gram negative bacteria, whether the same material will have similarly good bactericidal properties against gram positive bacteria or not; that question also needs to be answered.

The third one is that, does the phase composition, does the phase composition, that is the HAp TCP, or the mixture of HAp and TCP, have any influence on bactericidal property? Fourth one is that, does the synthesis route to incorporate antibacterial agent in HAp lattice have any effect on the antimicrobial property? Now, this synthesis route can be, either you can mix this two powders, hydroxyapatite with any antimicrobial agent by mechanical mixing route, or you can mix them via or you can dope, dope hydroxyapatite antimicrobial agent by chemicals synthesis route. In both the cases, it will be shown that, how antimicrobial property can be improved.

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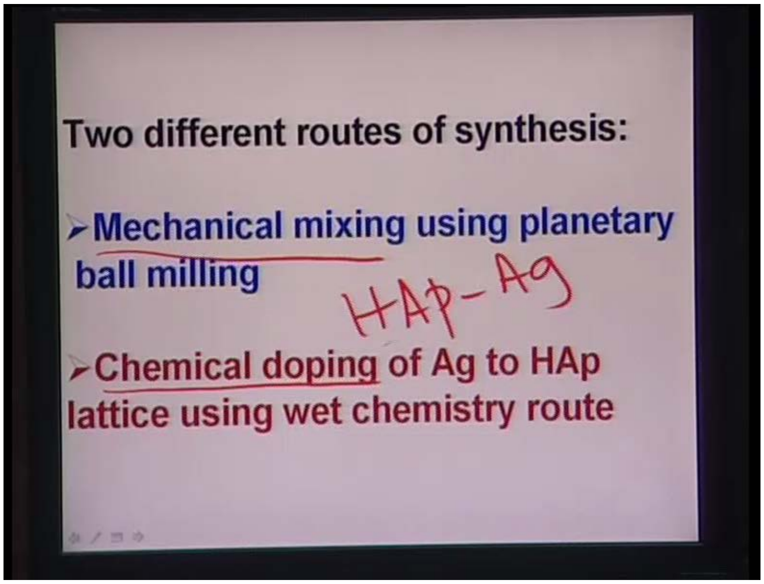

Two different routes of synthesis:

- Mechanical mixing using planetary ball milling
- Chemical doping of Ag to HAp lattice using wet chemistry route

HAp-Ag

Now, there are two different routes of synthesis; one is the mechanical mixing using planetary ball milling and second one, is the chemical doping of silver to hydroxyapatite lattice, using wet chemistry route; and in this both cases, it will be shown that, for the same HAp silver system, now, it will be shown for the hydroxyapatite silver system that, how both these routes can equally impart good antimicrobial property in this system.

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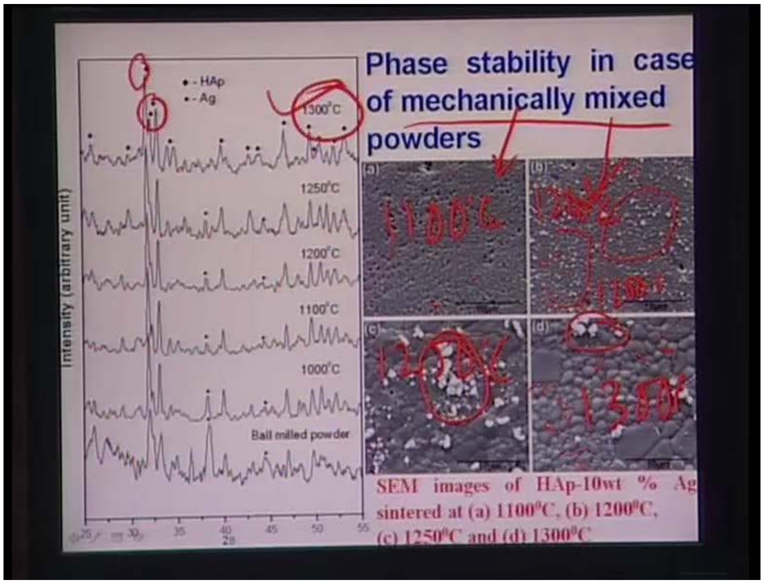

Phase stability in case of mechanically mixed powders

SEM images of HAp-10wt % Ag sintered at (a) 1100°C, (b) 1200°C, (c) 1250°C and (d) 1300°C

This is, the first one is the mechanically mixed powders. So, you take hydroxyapatite with 10 percent silver, and then, you mix it in a planetary ball milling and thereby, after that, you sinter them at different temperature, right up to 1300 degrees Celsius. What interestingly you see in the XRD fraction plot that, this hydroxyapatite does not dissociate to tri-Calcium Phosphate, even at 1300 degrees Celsius. Because, you can see characteristics peaks of, (( )) major character peaks of hydroxyapatite, which are present very well at high temperature of 1350 degrees Celsius. Now, if you look this SEM image, what you see at 1200 degree Celsius, it is more or less porous microstructure; at 1250 and 1300, you can see that silver distribution and this silver distribution, silver particulars are dispersed more or less homogeneously in this b, that is the 1200 degree Celsius.

So, this is at 1200 degrees Celsius; this is at 1100 degree Celsius; this is at 1250 degree Celsius, and this is at 1300 degree Celsius. Now, you do notice certain differences in the distribution of the silver, or the shape, or morphology of the silver. What is this difference? At 1250 degree Celsius, you see that, silver clusters are forming, or silver are forming some aggregates in the 1250 degree Celsius. However, at 1300 degree Celsius, what you see that, silvers are clearly distributed along the grain boundary region. Do you notice that, you know, bright silver phase, they are clearly distributed in the grain boundary region. So, these are the, like a distribution part. At 1200 degree Celsius, what you see here that, this silvers are homogeneous distributed as individual particulates, not essentially at the grain boundaries, but at different locations, microstructure locations within the grain.

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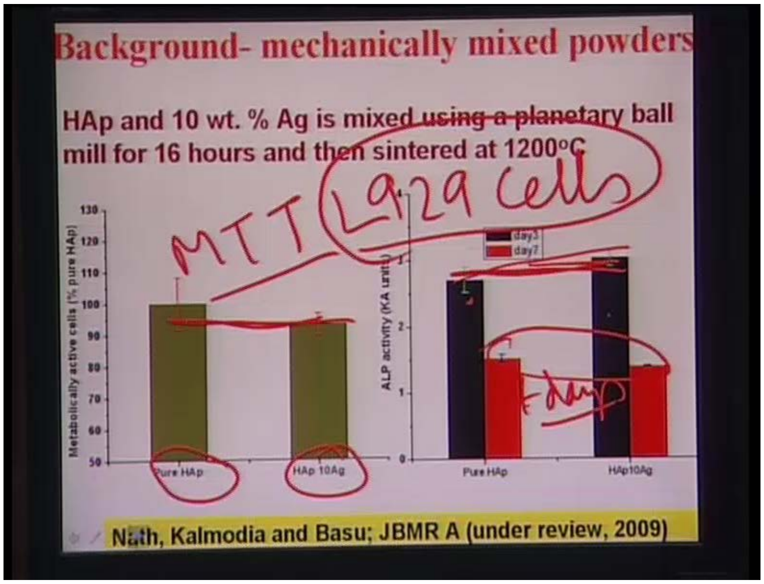

Background- mechanically mixed powders

HAp and 10 wt. % Ag is mixed using a planetary ball mill for 16 hours and then sintered at 1200°C

Nath, Kalmodia and Basu; JBMR A (under review, 2009)

Now, we have done some initial study on the cytocompatibility, or the cell-fate processes. Now, first one, we have done the MTT test. As you know, this MTT test is largely used for cell viability, or it is for assessing the cell viability. Now, here, we have done the pure hydroxyapatite and HAp 10 percent silver. Now, what you notice that, their error was slightly overlap with each other; so, that means, that as far as the cell viability is concerned, the 10 percent silver does not affect to any statistically significant manner, as in terms of the cell viability property. In other words, the cells are as viable in the hydroxyapatite, like the way they are as viable in the hydroxyapatite 10 percent silver.

The second one is that ALP. Now, ALP activity, Alkaline Phosphate activity, what you see here, this is pure hydroxyapatite after 3 days and this is hydroxyapatite 10 percent silver. In both the cases, again this error was overlapped, roughly overlap with each other. So, you do not see any statistically significant difference. The similar thing you have also noticed that the 7 days; so, red ones are 7 days. So, after the 7 days of culture of this, all these results are obtained with L929, that is mouse fibroblast cells. So, after 3 days and 7 days, the culture of that ALP and then, osteogenic differentiation activity states that, what you find, that is no difference that you can find which is statistically important, when you add 10 percent silver to the hydroxyapatite.

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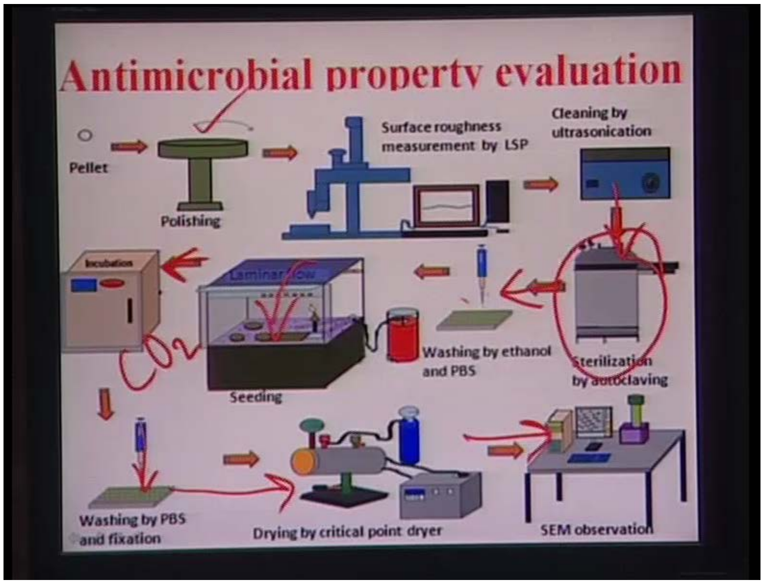

Antimicrobial property evaluation

We have done this antimicrobial property, which is the key property that was investigated, that was investigated in this materials. Now, what you do in antimicrobial property that, what were the protocols that were used in antimicrobial property is that, you start with this polishing the pellets; then, you do this LSP, rap surface roughness measurement, this ultrasonication; subsequently, you do the autoclaving; after the autoclaving, you wash by ethanol as well as PBS, that is Phosphate Buffer Solution. Then, you put it in the vertical laminar flow; then you seed the bacteria that you want to see; like, you can, in our experiments, we have used three types of bacteria; one is the gram negative, that is the Ecoli bacteria (( )); two are the gram positive bacteria; they are staphylococcus epidermitis and staphylococcus aureus; and, after seeding this bacteria there, then, we can incubate and this is CO 2 incubator; and in this CO 2 incubator, we will leave this bacterial culture solution for 4 hours, roughly, and after four hours, again you have to fix this bactericidal cells on the materials and this fixing is done by PBS again, and then, serial dilution and then, you can dry it by critical point dryer and after critical point dryer, you can use either (( )) microscopy, or standing electron microscope.

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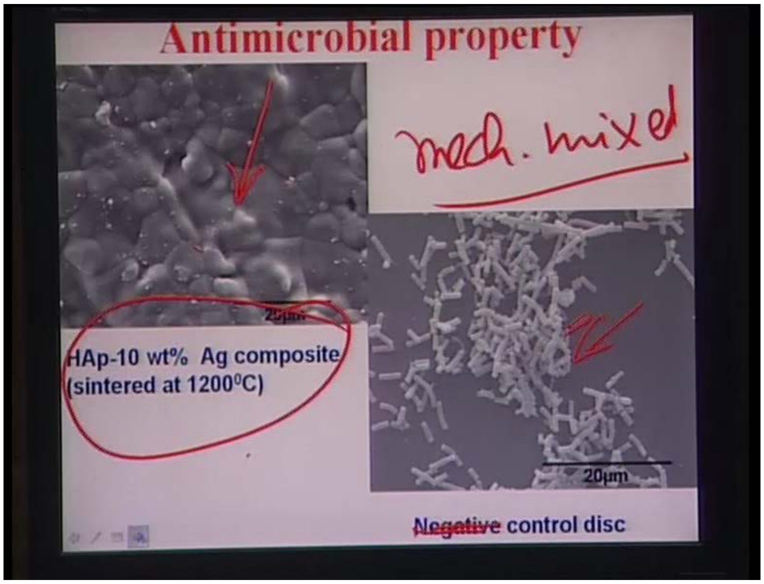

Antimicrobial property

mech. mixed

HAp-10 wt% Ag composite
(sintered at 1200⁰C)

~~Negative~~ control disc

Now, this is the antimicrobial property of the mechanically mixed powder. This is that, you know, planetary, planetary ball milled powder; after that, you sintered them. Now, this is the e-coli bacteria, rod shaped e-coli bacteria; as you can see, on the negative control disc and you have this hydroxyapatite 10 percent silver composite which is sintered at 1200 degree Celsius; and after sintering, you can see that, after culturing the same e-coli bacteria for 4 hours, you do not see any bacteria which is adhering on this materials, which it shows, it shows that, you can only see the remnants of the bacteria, or the residue of the bacteria, after they are being killed. So, therefore, hydroxyapatite 10 percent silver composite has good antimicrobial property.

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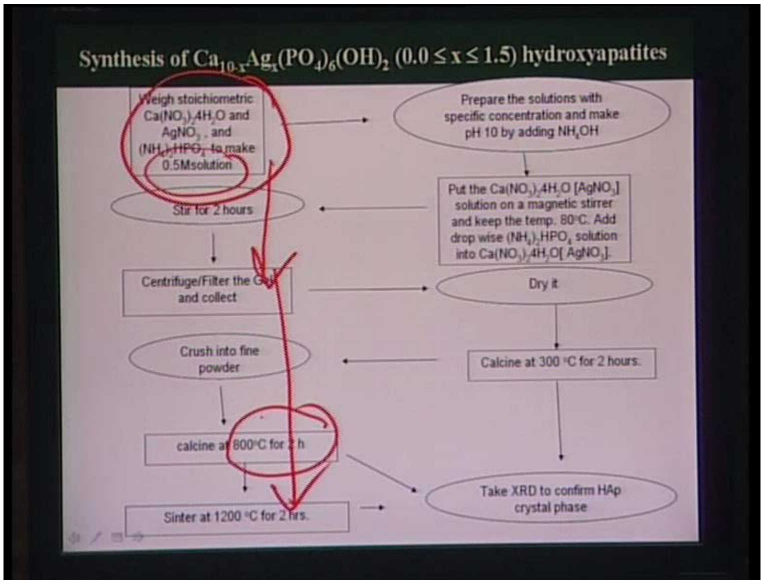

Synthesis of $Ca_{10-x}Ag_x(PO_4)_6(OH)_2$ ($0.0 \leq x \leq 1.5$) hydroxyapatites

Weigh stoichiometric $Ca(NO_3)_2 4H_2O$ and $AgNO_3$, and $(NH_4)_2HPO_4$ to make 0.5Msolution

Prepare the solutions with specific concentration and make pH 10 by adding NH_4OH

Put the $Ca(NO_3)_2 4H_2O$ [$AgNO_3$] solution on a magnetic stirrer and keep the temp. 80°C. Add drop wise $(NH_4)_2HPO_4$ solution into $Ca(NO_3)_2 4H_2O$[$AgNO_3$].

Stir for 2 hours

Centrifuge/Filter the Gel and collect

Dry it

Calcine at 300 °C for 2 hours.

Crush into fine powder

calcine at 800°C for 2 h

Sinter at 1200 °C for 2 hrs.

Take XRD to confirm HAp crystal phase

Now, we have done this chemical silver (( )) hydroxyapatite. We have also carried out the synthesis of silver (( )) hydroxyapatite using chemical synthesis route. And, using chemical synthesis route, what we have done, we have started with this precursor powders, that is Calcium Nitrate 4 H 2 O, Silver Nitrate and NH 4 whole 2 H P O 4 and to make this 0.5 molar solution, then, we have adopted the typical chemical synthesis route. And then, subsequently, we sintered them at 1200 degrees Celsius for 2 hours. And, this 1200 degrees Celsius, 2 hours after sintering, then, you do the XRD to confirm the hydroxyapatite crystal phase, as well as ACM analysis, etcetera, and in all this cases, we have used that chemically, high purity chemical precursors, and then, make sure that we calcine at 800 degree Celsius, 2 hours, so that, you get (( )) pure hydroxyapatite silver powder.

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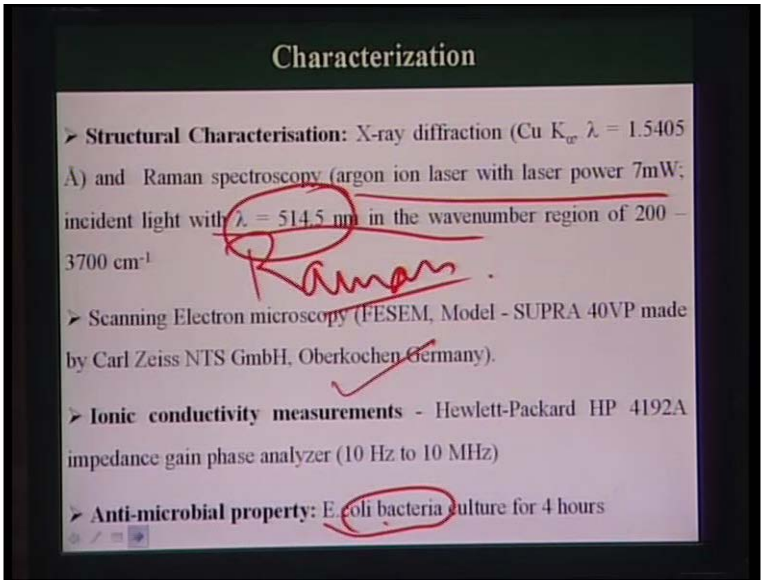

Characterization

- **Structural Characterisation:** X-ray diffraction (Cu K_{α}, λ = 1.5405 Å) and Raman spectroscopy (argon ion laser with laser power 7mW; incident light with λ = 514.5 nm in the wavenumber region of 200 – 3700 cm^{-1}
- Scanning Electron microscopy (FESEM, Model - SUPRA 40VP made by Carl Zeiss NTS GmbH, Oberkochen Germany).
- **Ionic conductivity measurements** - Hewlett-Packard HP 4192A impedance gain phase analyzer (10 Hz to 10 MHz)
- **Anti-microbial property:** E.coli bacteria culture for 4 hours

Now, apart from XRD fraction, we have done this, the ionic conductivity measurements, as well as Raman spectroscopy. And, Raman spectroscopy, we have done at, using Argon ion laser, with the laser power typically kept at 7 milliwatts and incident light has an wavelength of 514.5 nanometer; and then, we have done this antimicrobial property with e-coli bacteria, as well as other bacteria, as I have mentioned already.

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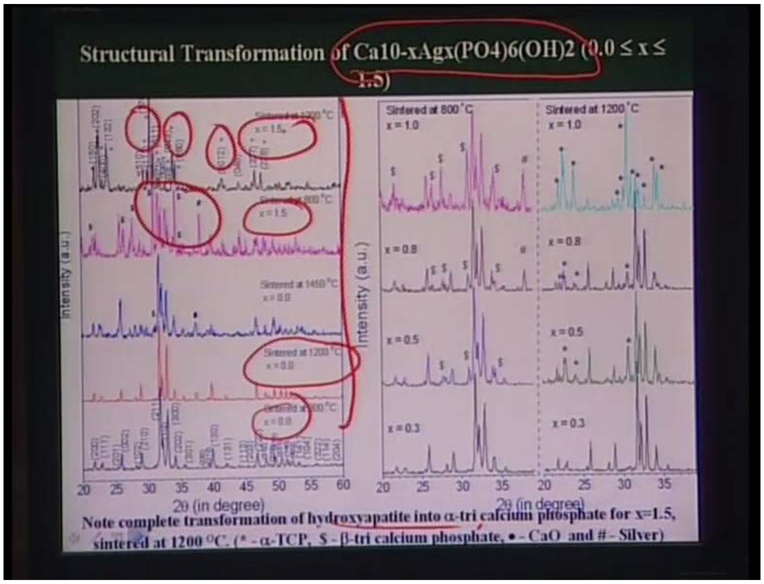
Structural Transformation of Ca10-xAgx(PO4)6(OH)2 (0.0 ≤ x ≤ 1.5)



Note complete transformation of hydroxyapatite into α-tri calcium phosphate for x=1.5, sintered at 1200 °C. (* - α-TCP, $ - β-tri calcium phosphate, • - CaO and # - Silver)

Now, these are like, you know, very busy slides, which essentially tells you that Ca 10… So that, you, when you dope this silver in this hydroxyapatite lattice, that means, some amount of Calcium will be replaced by Silver and thereby, they are being incorporated into the solid solution. Now, when we are sintered at 1200 degree Celsius, or 800 to 1200 degree Celsius, then, what you notice that, if your x is equal to 0, at 800, x equal to 1.5 and then, you can see that, there are beta tri Calcium Phosphate at 12, x is equal to 1.5. So, when the replacement, or the substitution level, where, which is denoted by x, if x is equal to 1.5, then, you can form lot of beta tri Calcium Phosphate. Now, similar observation also be noticed at, sintered, when they are sintered at 1200 degree Celsius, when x is equal to 0, and x is equal to 1.5. In both the cases, we do see hydroxyapatite, but at the same time, we do see alpha tri Calcium Phosphate, as well as beta tri Calcium Phosphate. So, what we notice here that, while substituting (( )) hydroxyapatite lattice by silver, there is potential formation of both alpha tri Calcium Phosphate and beta tri Calcium Phosphate, and their formation critically depends on the sintering temperature.

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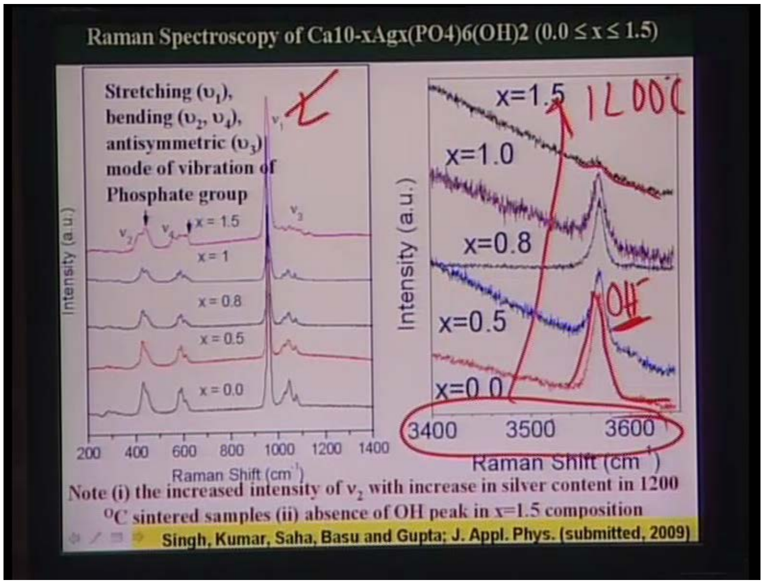

Raman Spectroscopy of Ca10-xAgx(PO4)6(OH)2 (0.0 ≤ x ≤ 1.5)

Note (i) the increased intensity of v_2 with increase in silver content in 1200 °C sintered samples (ii) absence of OH peak in x=1.5 composition

Singh, Kumar, Saha, Basu and Gupta; J. Appl. Phys. (submitted, 2009)

The other clear trend that you can notice here, from x equal to 0 to x equal to 1.5, when you vary this, after sintering at 1200 degrees Celsius, the Raman band which corresponds to OH minus vibration band, what you see very clear OH minus vibration beta texture to point 0.0; it clearly disappears at x is equal to 1.5. What it means that, OH minus essentially indicates that, whether you have hydroxyapatite in the lattice or not. Now, when this OH minus completely vanishes, or completely disappears, that essentially tells you that, hydroxyapatite is completely lost from the material and you only have that tri Calcium Phosphate phase. If you see that Phosphate peaks here, in this hydroxyapatite lattice and these are observed at the relatively higher Raman band, Raman shift, like 3400 to 3600 region.

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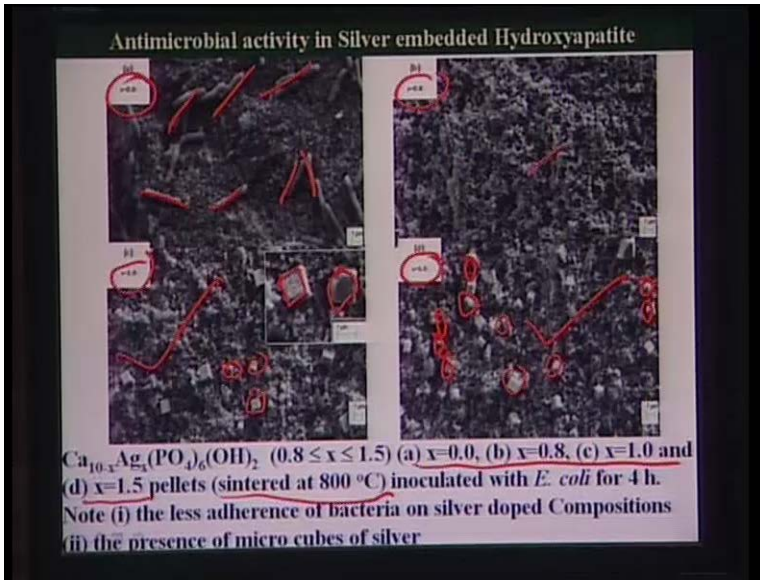

Antimicrobial activity in Silver embedded Hydroxyapatite

$Ca_{10-x}Ag_x(PO_4)_6(OH)_2$ $(0.8 \le x \le 1.5)$ (a) x=0.0, (b) x=0.8, (c) x=1.0 and (d) x=1.5 pellets (sintered at 800 °C) inoculated with *E. coli* for 4 h.

Note (i) the less adherence of bacteria on silver doped Compositions

(ii) the presence of micro cubes of silver

Now, this antimicrobial property, as I said, we first check with the, using this e-coli bacteria. Now, these are the materials which are sintered at 800 degrees Celsius and where that substitution level was varied between x is equal to 0.0, to x is equal to 1.5. Now, if this is x is equal to 0.0, what you see you, have this long rod like bacteria, these are present on the material. Now, at x equal to 0.8, we do see some bacteria, but it is much less in number; at x is equal to 1 and x is equal to 1.5, what you notice, you have this silver tube and this silver tube is coming out of the material and we have found a number of silver tubes on the surfaces. And, these number of silver tubes also increases, when you increase the substitutional level from x is equal to 1 to x is equal to 1.5. Now, this is what you can see here on this biomaterial surface, after culturing with e-coli bacteria and what we believe that, this presence of this silver tubes, essentially kills all the bacteria, as you do not see any evidence of e-coli bacteria on these two surfaces. So, essentially, when you substitute at x is equal to 1.0, or higher level, then, these materials have good antimicrobial property.

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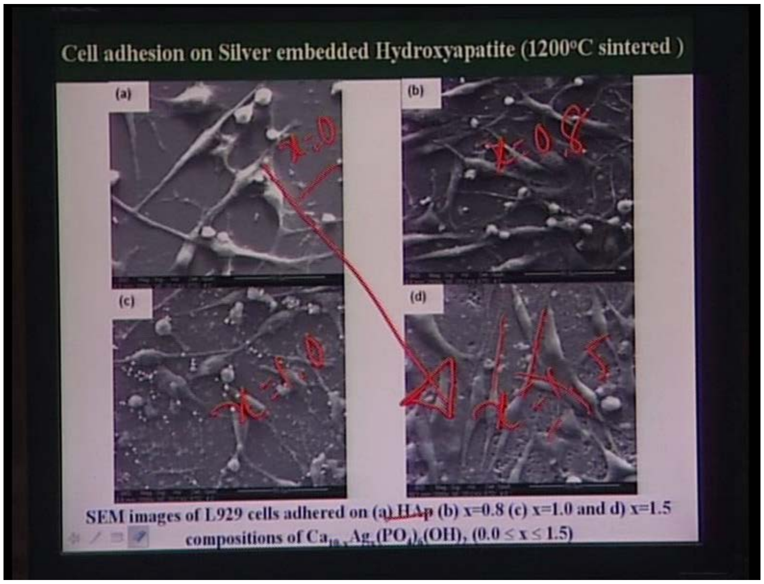

Cell adhesion on Silver embedded Hydroxyapatite (1200°C sintered)

SEM images of L929 cells adhered on (a) HAp (b) x=0.8 (c) x=1.0 and d) x=1.5 compositions of $Ca_{10-x}Ag_x(PO_4)_6(OH)_2$ $(0.0 \leq x \leq 1.5)$

Now, this set of 4 SEM images essentially tells you that, how this cell adhesion on the silver embedded hydroxyapatite, they take place. And, this cell adhesion property essentially tells, shows you that, this is for pure hydroxyapatite, x is equal to 0; b is x is equal to 0.8; c is x is equal to 1 and d is x is equal to 1.5. Now, what you notice, therre is not much difference, when you go from x is equal to 0 to x is equal to 1.5 and the cells are equally proliferate and cells also expand on the material surface by filopodia extension; there is cell to cell breach formation and cellular network formation, which are equally observed at x is equal to 1.5. So, that essentially tells you that, you can kill the bacteria at x is equal to 1, or higher level of substitution; at the same time, you can have good cell cellular fate processes, which are also observed in this material.

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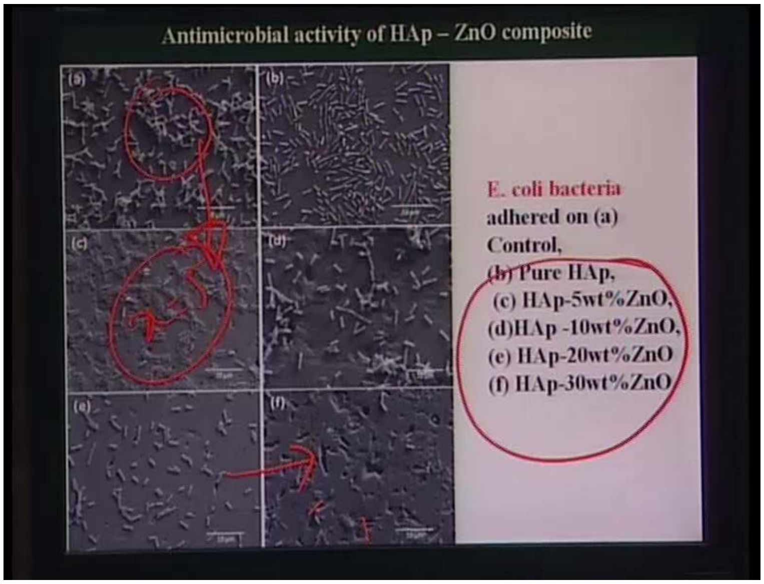

Antimicrobial activity of HAp – ZnO composite

E. coli bacteria
adhered on (a)
Control,
(b) Pure HAp,
(c) HAp-5wt%ZnO,
(d)HAp -10wt%ZnO,
(e) HAp-20wt%ZnO
(f) HAp-30wt%ZnO

Now, next set of composites that were developed to check the antimicrobial property is the hydroxyapatite zinc oxide composite. Now, hydroxyapatite zinc oxide composite. Now, here, that one of the postulate was that, whether the addition of the zinc oxide essentially, enough to kill the, all the bacteria. In order to test that hypothesis, we have added that zinc oxide content, upto 30 weight percent. Now, these experiments are, first set of experiments were carried out using e coli bacteria, where you can see at x is equal to 0, as well as upto c x is equal to 5, or additional 5 percent zinc oxide, there the colony formation is equally present; but when you increase the zinc oxide content by upto 20 or 30 percent, what you seen interestingly that, the number of bacterial cells are much less in at x is equal to 20, or x is equal to 30; essentially indicates that, it is possible to improve the antimicrobial property, with increase in the zinc oxide content.

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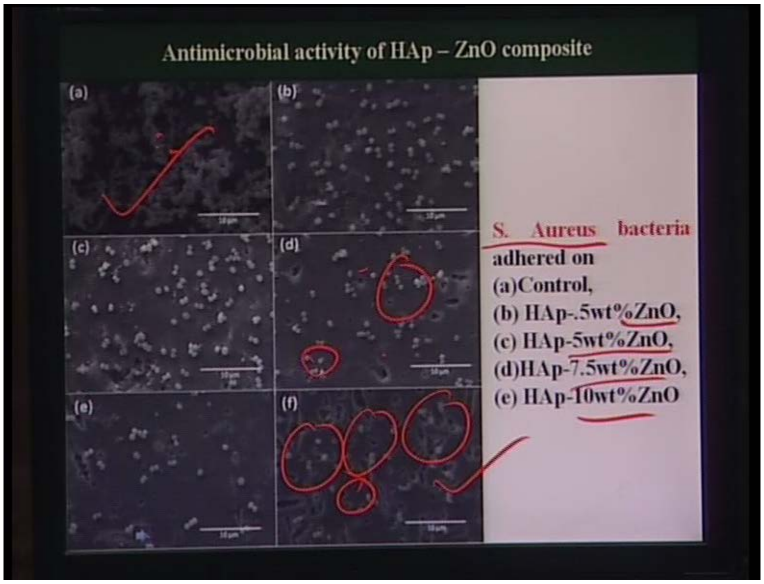

Antimicrobial activity of HAp – ZnO composite

S. Aureus bacteria adhered on
(a)Control,
(b) HAp-.5wt%ZnO,
(c) HAp-5wt%ZnO,
(d)HAp-7.5wt%ZnO,
(e) HAp-10wt%ZnO

Now, similar observation was also made using that staphylococcus aureus, that is the gram positive bacteria, which are cultured on the HAp 0.5 percent zinc oxide, 5 weight percent zinc oxide, 7.5 and 10 weight percent zinc oxide, and in all this cases, you can see that, number of bacteria cells, there are also equally number of bacterial cells are less and there is not much bacterial colony which are formed, to what we have seen at controlities as well as lower amount of zinc oxide content.

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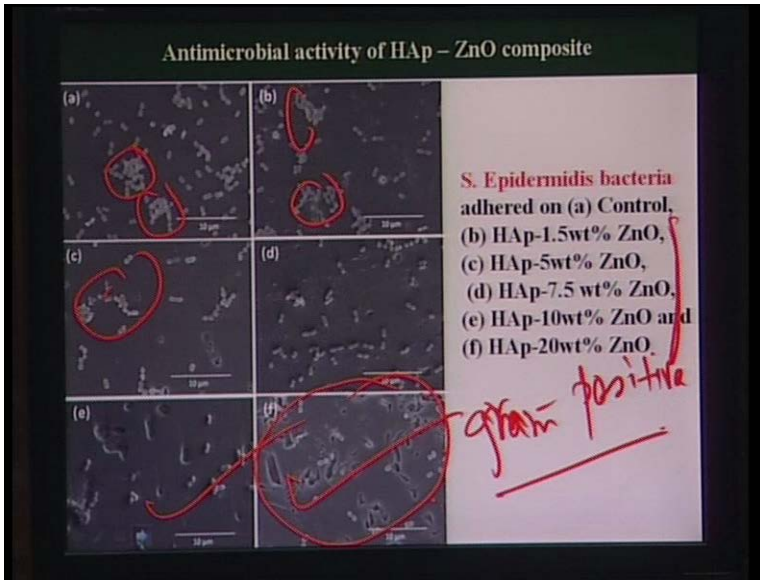

Antimicrobial activity of HAp – ZnO composite

S. Epidermidis bacteria adhered on (a) Control, (b) HAp-1.5wt% ZnO, (c) HAp-5wt% ZnO, (d) HAp-7.5 wt% ZnO, (e) HAp-10wt% ZnO and (f) HAp-20wt% ZnO.

gram positive

So, with increase in zinc oxide content, we do see noticeable change in the antimicrobial or bacterial activity on the material surface. Another bacterial strain that we have used, that is a staphylococcus epidermidis. So, all these staphylococcus species, are important gram-positive bacteria. So, this is like gram-positive bacteria, gram, gram-positive bacteria and this gram-positive bacteria were cultured, or were seeded on this hydroxyapatite, series of hydroxyapatite with zinc oxide composite and then, again, you have this large colony and this colony are also present to some extent at the lower amount of zinc oxide content. But at higher amount of zinc oxide content, this number, as well as the colony formation is, this is much much less significant, I mean therefore, that this materials which have very high zinc oxide content, they have good antimicrobial property.

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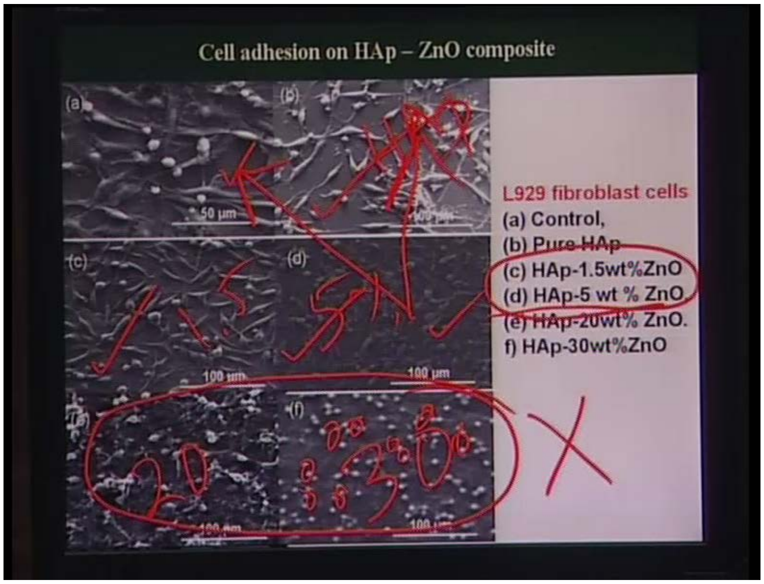

Cell adhesion on HAp – ZnO composite

L929 fibroblast cells
(a) Control,
(b) Pure HAp
(c) HAp-1.5wt%ZnO
(d) HAp-5 wt % ZnO.
(e) HAp-20wt% ZnO.
f) HAp-30wt%ZnO

Now, problem is that, with the zinc oxide content, that is the cellular adhesion or cell-fate processes which takes place on this HAp zinc oxide composite. Now, what you notice on this HAp zinc oxide composite is that, this is the control disk; this is the pure Hap; this is 1.5 percent zinc oxide; this is 5 percent zinc oxide; this is 20 percent zinc oxide and this is 30 percent zinc oxide. Now, both 20 and 30, you cannot use for any applications, which require good cytocompatibility property, because, what you see here, this cells are much spherical in shape, which essentially indicates that, all the cells are apoptotic, or all the cells have died in contact with higher amount of zinc oxide; however, at lower amount of zinc oxide, upto 5 weight percent zinc oxide, we do see the cell proliferation and cell-fate processes which are comparable with the controlled, or with the pure hydroxyapatite material.

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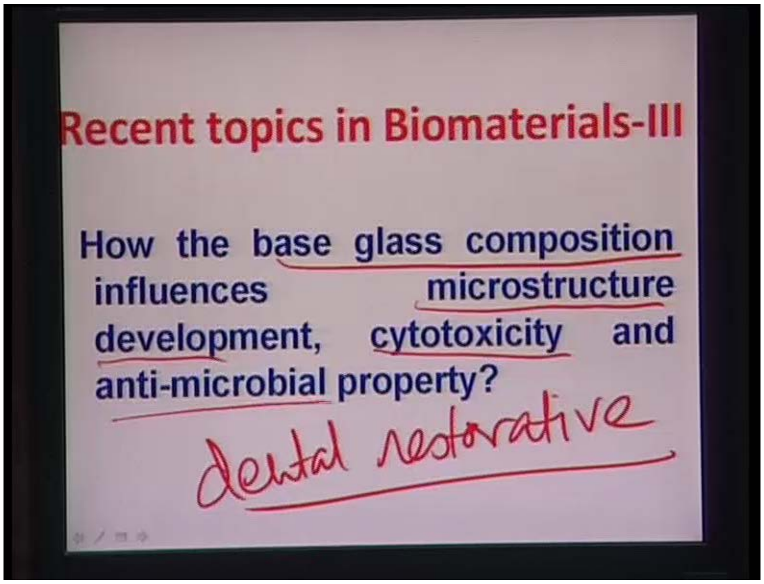

Recent topics in Biomaterials-III

How the base glass composition influences microstructure development, cytotoxicity and anti-microbial property?

dental restorative

So, next topic that I would like to discuss, that relates to the materials for dental restorative application. And, these dental restorative applications are materials, these were developed in our laboratory is that, glass ceramic based materials and I want to show you that, you know, how the base glass composition, if that changes and if the heat treatment condition changes, then, how that changes the microstructure in this materials and cytotoxicity and antimicrobial property of this material.

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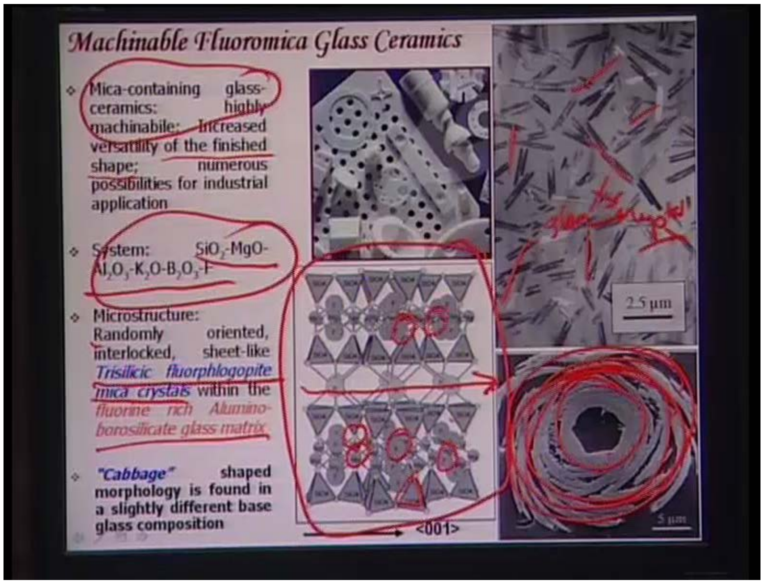

Machinable Fluoromica Glass Ceramics

- Mica-containing glass-ceramics: highly machinabile: Increased versatility of the finished shape; numerous possibilities for industrial application
- System: SiO_2-MgO-Al_2O_3-K_2O-B_2O_3-F
- Microstructure: Randomly oriented, interlocked, sheet-like *Trisilicic fluorphlogopite mica crystals* within the *fluorine rich Alumino-borosilicate glass matrix*
- **"Cabbage" shaped morphology is found in a slightly different base glass composition**

Now, the system that we have worked, which is very similar to that of the macro glass ceramics and this is fluoromica glass ceramics and this Si O 2 Mg O Al 2 O 3 K 2 O B 2 O 3 fluorine and this mica-containing glass ceramics, they are highly machinable. So, therefore, because the human tooth has different, complex shapes; so, whatever materials that you try to develop, that needs to be machined to give particular shape and therefore, this increased versatility of the finished shape is only possible, if the material is highly machinable. Then, microstructure, typically they contain the randomly oriented, you can see here, this is the transmission electron micrograph and this transmission electron micrograph you see that, essentially, this is your glass matrix and this is your crystals; and this crystals are randomly oriented and they are mostly the interlocked, or sheet-like crystals and this crystals are Trisilicic fluorphlogopite mica crystals and they are present in a borosilicate glass matrix.

It is fascinating to note here that, in this particular glass ceramic system, you can develop different morphology of the crystals. For example, this morphology is called the cabbage shaped morphology. This looks like, more like a cabbage, right; cabbage is a vegetable and in this cabbage shaped morphology, is observed by change, or can be developed in this materials, by changing the heat treatment conditions. This is the characteristic structure of this crystals and what you see, this characteristic structure, the Potassium is characteristically located at the central region of this lattice, and then, you have this Si O 4 silicate tetrahedron and then, you have Fluorine and Magnesium, are located in between the silicate tetrahedron.

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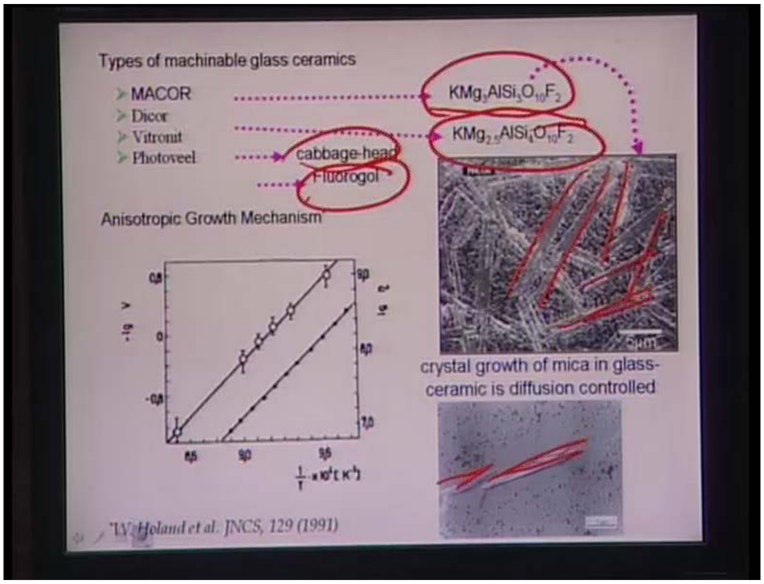

Types of machinable glass ceramics

- MACOR
- Dicor
- Vitronit
- Photoveel

$KMg_3AlSi_3O_{10}F_2$

$KMg_{2.5}AlSi_4O_{10}F_2$

cabbage-head

Fluorogol

Anisotropic Growth Mechanism

crystal growth of mica in glass-ceramic is diffusion controlled

W. Holand et al. JNCS, 129 (1991)

Now, there are different type of commercially available, machinable glass ceramics are present. One is the MACOR glass ceramic and where the crystals, they have a typical composition K Mg 3 Al Si 3 O 10 F 2 and you have the Dicor glass ceramics; again, that although the crystal phase has similar type of composition, but is slightly different in terms of that, that it has a K Mg 2.5 Al Si 4 O 10 F 2; and, what you notice in this kind of glass ceramic, the crystalline phase which is formed, which has a very complex composition; and this complex composition, forms during the heat treatment process of the base glass. Now, Photoveel and Vitronit, they have a cabbage head, or fluorogol type of morphology; and here, you can see that kind of, very different type of morphology of the crystals; and here again, this is a interlocking type of morphology of this mica crystals.

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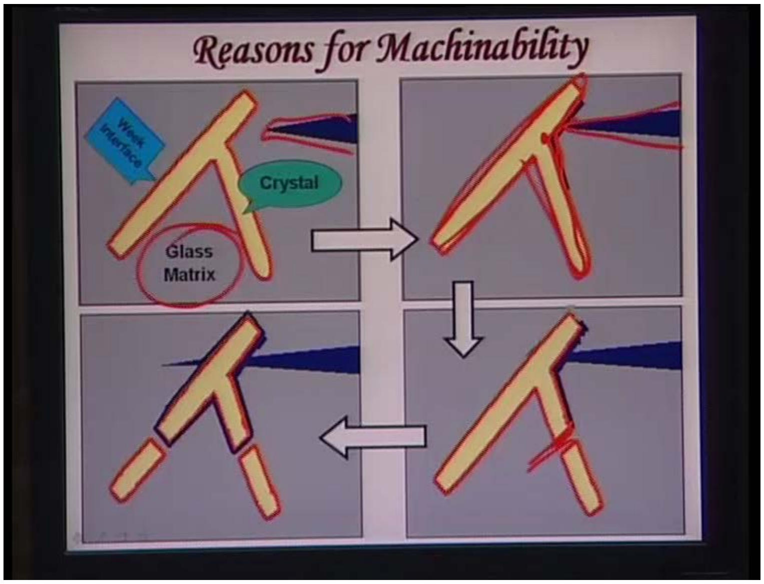

Reasons for Machinability

Now, reasons for machinability, when you have interlocked type of crystals, like this, it is shown, as shown here; now, when a machinery cracks will come, they will interact with this interlock type of crystals and then, this crystals, they can break at some preferred pH plane here and it therefore, it gives more resistance to crack propagation, in contrast to the, in contrast to the fact, than when you have a simple glass matrix itself.

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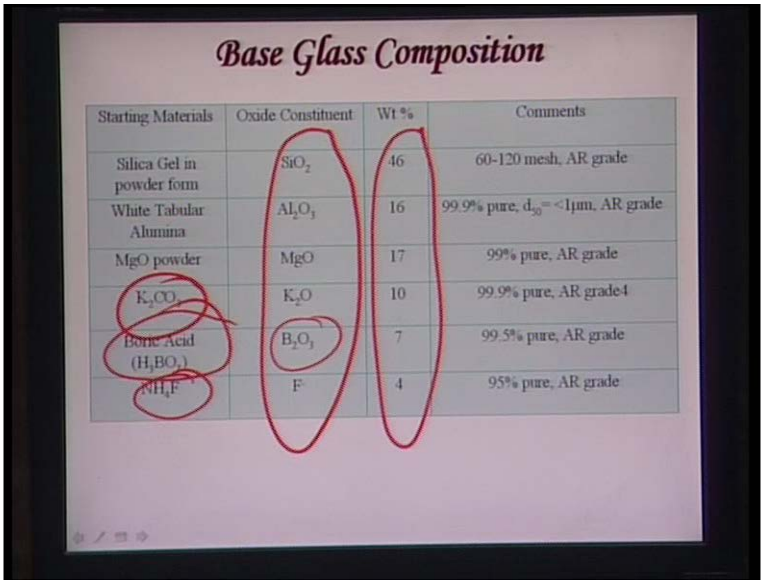

Base Glass Composition

Starting Materials	Oxide Constituent	Wt %	Comments
Silica Gel in powder form	SiO_2	46	60-120 mesh, AR grade
White Tabular Alumina	Al_2O_3	16	99.9% pure, d_{50}= <1μm, AR grade
MgO powder	MgO	17	99% pure, AR grade
K_2CO_3	K_2O	10	99.9% pure, AR grade4
Boric Acid (H_3BO_3)	B_2O_3	7	99.5% pure, AR grade
NH_4F	F	4	95% pure, AR grade

Now, the various precursor powders which are used to develop this glass ceramics. You start with the silica, alumina, Magnesium oxide, K 2 O, B 2 O 3, Fluorine. So, these are like and then Fluorine, you use that NH 4 F as a precursor; for B 2 O 3, you use that boric acid as a precursor; and these cases, that composition of this glass ceramics materials, they vary like, over wide range like, 46 weight percent silica gel, 16 weight percent alumina, 17 weight percent Magnesium oxide, 10 weight percent K 2 C O 3, which will finally, give rise to K 2 O, then, 7 weight Boric oxide B 2 O 3, and 4 percent N H 4 F, that is the Fluorine.

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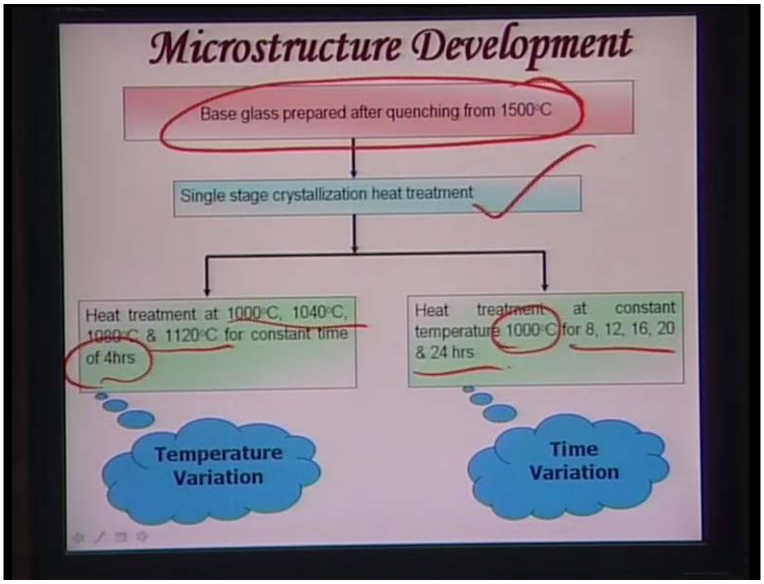

Microstructure Development

Base glass prepared after quenching from 1500°C

Single stage crystallization heat treatment

Heat treatment at 1000°C, 1040°C, 1080°C & 1120°C for constant time of 4hrs

Heat treatment at constant temperature 1000°C for 8, 12, 16, 20 & 24 hrs

Temperature Variation

Time Variation

Now, how this glass ceramic materials, they are processed? They are processed, like, you start with the base glass, which is prepared after quenching from 1500 degree Celsius. So, you first heat it to, you first mix them for, mix this powders and then, heat it them and heat them to high temperature, they will melt; and, this heating is done in platinum crucible, so that, it does not react with that; other crucible, like, if you use the alumina, then, it will re-react with the alumina. So, you cannot use alumina. So, platinum is more noble. So, it does not react with this glass ceramic melt. So, after you melt, then, you can cast it and then, you make a base glass; then, you grind it again, then again, you remelt. So, melting and remelting is done, two to three times. Why, because, it is a multi component system; if you do melting once, then, this, all this glass composition may not be homogeneously distributed. So, if you do melting and remelting twice or thrice, then, that will essentially facilitate good mixing of, in this multicomponent systems. Then, you can do this single stage crystallization heat treatment and this heat treatment can be done at 1000 to 1120 degree Celsius for 4 hours; and then, other case you can do this heat treatment from 8 to 24 hours time scale. So, in one case, you are changing the temperature; in other case, you are changing the time.

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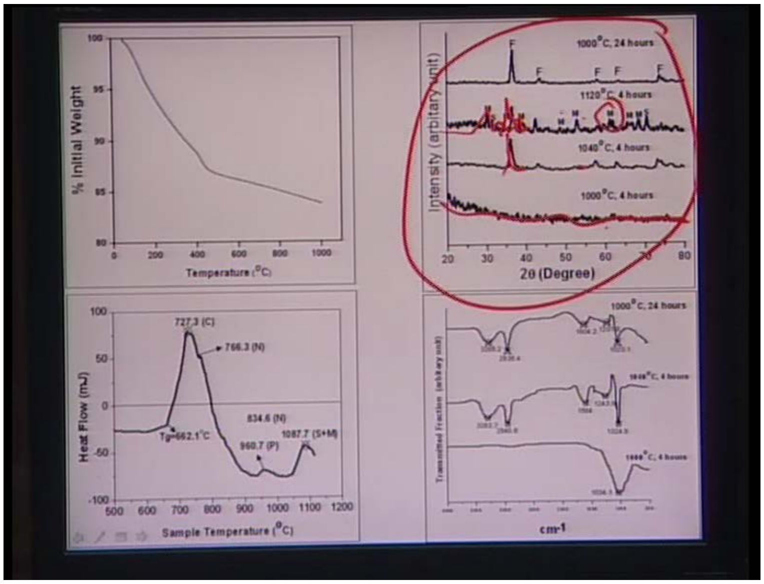

Now, this is your XRD spectra. What it shows that, you have a base glass here; there is no crystalline peak when you heat it at 1040 degree Celsius, you do see the fluorphlogopite peak; if you heat it at, heat it at 1120 degree Celsius, you see the fluorphlogopite peak, as well as m, that is the mullite peak.

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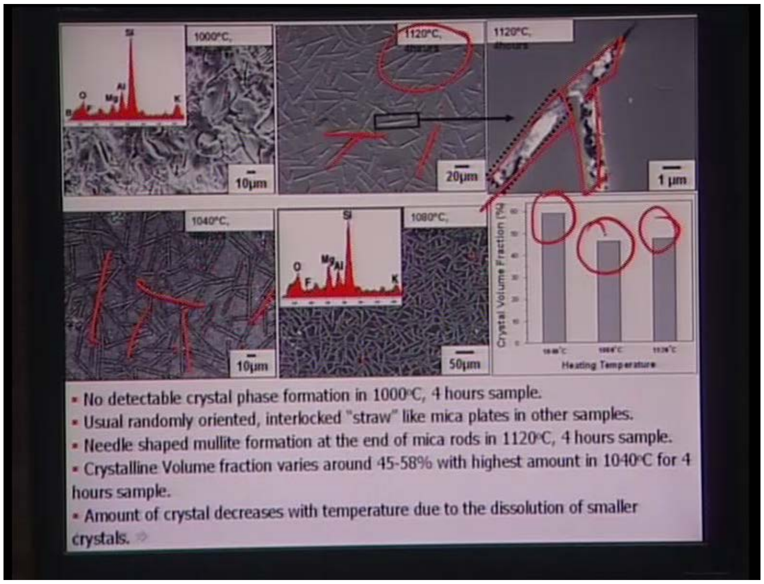

- No detectable crystal phase formation in 1000°C, 4 hours sample.
- Usual randomly oriented, interlocked "straw" like mica plates in other samples.
- Needle shaped mullite formation at the end of mica rods in 1120°C, 4 hours sample.
- Crystalline Volume fraction varies around 45-58% with highest amount in 1040°C for 4 hours sample.
- Amount of crystal decreases with temperature due to the dissolution of smaller crystals.

Now, this is the microstructure, typically, what has been observed, when you change the temperature; like, you start with the 1000 degree Celsius, it is the amorphous matrix; you heat it at 1120 degree Celsius for 4 hours; what you see here, this microcrystals, they form; 1120 degree Celsius, similar microcrystals form and then, you can see, that is interlocking type of crystals; at other temperature 1040 and 1080 also, you see, you do see that interlocking type of crystals; the major difference is the volume fraction of the crystals that form. Now, what you see at 1040, is around 50 to 60 percent volume percent; at around 1080, it is little less, or it is around 45 percent of crystals, those are formed.

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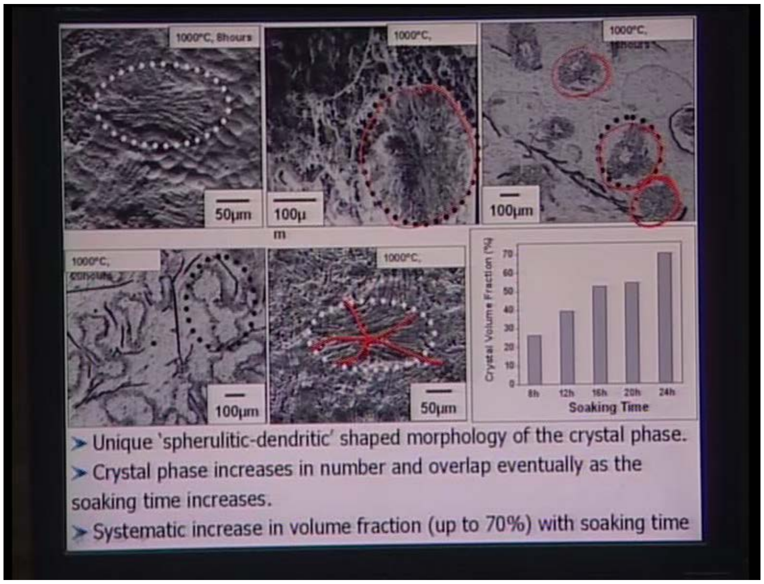

- Unique 'spherulitic-dendritic' shaped morphology of the crystal phase.
- Crystal phase increases in number and overlap eventually as the soaking time increases.
- Systematic increase in volume fraction (up to 70%) with soaking time

Now, in case when the time is varied, for 8 hours to 24 hours, then, what you notice here that, and the 8 hours and subsequently higher time, you do see that, some kind of butterfly type of crystals; why I, why do I see, why do I say butterfly, because, it has a very characteristically shaped crystals, which are generated from central nucleus.

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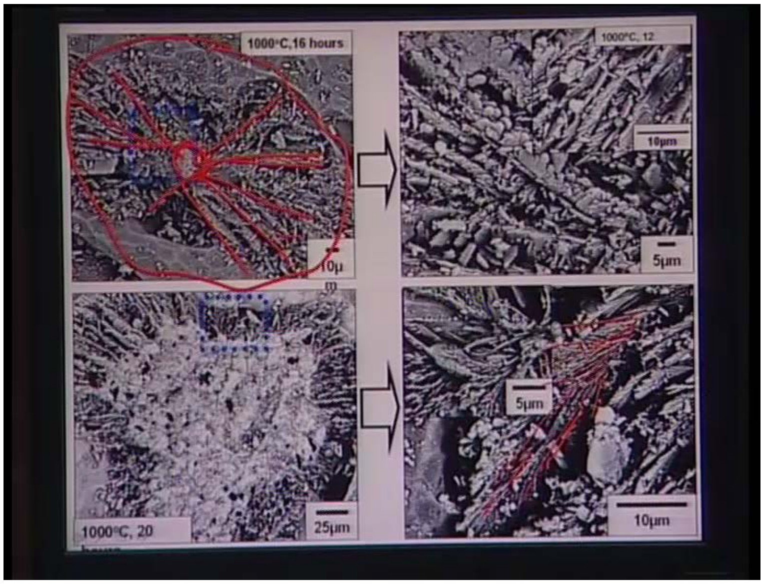

You can see more such crystals here. Let us say, this is your starting point. So, this is like nucleus, and from this nucleus, this crystals, they are growing at different directions and this crystals are growing at different directions and subsequently, they are like enclosed in a large spherical shaped area, which is called crystal domain. Now, if you look at this crystals more carefully, what you notice, they also have a tree leaf type of patterns; tree leaf, like, you know, you have a one main branch and from that other branches are also formed; and, this is a very fascinating type of crystals and this crystals are only formed, when you change the time, or when you vary the time.

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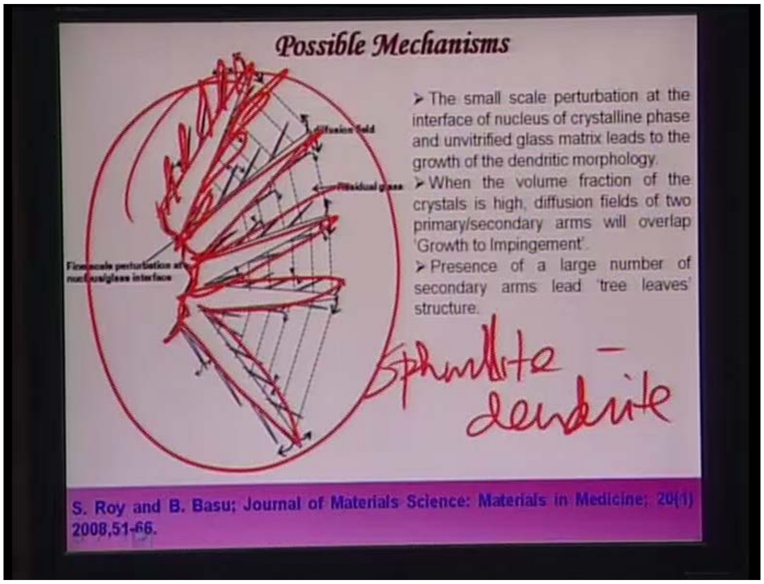

Possible Mechanisms

- The small scale perturbation at the interface of nucleus of crystalline phase and unvitrified glass matrix leads to the growth of the dendritic morphology.
- When the volume fraction of the crystals is high, diffusion fields of two primary/secondary arms will overlap 'Growth to Impingement'.
- Presence of a large number of secondary arms lead 'tree leaves' structure.

Spherulite –
dendrite

S. Roy and B. Basu; Journal of Materials Science: Materials in Medicine; 20(1) 2008,51-66.

Now, what are the possible mechanisms? Possible mechanism is that, when the crystals are nucleated at, from the amorphous glass matrix, and if there is fine scale perturbation in the nucleus glass matrix, so, this perturbations will subsequently develop some kind of dendrites here; and, these dendrites will grow in the glass matrix and from these dendrites, primary dendrites, you also have secondary dendrite; and, these secondary dendrites are also growing in this glass matrix and as a result, you form more like a tree leaf type of pattern and that, what we call as a butterfly type of crystals.

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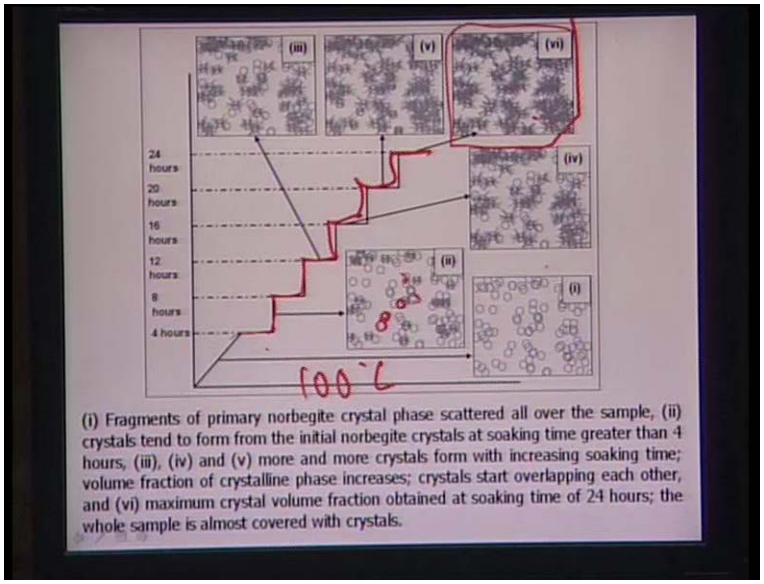

(i) Fragments of primary norbegite crystal phase scattered all over the sample, (ii) crystals tend to form from the initial norbegite crystals at soaking time greater than 4 hours, (iii), (iv) and (v) more and more crystals form with increasing soaking time; volume fraction of crystalline phase increases; crystals start overlapping each other, and (vi) maximum crystal volume fraction obtained at soaking time of 24 hours; the whole sample is almost covered with crystals.

Now, in the literature, this kind of crystals are also known as spherulite - dendritic type of crystals. Now, what we believe, when you do this heat treatment at different time scale, at the same temperature of 1000 degree Celsius, let us say, now, when you are heat treat at 4 hours, then, some volume fraction of the glass will occupy of this spherulite - dendritic type of crystals; if you do stage-wise, more and more time, if you have, allow more and more time for this glass ceramic to spend at the 1000 degree Celsius, upto 24 hours, then, you can get more densely packed crystals and where this growth of one butterfly crystals will be stopped, as soon as it impinges on the other butterfly crystals.

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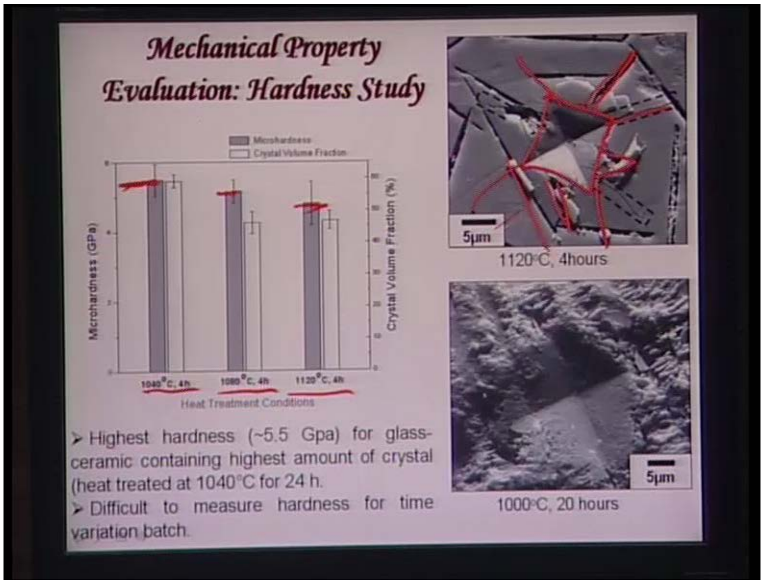

Mechanical Property Evaluation: Hardness Study

- Highest hardness (~5.5 Gpa) for glass-ceramic containing highest amount of crystal (heat treated at 1040°C for 24 h.
- Difficult to measure hardness for time variation batch.

Now, some comments on the hardness property of this materials. Now, what you see, as far as the hardness property is concerned, at 1044 hours, 1080 and 1124 hours, when you vary the temperature, the hardness of this materials, which is the shaded bar and this materials has a (( )) hardness, little higher than 5 gigapascal. Now, 5 gigapascal is not a bad number; it is actually a good value, as far as the glass ceramics are concerned. Now, what you notice here that, although the indents are very sharp in these glass ceramics, but they do, they will show extensive crack propagation because of their brittleness, and also, there is fractures observed as this indents will also interact with this mica, glass mica crystals.

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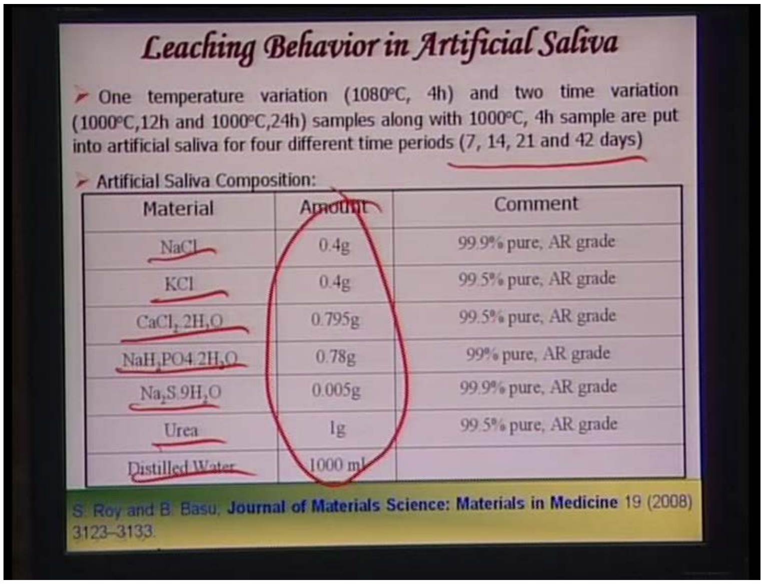

Leaching Behavior in Artificial Saliva

- One temperature variation (1080°C, 4h) and two time variation (1000°C,12h and 1000°C,24h) samples along with 1000°C, 4h sample are put into artificial saliva for four different time periods (7, 14, 21 and 42 days)
- Artificial Saliva Composition:

Material	Amount	Comment
NaCl	0.4g	99.9% pure, AR grade
KCl	0.4g	99.5% pure, AR grade
$CaCl_2 2H_2O$	0.795g	99.5% pure, AR grade
$NaH_2PO4 2H_2O$	0.78g	99% pure, AR grade
$Na_2S 9H_2O$	0.005g	99.9% pure, AR grade
Urea	1g	99.5% pure, AR grade
Distilled Water	1000 ml	

S. Roy and B. Basu, **Journal of Materials Science: Materials in Medicine** 19 (2008) 3123–3133.

Now, in order to assess that, whether this glass ceramic materials, they do have good in-vitro biomineralization property, and these was assessed by immersing them in the artificial saliva. Now, artificial saliva actually, closely, simulate the oral environment; oral environment means, inside the mouth, what is the content of this aqueous environment, that is what has been mimicked by artificial saliva. Now, what is this environment? They look like, you have the Na Cl; you have K Cl; you have Calcium fluoride; NH NH 2 PO 4, you have Na 2 S, urea and distilled water. Now, this, these composition has been taken from the text book and other literatures, and then, we have done this testing upto 42 days; so, that means, 6 weeks and then, we have selected, based on the mechanical properties on the microstructure, we have selected a few samples, which are further tested in artificial saliva.

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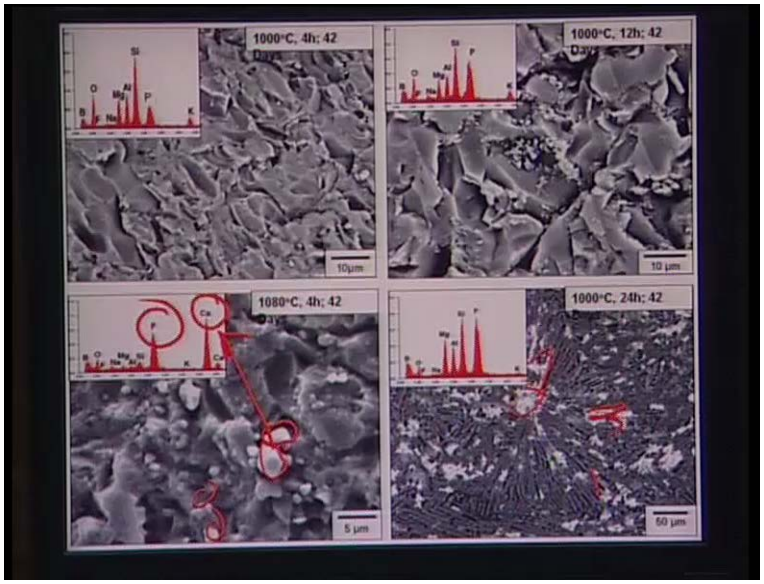

Now, what you notice here, in the, after this artificial saliva testing, that for the 1080 degrees as a 4 hours, you do notice these crystals, which are brighter in contrast and when you test this ((Eder)) spectra from this brighter crystal, then you see Calcium peak, as well as Phosphorous peak. The presence of the strong Calcium and Phosphorous peak essentially tells you, they are like Calcium Phosphate rich phase. Now, similar things, what we also notice, at the other materials, that also you have a bright appearing crystals phases, which are not present prior to the in-vitro dissolution.

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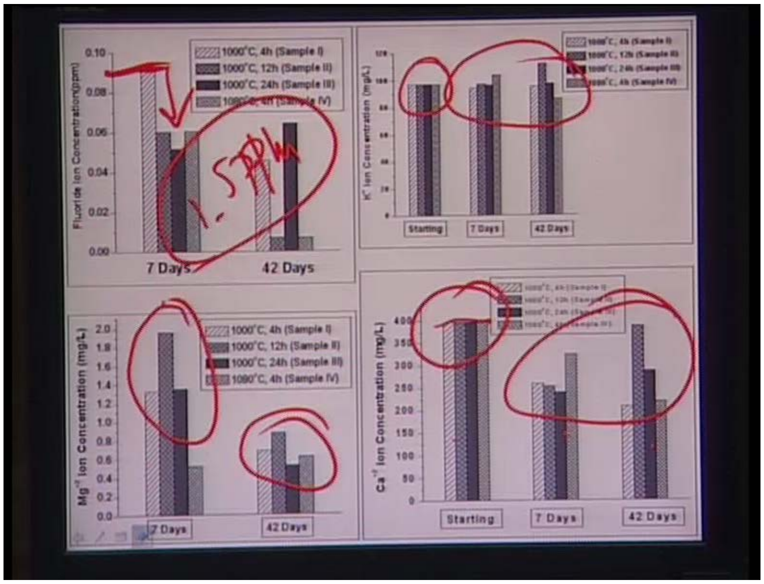

Now, this leaching of the ions is important, as I mentioned during the discussion on the in-vivo test, that both the in-vivo biodegradation and in-vitro biomineralization is equally important; that means, some materials can be always leached out from the biomaterial, depending on the composition of the biomaterial. Now, here, you can see that, how this Fluoride ion, they are getting leached from this material. This Fluorine ion, they are leached upto 0.1 ppm, or around 0.1 p p m, that is the maximum and it is less than 0.1 ppm. According to the WHO standards, that is the World Health Organization, this fluorine ion, which can be tolerated in the solution, or the leaching of the Fluorine ion that can be tolerated in the solution is 1.5 ppm. So, the amount of Fluorine ion which is leached from our materials, which is much less than that, what is recommended by WHO. So, therefore, this glass ceramics materials, although they are tested up to 42 days, this Fluorine ion leaching cannot cause any additional concern.

The other things, that is the Potassium ion concentration…Now, Potassium, your starting solution has somewhat this level and after 7 days, or 42 days, even 6 weeks, there is not much increase in the Potassium ion concentration; that means, this Potassium ion is not released to a large extent. As for the Magnesium ion concentration is concerned, your Magnesium ion is roughly less than 2 mg per liter and it decreases at 42 days. So, the other, now, important thing that you will notice is Calcium ion concentration. Now, calcium ion in the solution was much higher; but after 7 days, and after 42 days, Calcium ion concentration largely reduced from the starting solution. What it means? This means that, this Calcium ions, they forms Calcium Phosphate on the material and therefore, from the solution, Calcium ion content is reduced.

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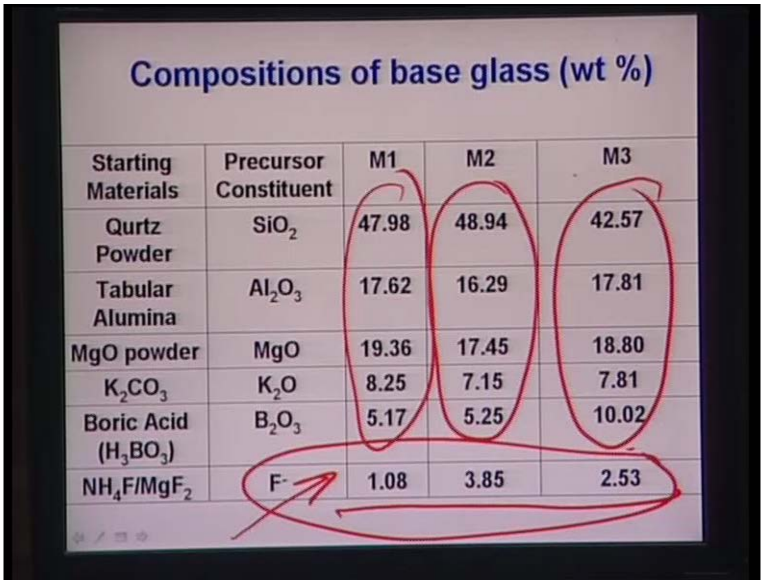

Compositions of base glass (wt %)

Starting Materials	Precursor Constituent	M1	M2	M3
Qurtz Powder	SiO_2	47.98	48.94	42.57
Tabular Alumina	Al_2O_3	17.62	16.29	17.81
MgO powder	MgO	19.36	17.45	18.80
K_2CO_3	K_2O	8.25	7.15	7.81
Boric Acid (H_3BO_3)	B_2O_3	5.17	5.25	10.02
NH_4F/MgF_2	F^-	1.08	3.85	2.53

Now, in the next set of experiments, what we have done in this glass ceramics is that, we have varied the fluoride ion concentration and by varying the Fluorine, fluoride ion concentration, we are trying to see that, whether this changing in the base glass composition will have any important effect as far as the hardness property is concerned, as far as the biocompatibility property is concerned, or as far as the cellular adhesion property is concerned. And this, by changing the fluorine ion over 1 to 4 percent, that other amount of this, the other oxides are also varied, in a relatively narrow window.

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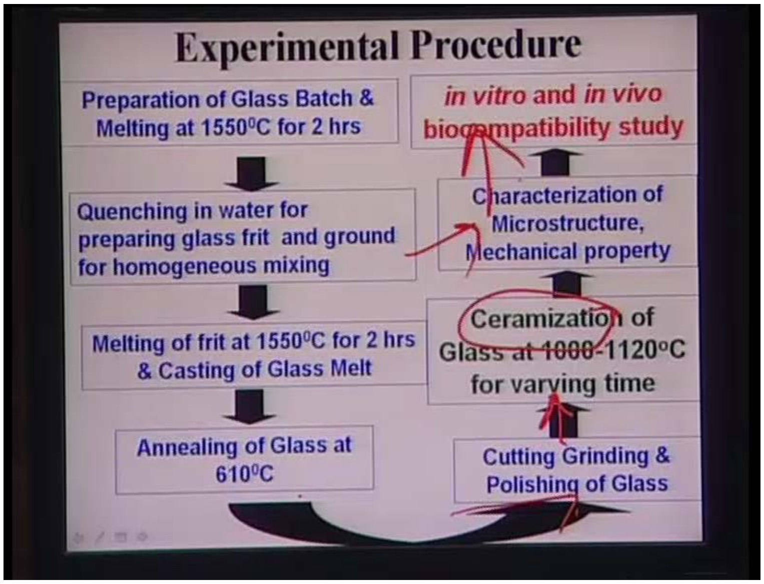

Experimental Procedure

Preparation of Glass Batch & Melting at 1550^{0}C for 2 hrs

Quenching in water for preparing glass frit and ground for homogeneous mixing

Melting of frit at 1550^{0}C for 2 hrs & Casting of Glass Melt

Annealing of Glass at 610^{0}C

Cutting Grinding & Polishing of Glass

Ceramization of Glass at 1000-1120^{o}C for varying time

Characterization of Microstructure, Mechanical property

in vitro and *in vivo* biocompatibility study

Now, the same protocol has been used to fabricate this materials, that is the preparation of the glass batch and melting at 1550 degree Celsius for 2 hours. So, typically, this melting was done in platinum crucible and then, as I have explained to you just few minutes back that, melting and remelting were carried out altogether and therefore, to homogenize this mixture, this, or to ensure homogeneity as far as the composition of the base glass is concerned. After that, they are all quenched in water; basically they are cast into the mould and then, you can take out this base glass and then, you can further anneal this glass at 610 degree Celsius to reduce any thermal induced stresses; then, you can cut, grind and then, after that, each pieces you can ceramize. Ceramize means, this is a heat treatment which will essentially form that ceramic particles in the glass matrix; that is why it is called as glass ceramics material. And then, we can characterize the mechanical and microstructure properties and subsequently, in-vitro and in-vivo properties were characterized.

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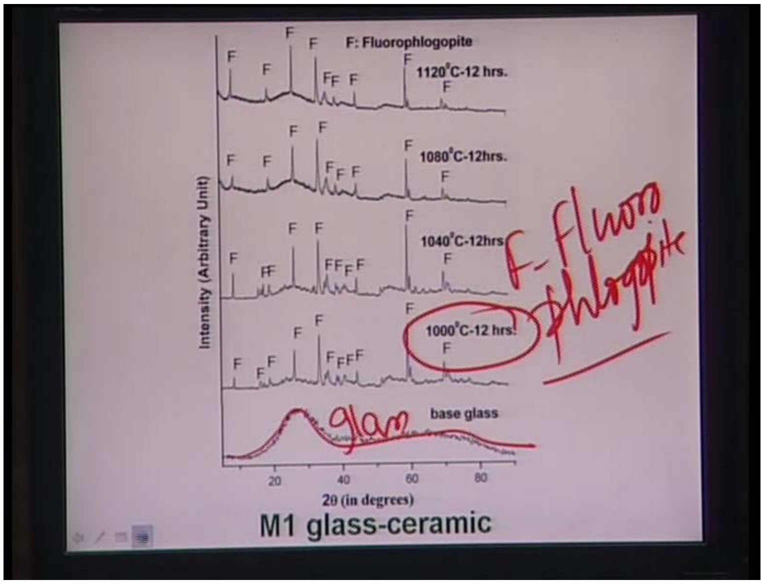

M1 glass-ceramic

So, this glass ceramic materials which are heat treated at different temperature, right from 1000 degree Celsius to 1120 degree Celsius. Now, what you notice here that, at base glass, this is a very broad amorphous peak. So, it is essentially pure glass. So, this amorphous nature, or broadness of the peak essentially tells you that, it is a pure glass phase. Now, when you heat treat at 1000 degree to 1120 for 12 hours, then you see the characteristic short peak of the fluorophlogopite. So, F stands for fluorophlogopite and this is the characteristic crystalline phase which forms and you do see that, it forms right at 1000 degree Celsius.

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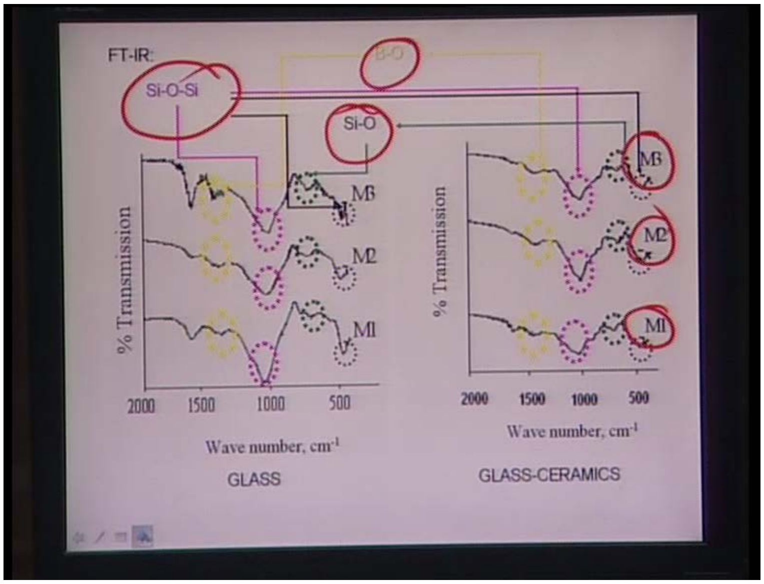

Now, we have done this FTIR and that is the Fourier Transform Infra-Red spectroscopy. Now, what you see in the FTIR, you do notice that, there is a B O stretching peak, you have a Si O bond peak and they have a Si O Si peak. So, all these different type of peaks, FTIR bands are observed in different glass ceramics from M 1, M 2, M 3 essentially tells you that, different chemical bondings, which are present in this glass ceramic material.

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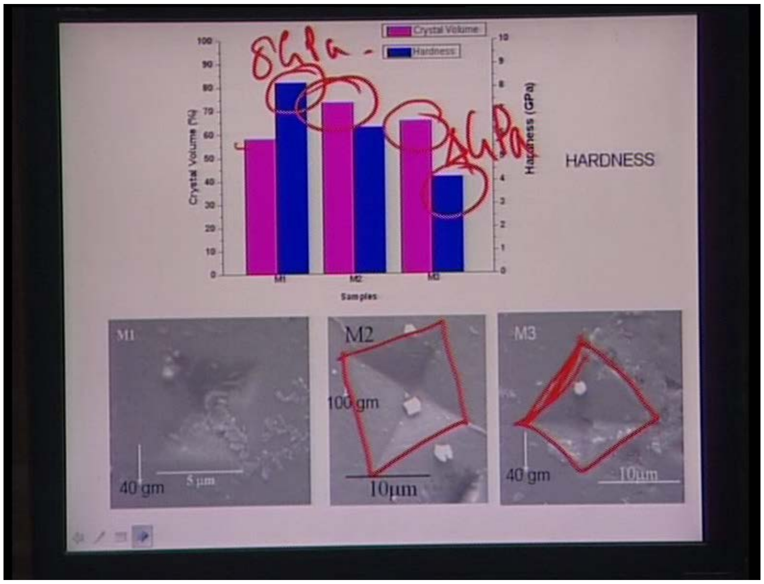

Now, as far as the crystal volume fraction is concerned, this materials contains the crystals upto 60 to 70 percent, 70 volume percent of the crystals; and, as far the hardness is concerned, these materials, they have a very good hardness values which is around 8 gigapascal. Now, 8 gigapascal to roughly 4 gigapascal, that is the hardness values, they are measured in this glass ceramic materials. To obtain reliable hardness properties, what we have done, on the Vickers hardness were also observed in the standing electron microscope; then, you can calculate with more accuracy and precision, the Vickers diagonal and then, thereby determine this hardness properties.

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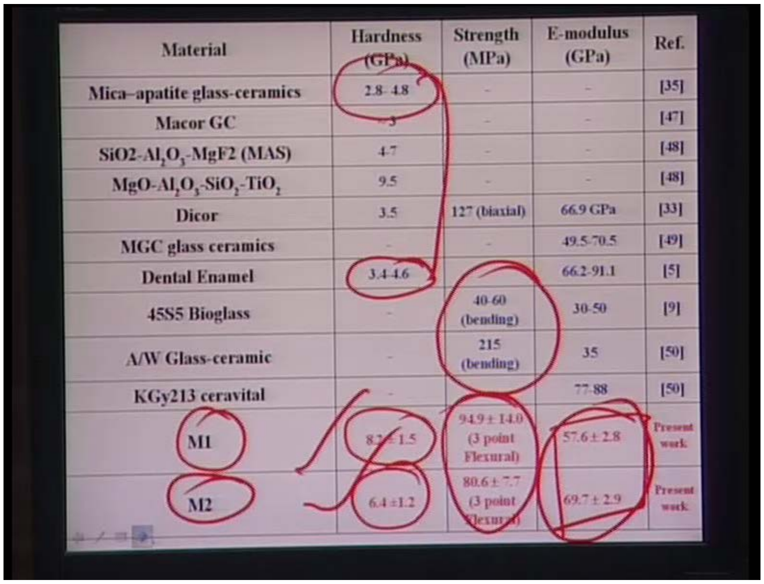

Material	Hardness (GPa)	Strength (MPa)	E-modulus (GPa)	Ref.
Mica–apatite glass-ceramics	2.8-4.8	-	-	[35]
Macor GC	~3	-	-	[47]
SiO2-Al_2O_3-MgF2 (MAS)	4-7	-	-	[48]
MgO-Al_2O_3-SiO_2-TiO_2	9.5	-	-	[48]
Dicor	3.5	127 (biaxial)	66.9 GPa	[33]
MGC glass ceramics	-	-	49.5-70.5	[49]
Dental Enamel	3.4-4.6		66.2-91.1	[5]
45S5 Bioglass	-	40-60 (bending)	30-50	[9]
A/W Glass-ceramic	-	215 (bending)	35	[50]
KGy213 ceravital	-		77-88	[50]
M1	8.2 ± 1.5	94.9 ± 14.0 (3 point Flexural)	57.6 ± 2.8	Present work
M2	6.4 ± 1.2	80.6 ± 7.7 (3 point Flexural)	69.7 ± 2.9	Present work

Now, if you compare the properties of this glass ceramic materials with the other existing materials, what we notice here that, either the mica based apatite glass-ceramic or MGC glass ceramic or dental enamel, they have the hardness values which is less than 5 gigapascal. At the same time, you have this hardness values, which is 8 gigapascal or 6 to 8 gigapascal, which you are observed in our glass ceramic, by varying this fluorine content. So, therefore, these values are much better than those of the earlier developed materials, which are already reported in the literature. Other thing that you notice that, in our glass ceramic materials, elastic modulus is somewhere 57 to 70 gigapascal; and your cortical bone elastic modulus is around upto 3 to 80 gigapascal. So, our glass ceramic materials, they have the elastic modulus which is just sufficient for the cortical bone replacement, or the hard tissue replacement. As far as the strength property is concerned, one can get strength in this materials upto 95 megapascal, and 95 megapascal strength is quite good, as far as that, if you consider the other materials, which is 40 to 60 or 215 megapascal is concerned.

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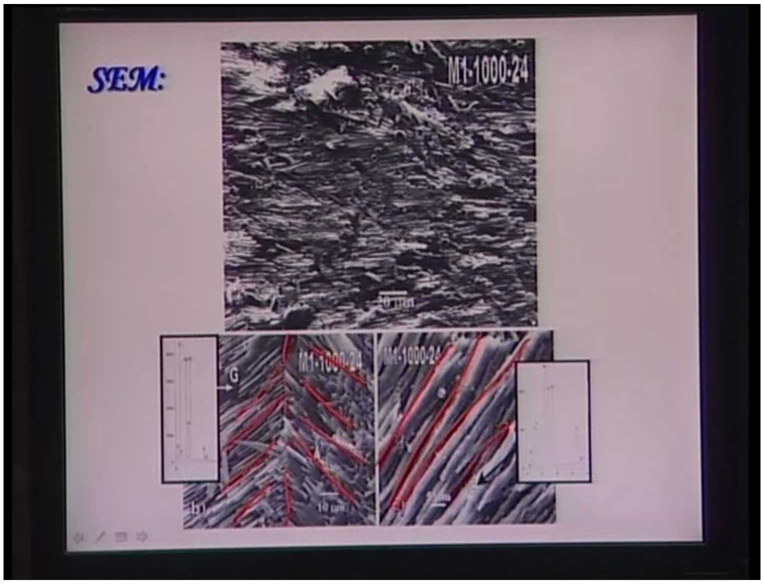

Now, these materials which are developed by varying this Fluorine content in this glass ceramics, important things that you can notice is that, this kind of domain, crystal domain and in this individual crystal domain, the glass, the crystals are oriented differently and they are much like rod like structure and this rods, they experience anisotropic growth and anisotropic growth has been shown also here.

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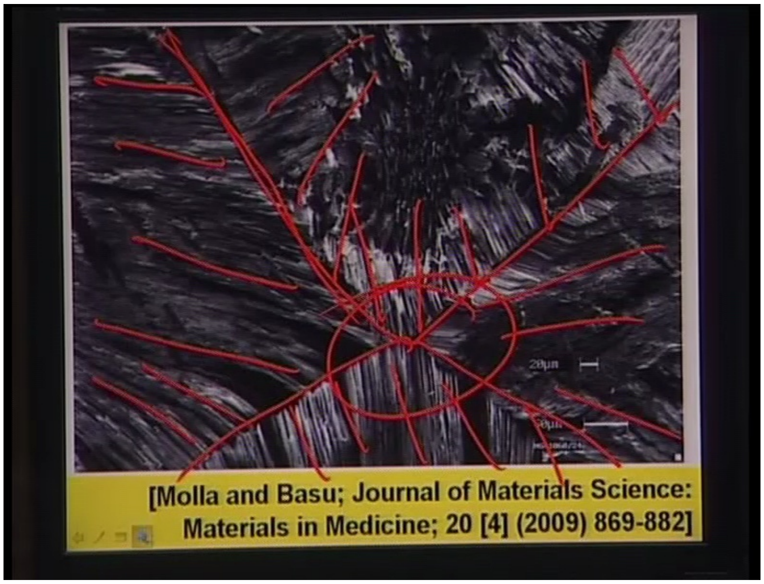

[Molla and Basu; Journal of Materials Science: Materials in Medicine; 20 [4] (2009) 869-882]

The more interestingly, the high magnificent SEM image tells you that, these glass ceramic materials, glass ceramic materials, they have a, what we call envelope type of crystals. If you remember the postal envelope, in this postal envelope, they also have typical this kind of shape and in individual envelope, you have this glass ceramic crystals and these ceramic crystals, they are oriented differently, in different directions and therefore, these crystal domains, they meet at a particular locations here and thereby forming that envelope like crystals.

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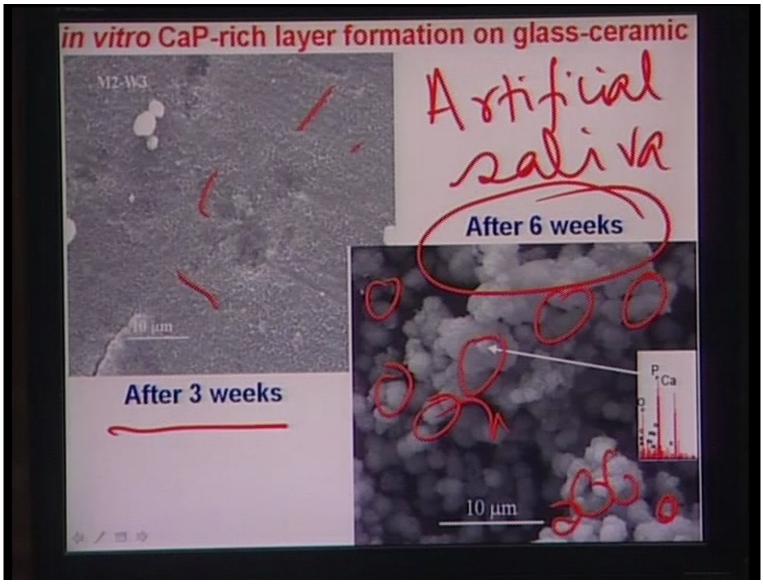

in vitro CaP-rich layer formation on glass-ceramic

Artificial saliva

After 3 weeks

After 6 weeks

In-vitro property, we have carried out again, this in-vitro dissolution test in this artificial saliva. And, in this artificial saliva, as I mentioned earlier, the composition of artificial saliva, after 3 weeks, we do see the signs of CaP like layer formation; after 6 weeks, or 42 days, you can see this Calcium Phosphate rich, you know, precipitates are formed over uniformly and homogenously, over the entire surface and thereby this CaP rich layer formation essentially indicates good biomineralization ability of this materials.

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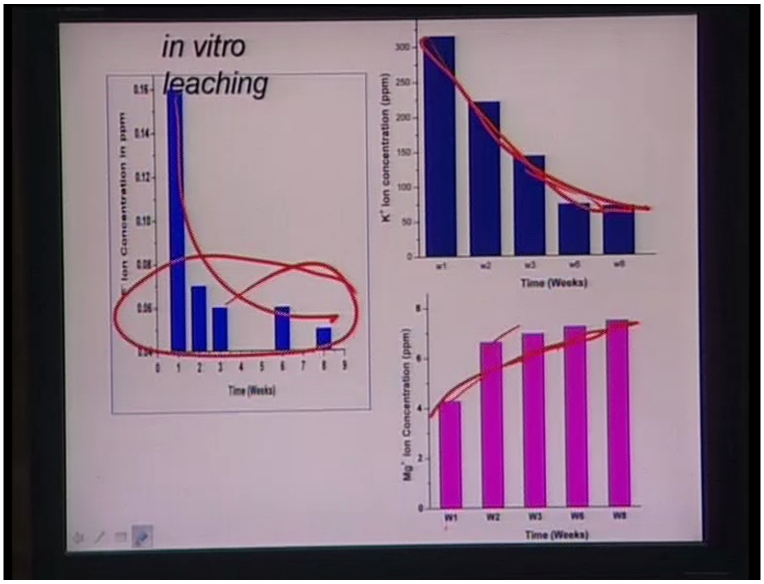

in vitro leaching

As far as the in-vitro leaching is concerned, in the fluorine ion concentration, in the, when it goes to upto 7 or 8 weeks, again, your fluorine ion leaching is much less than the WHO recommended value and this value is even less than 0.1 by ppm. As far as the Potassium and Magnesium ion concentration, it shows a systematically decreased value, with the Potassium concentration, you, if you go from week 1 to week 8; and similarly Magnesium ion concentration, it shows more or less systematically increased value, as you go from W 1 to W 8. What it means, that means, Magnesium concentration is getting increased, or Magnesium is more leached from the solution, whereas, Potassium concentration, it goes on decreasing with more time of leaching in the solution.

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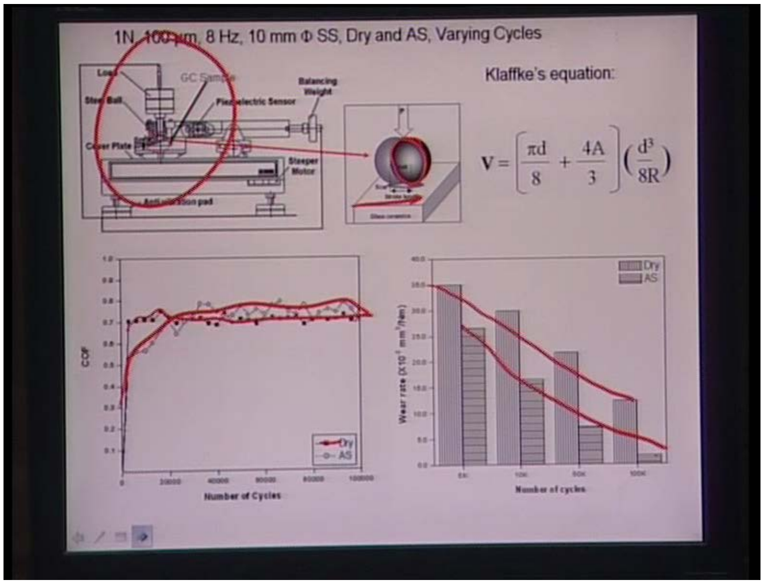

1N, 100 μm, 8 Hz, 10 mm Φ SS, Dry and AS, Varying Cycles

Klaffke's equation:

$$V = \left[\frac{\pi d}{8} + \frac{4A}{3}\right]\left(\frac{d^3}{8R}\right)$$

Now, some friction and wear experiments were carried out in this materials in artificial saliva and there this ball and flat type of configuration is used. So, this is a ball and this is your flat. So, flat is your glass ceramic and this is a typical setting wear set up where you can continuously measure the frictional force, and if you see the frictional force, how they will evolve in this glass ceramic materials. In the case of dry, which is this dry COF, which is around 0.7. In the artificial saliva also, the average COF is 0.7. So, there is not much difference in the dry condition and the artificial saliva condition. As far as the wear rate is concerned, we do see a interesting trend, like, you start with the 5000 cycles and to the more number of cycles like upto 100000 cycles. Now, what you notice, here, in the dry case also, the wear rate decreases and the artificial saliva case also, wear rate decreases. And, each time duration, the artificial saliva glass ceramic materials were less as compared to dry conditions. Now, that will be clear, when you look at the worn surfaces.

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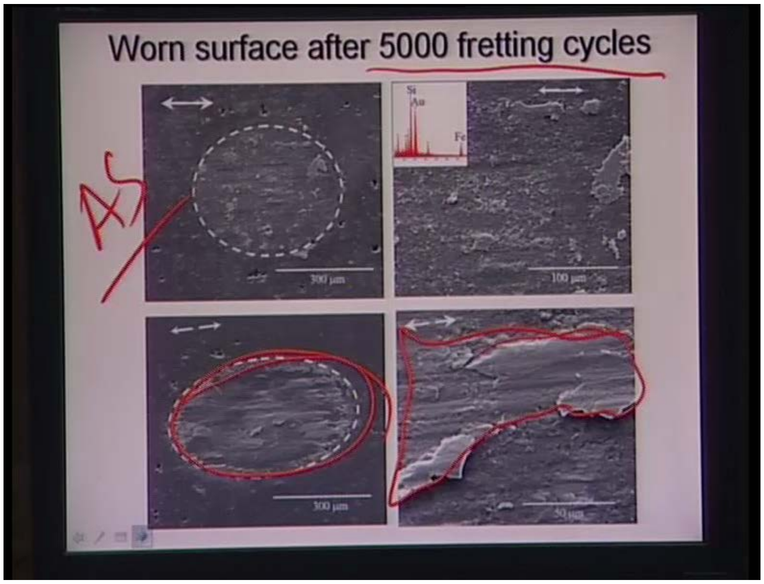

Worn surface after 5000 fretting cycles

Now, this, all this worn surfaces were taken after the artificial saliva testing. And, this after artificial saliva testing after 5000 fretting cycles what you notice here that, there is a tribochemical layer formation and this tribochemical layer formation, it is formed over the entire worn surface, thereby protecting the surface. And, after 50000 cycles, we do see this tribo layer, but there is extensive cracking and this cracking essentially can potentially (( )) these materials out of the system, so, thereby exposing the underlying material to further wear.

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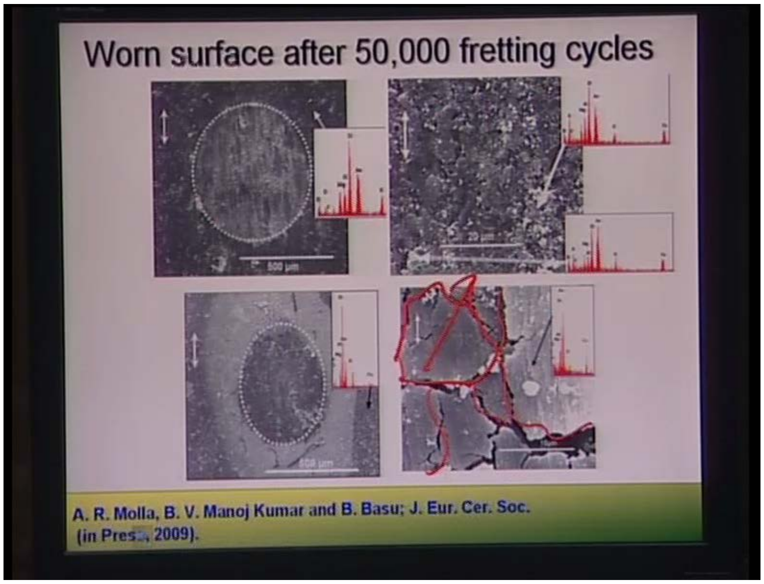

Worn surface after 50,000 fretting cycles

A. R. Molla, B. V. Manoj Kumar and B. Basu; J. Eur. Cer. Soc. (in Press, 2009).

Now, coming to the in-vitro cell compatibility testing, or cytocompatibility testing, we have used that SOS 2 cell lines; SOS 2 cell lines is that human osteoblast like cells; they are osteoblast like cells and this osteoblast like cells, were seeded for 24 hours on this materials.

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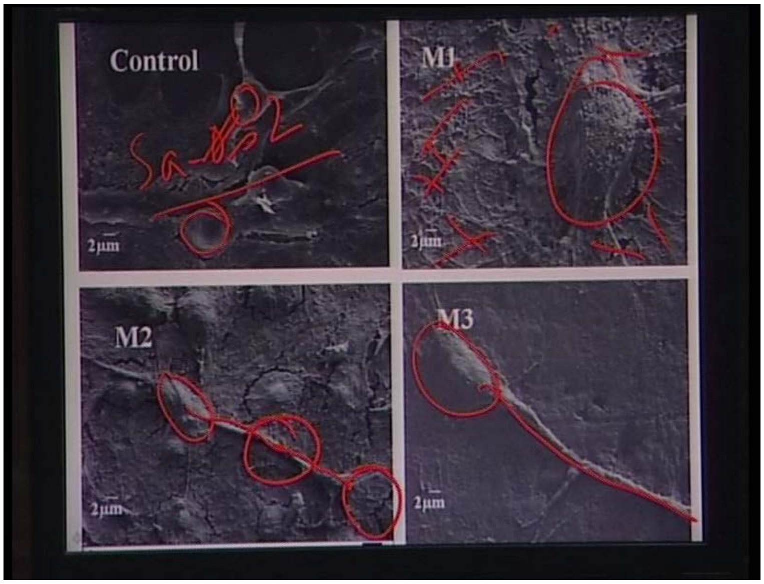

Now, this is your control sample. These cells or cell to cellular network form and this is a extensive filopodia extension and this is one cell, this is another cell and this is filopodia extension from this cell; this is filopodia extension from that cell and that is the cellular breach formation; and as you can see, that is the some, collagen like layer formation, that is the e c m formation also in this materials, when they are seeded for 24 hours.

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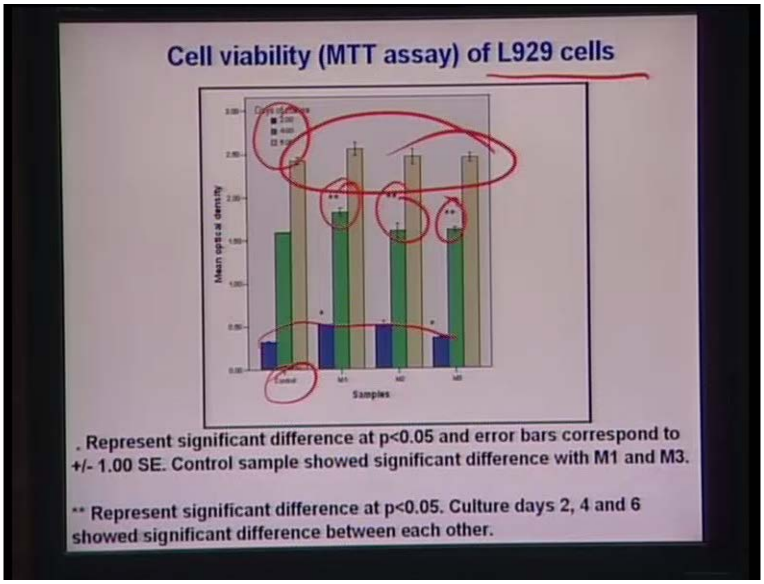

Cell viability (MTT assay) of L929 cells

. Represent significant difference at p<0.05 and error bars correspond to +/- 1.00 SE. Control sample showed significant difference with M1 and M3.

** Represent significant difference at p<0.05. Culture days 2, 4 and 6 showed significant difference between each other.

As far as the MTT assay is concerned, that is the cell viability testing, for L929 cells, that is the mouse fibroblast cells, what you see here, after 2 days, 4 days and 6 days culture, there is a significance difference in this MTT culture, MTT values compared to the control. Similar difference is also observed in the 4 days culture, and after 6 days culture, they are more or less comparable. So, what I am trying to emphasize here, by varying the fluorine content, you do not compromise much on the biocompatibility property, or cell viability property, because, you do see similar kind of MTT OD values, that is your Optical Density values, both after 6 days, 4 days and 2 days, with some cases, having some statistically, significantly, different value.

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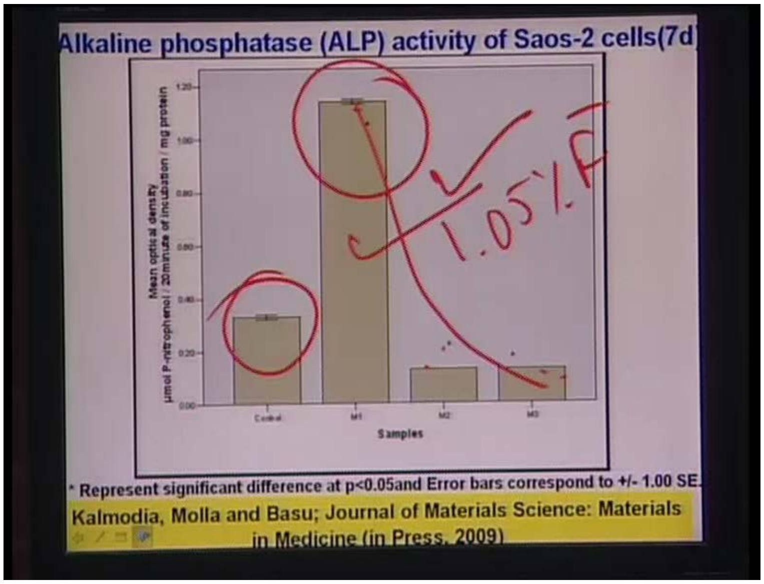

Alkaline phosphatase (ALP) activity of Saos-2 cells(7d)

* Represent significant difference at p<0.05and Error bars correspond to +/- 1.00 SE.

Kalmodia, Molla and Basu; Journal of Materials Science: Materials in Medicine (in Press, 2009)

As far the ALP expression, that is the Alkaline Phosphatase activity is concerned, now, in M 1 to M 3, we do see, that from the control to M 1, M 2 and M 3, in both the cases, corresponding to a control specimen, you do have significant, statistically significant difference. Now, other thing is that, you can notice that, in the case of M 1, the ostrigenic differentiation value is quite large; M 2 and M 3 it is comparable. So, what you see that, at lower, this is the 1.05 percent fluorine; so, at lower fluorine content, your ostrogenic differentiation is more expressed, or it is, it has much more value, compared to other glass ceramic composition.

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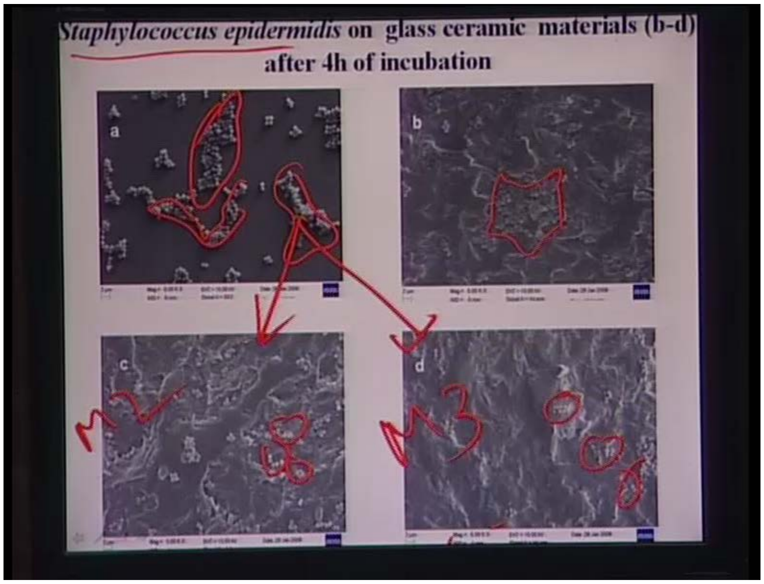

Staphylococcus epidermidis on glass ceramic materials (b-d) after 4h of incubation

As far as the antimicrobial property is concerned, we have seeded this staphylococcus epidermidis, that is the gram positive bacteria for 4 hours on this glass ceramic material. You can see very significant colony formation at different places in the control sample. Such colony formation is largely reduced, this is M 2 and this is M 3, with few bacteria only forming some clusters here and there, but this is certainly much improved in M 2 and M 3; whereas, in M 1, we do notice some aggregation of bacteria at different pockets in this microstructure region.

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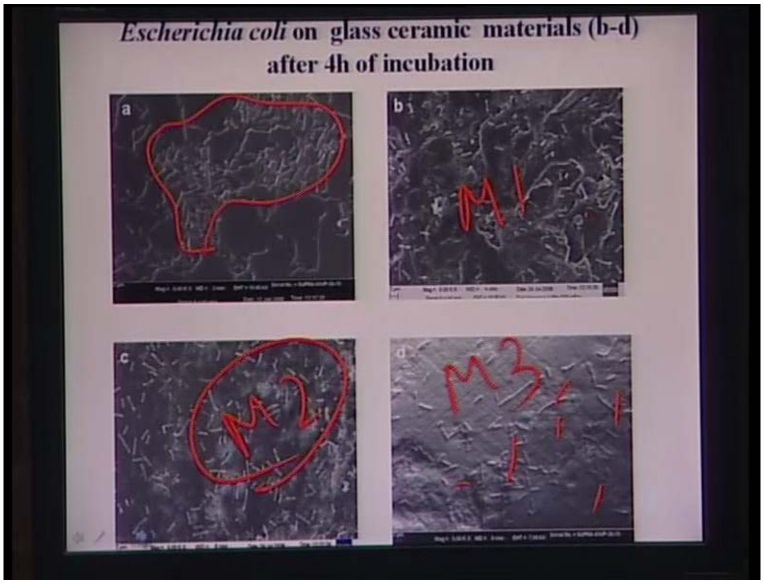

Escherichia coli on glass ceramic materials (b-d) after 4h of incubation

Similar observations were also made in case of the Escherichia coli, that E coli bacteria, after 4 hours of incubation. In the control sample, you do notice that, large aggregates, or large colony of the bacteria, that is formed; at M 3 surface, there is much less number of bacteria, compared to this is M 1, or this is M 2.

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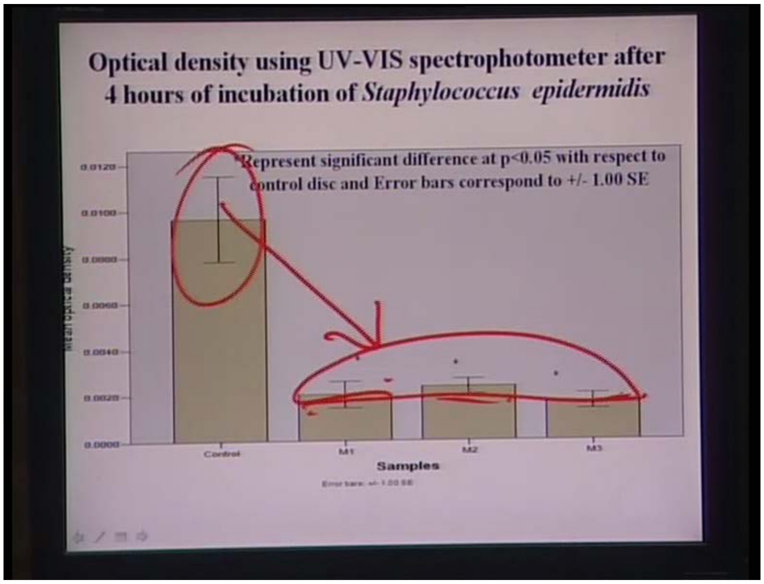

Optical density using UV-VIS spectrophotometer after 4 hours of incubation of *Staphylococcus epidermidis*

*Represent significant difference at $p<0.05$ with respect to control disc and Error bars correspond to +/- 1.00 SE

So, M 2 here is shown. It does not show much improvement in antimicrobial property, compared to M 3 or M 1 per se. That UV visible spectrophotometry values, that is optical density values tells you that, in the control sample, you have that very strong, the lack of any antimicrobial activity; whereas, the M 1, M 2, M 3, have significant, statistically different value, compared to the control sample; and from these values, you can say that, M 1, M 2, M 3, they are both equally having antimicrobial property. So, I think, I will stop here and then, in the next lecture, I will start with other recent values, other recent topics (( )).