Biomaterials for Bone Tissue Engineering Applications
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Materials Research Centre
Indian Institute of Science Bangalore
Module 7
Lecture 33

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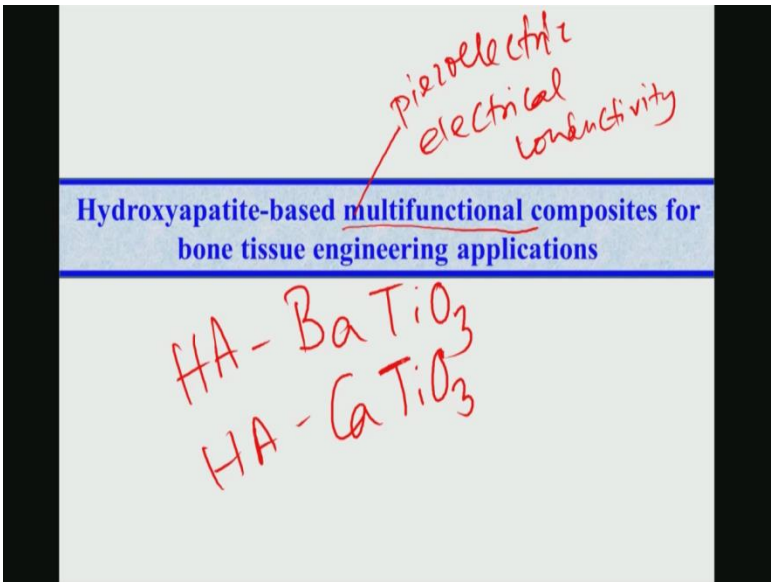

So, in this module I'll discuss that Hydroxyapatite based multifunctional composites I have emphasis that multifunctional because it does not only have a A biocompatibility property but also it has certain functional properties like piezoelectric properties, then electrical conductivity property and together with better toughness of this property with its composites. Now two classes of materials that we have developed in our laboratory as I said this is a series of case study, we have we will be I'll be presenting one after the other just to illustrate that opportunities for development of Hydroxyapatite based new composites we get a physical as well as um Biocompatibility properties.

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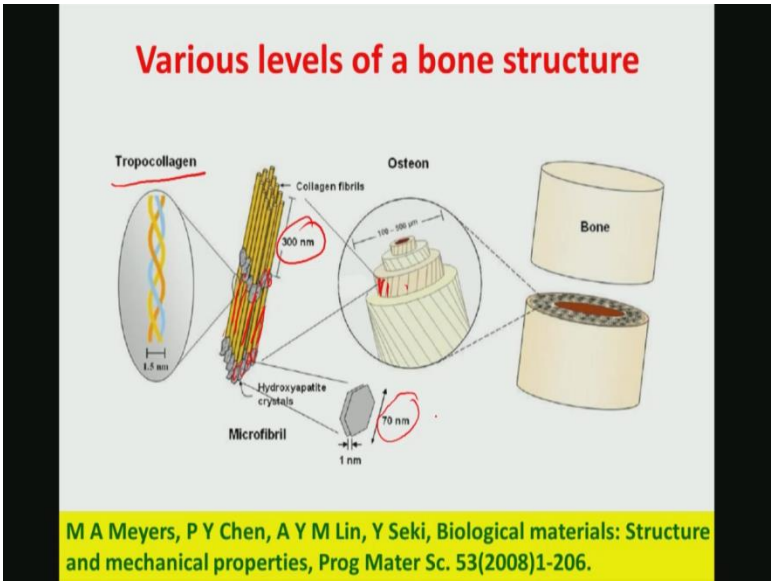

So, the first of this one is we will be discussing about the (())(1:18) these composites. So, now why a multifunctional properties is important in the natural bone structure this this slide is essentially describes you hierarchical structure of the bone what you see that bone is the composite of the Hydroxyapatite and collagen. So, this is a characteristics of procollagen that is the collagen triple helix pattern and this is this collagen bundles are oriented in this particular manner and then you have that Hydroxyapatite crystals and this Hydroxyapatite nano crystals were, the nano crystals typical dimension is 70 nano meter in the long axis and 1 nano meter is the thickness. So, this is the typical platelet shaped nano crystals this there embedded in hydro collagen bundle and then these together will form some osteon structure. This osteon structure is 100 to 500 micro meter diameter.

So, from this diameter you can from this dimension 300 nano meter and also this 70 nano meter how does the crystals you can see that what is the huge number of collagen fibril bundles are there micro fibril bundles are there in the osteon structure and this individual osteon structure is one of the structure in unit of the natural bone as it has been shown here.

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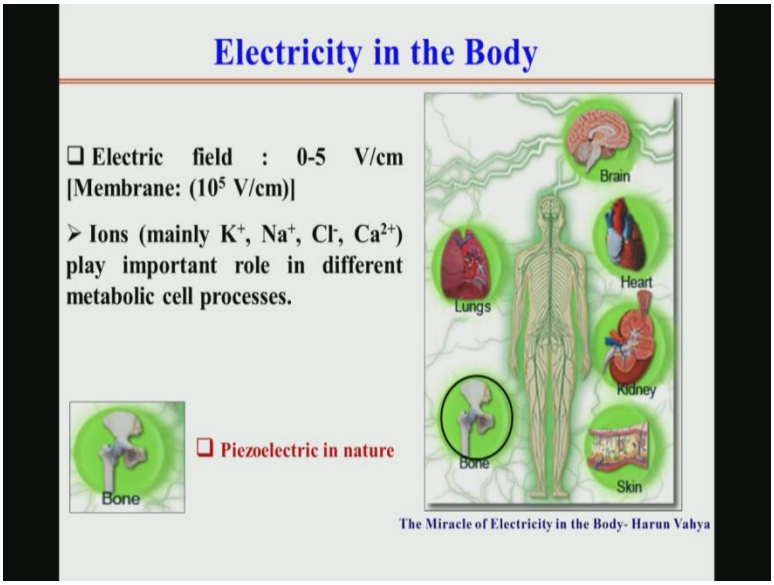

Now because of the presence of the collagen in the bone, bone has certain piezoelectric properties and conductivity properties. Hydroxyapetite is insulate in nature but hydroxyapatite actually contributes to the mineral content of the bone that means Hydroxy the presence of Hydroxide gives you gives the bone the elastic stiffness property as well as strength property as well as further physical properties.

Now, electricity in the bone typically you know biological cell it has the transmembrane potential and the transmembrane potential is fearly high because of the smaller thickness of the cell membrane. And this transmembrane potential is of the order 5 volt per centimeter. And there are several ions which have mentioned before, that cells itself has Sodium, Potassium channel as well as Calcium channels now because of the imbalance in the content of the ions and extracellular region with respect to the intracellular region this also plays the electricity in the bone.

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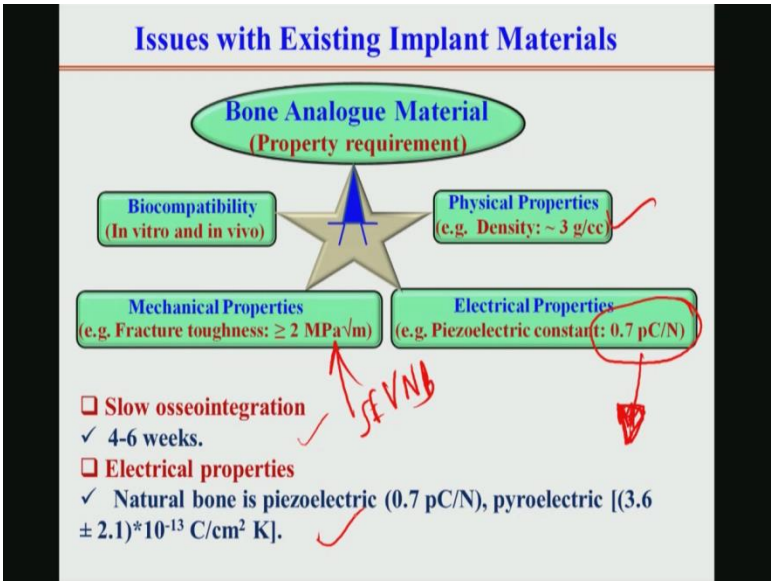

Now, these mechanical properties of the Hydroxyapatite which has been emphasized in the last module while discussing the hydroxide-titanium composites, so, let me repeat that. Typically bone material should have a fracture toughness of more high better than 2 mps square root meter and this should be used single H v notch that is that reliable fracture toughness. Whether it is 10 coefficient it is not that much demanding like functional applications it should be in a range of 0.7 picocoulomb per newton

And it should have a biocompatibility both In-vitra and In-vivo and physical and then other problem other problem other property that is required for bone replacement materials is the slowest integration like 4 to 6 weeks that is very slowest in most of the natural bone materials and second one natural bone is piezoelectric and pyroelectric in nature. So, pyroelectric coefficient also has been mentioned in the slide.

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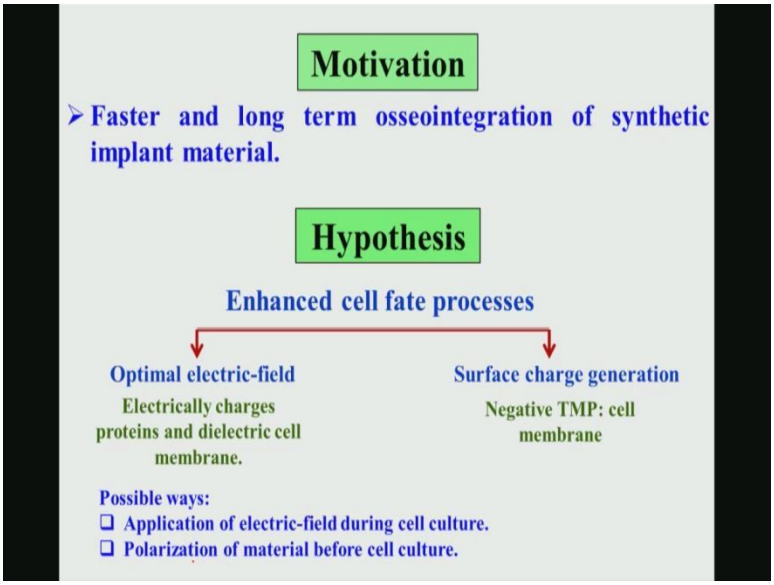

So, therefore the definition so, therefore the motivation is that you need to have a faster and long term Osteointegration synthetic implant materials. So, in this context what I'll show you next 20 minutes or so this part of the Spark Plasma Sintering of this, or related development of this Hydroxy Barium titanate composites. So, what is the, what are the issues in this Barium titanate Hydroxy based composite material, first is that if you need to optimize the functional properties you must retain Barium titanate in the microstructure.

Second one is that you should prevent the dissociation of Hydroxyapatite, because Hydroxyapatite can dissociate Tri Calcium Phosphate and various other phases, so those dissociation is to be prevented. So, A. Hydroxyapetite dissociation. B. Retention of Barium titanate. And these is very difficult to be accomplished which is very easier to be said. So, keeping that in mind these major two challenges what we have done, we have used Spark Plasma Sintering as a process to consolidate Hydroxyl Barium titanate composites and to retained them in the microstructure.

And what we have done we have (())(5:49) Spark Plasma Sintering which is not a very easy thing to adopt particularly in case of reactive system like Hydroxyl Barium Titanate. So, what we have done we have first heated this materials up to 670 degree Celsius in a an adopted a multi staged spark Spark Plasma Sintering, so 670 degree Celsius we adopted then

we have seen we have the sample at 850 degree Celsius followed by 950 degree 0 minute and then followed by cooling in room temperature.

So, why why this kind of sintering strategy was adopted because essentially you are not giving the system enough time to react. So, if you hold them for if you do not hold them at 950 degree Celsius you simply push it to 950 and cool it down. So, thereby you are essentially restricting any sintering reaction between Hydroxyapatite and Barium Titanate and that is what has been done here. And then second thing that we have mentioned is that there is two stage it is also possible that one is the 670 degree Celsius and 850 degree Celsius followed by cooling to room temperature.

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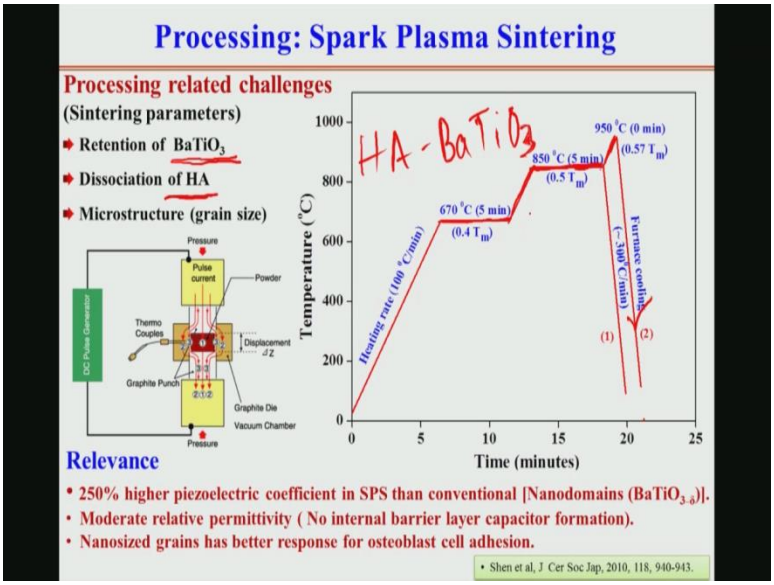

So, this is the Spark Plasma Sintering that we have I've, I've mentioned you before while discussing that ceramic processing.

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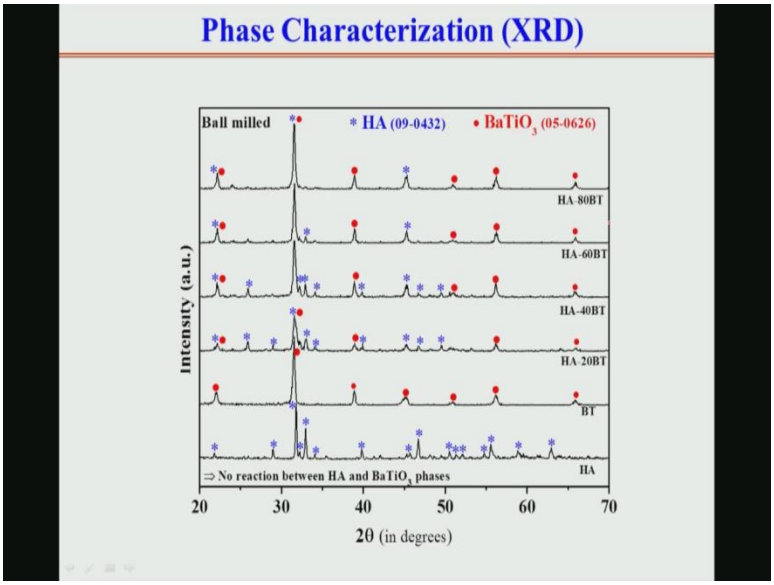

Now we have done this hydroxyapatite Barium Titanate composite at different temperatures then after that when we have done this extra diffraction, we have seen some minute amount of Beta Tri Calcium and Alpha Tri Calcium Phosphate as well as Calcium Titanate. So, it does show small detectable amount of these phases. A

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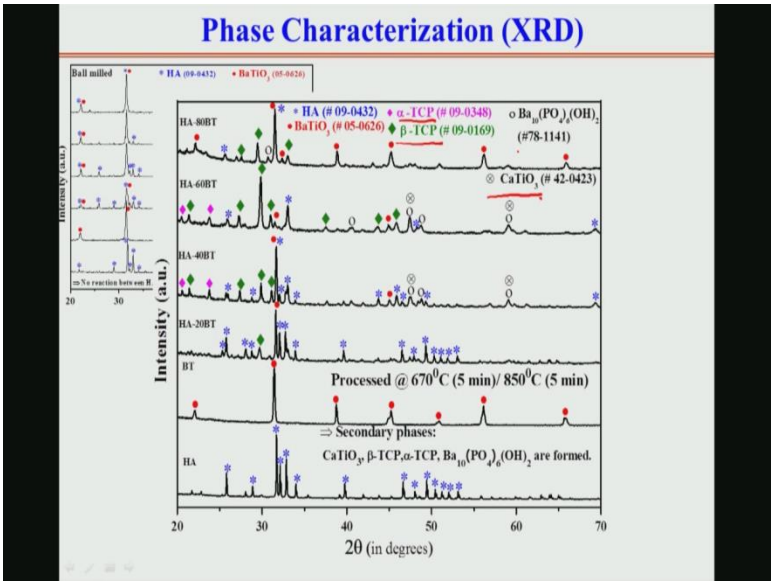

nd then we trying to understand that how these detectable formation of this detectable amount of Calcium Titanate and other phases can be explained. So, therefore we have proposed these kind of thermo dynamic reactions where Hydroxyapatite can be dissociate to

Calcium Phosphate and Calcium Phosphate can react with Barium Titanate to form Calcium Phosphate and Calcium Titanate and Barium Oxide and then Calcium Phosphate also can react with Barium Titanate what the beauty of this reaction you can see that it forms Ba10 (Po4)6 (OH)2 that means barium will simply replace the Calcium and it forms this one and then as a result Calcium also will replace Barium Titanate to form this Calcium Titanate. So, this is kind of an exchange reaction kind of reaction that is that can potentially take place during sintering.

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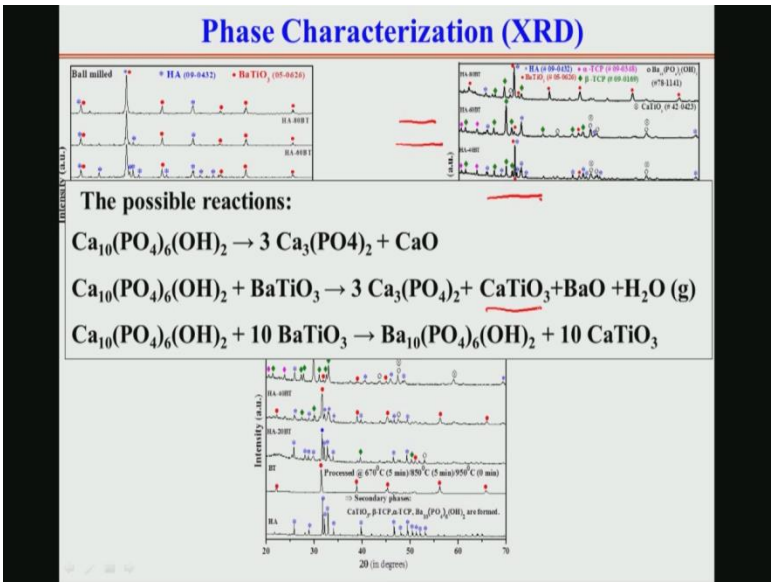

Now, these second phase formation also has been observed in this (())(8:25) transmission, microscopy observation you see in this SADP pattern analysis. Now what you see in the SADP pattern analysis, you see hydroxyapatite is one and barium Titanate is second phase and this one and second phase you can see that they are essentially formed and then that can be confirmed using that selected area diffraction pattern analysis.

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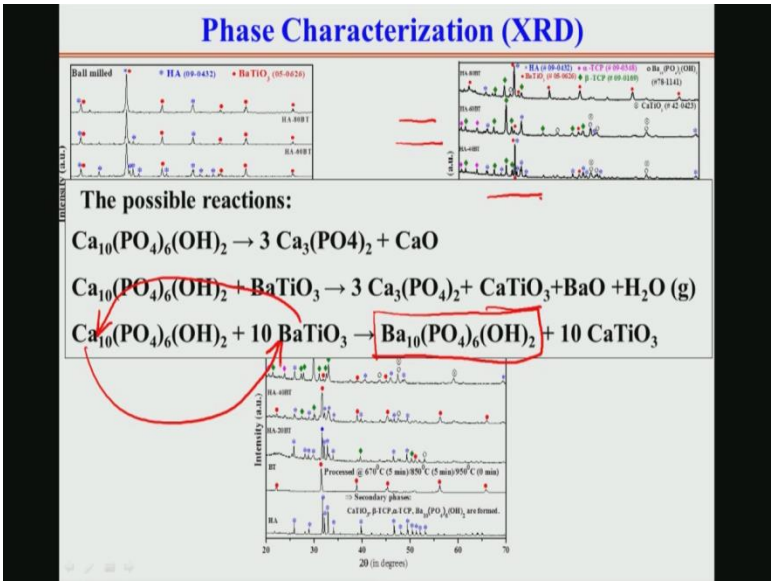

Now, all these functional properties that we have measured that includes dielectric constant, see electrical conductivity, piezoelectric scale B3 values and pyroelectric coefficient. What we have observed here that depending on the Barium Titanate content the Hydroxyapatite matrix is pyro and piezoelectric strain coefficient can go upto 0.7 hollows and this 0.7 is close to that of the 30% of barium Titanate. But we have got that few composition 20%, 40%, 60% and this 0.7 piezo strain coefficient is somewhere in the middle. The same is true for that pyroelectric strain coefficient and conductivity, but whatever you can see here that bone like conductivity or piezo strain coefficient is indeed possible to get in in this Hydroxyl Barium Titanate system with as low as 3o to 40 % of Barium Titanate that you can achieve very comfortably that bone mimicking properties. So, this is the major take home message from this slide.

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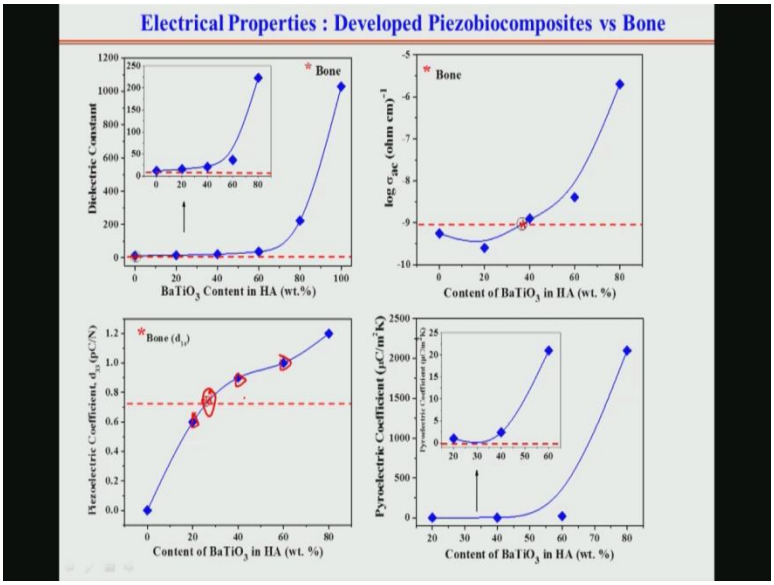

Now, other things that we have measured as part of the physical property measurements like thickness, hardness and fracture toughness of this materials and hardness and fracture toughness as you see that this is this shows very normal moderate values first of all fracture toughness was measured here is indentation toughness not SEVNB and it does not really show very high indentation toughness like 1 to 1.1 mpa square root meter where as beaker hardness is around 6.5 giga pascal beakers.

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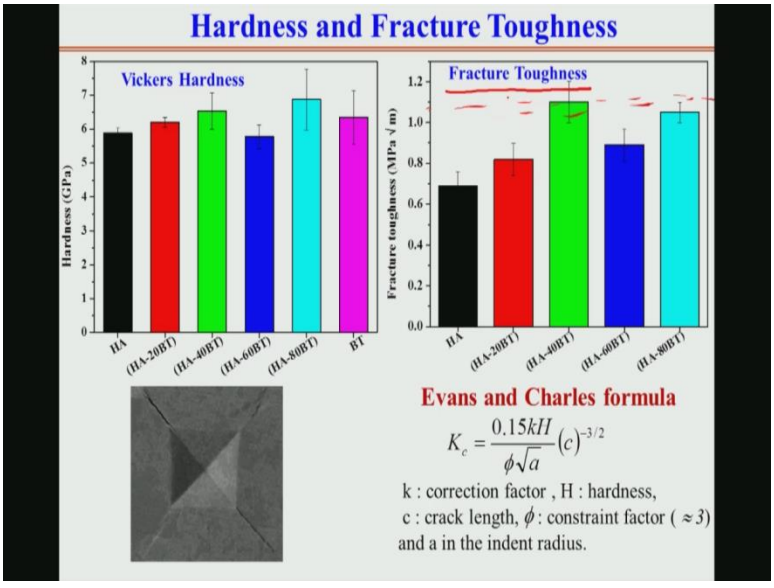

Now the compressive strength compressive strength of this materials can go up to 138 megapascal which is which is quite a respectable number in the ceramics community, flexural strength is also more than 125 megapascal fracture toughness SEVNB is low. It is less than 1 mpa square root meter. In an earlier module I've mentioned that fracture toughness Hydroxyapatite composite can be significantly enhance too close to 5 mpa square root meter, when we when one can add 10% of titanium.

So, it's such a small percent of Titanium addition fracture toughness can go almost 5 times higher than what we have achieved here in case of Hydroxyapatite Titanium composite. So, physical property wise certainly this material is not at the very high level but certainly what we see here that compressive strength, flexural strength these kind of strength combination is quite moderately higher than even other materials not even not not only hydroxyapatite titanium.

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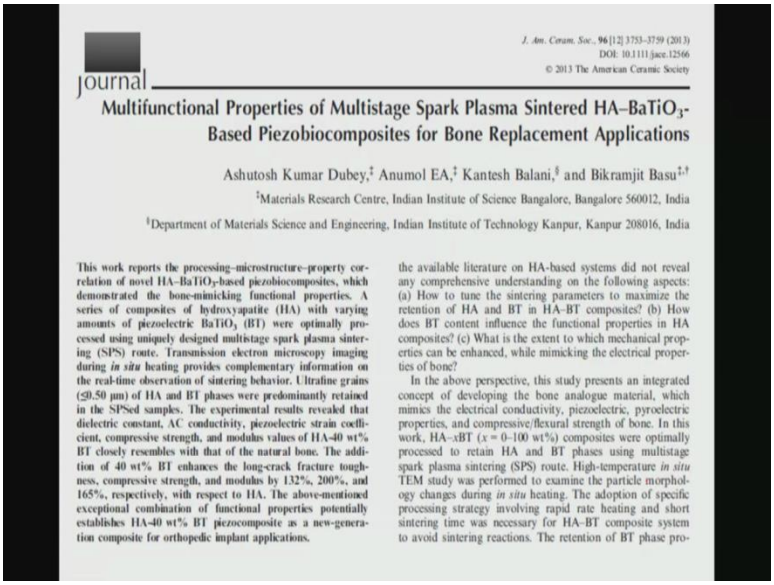

So, these work is published in Journal Ceramic Society in 2013 and one can see that what is that typical mode details of this whatever I have discussed now.

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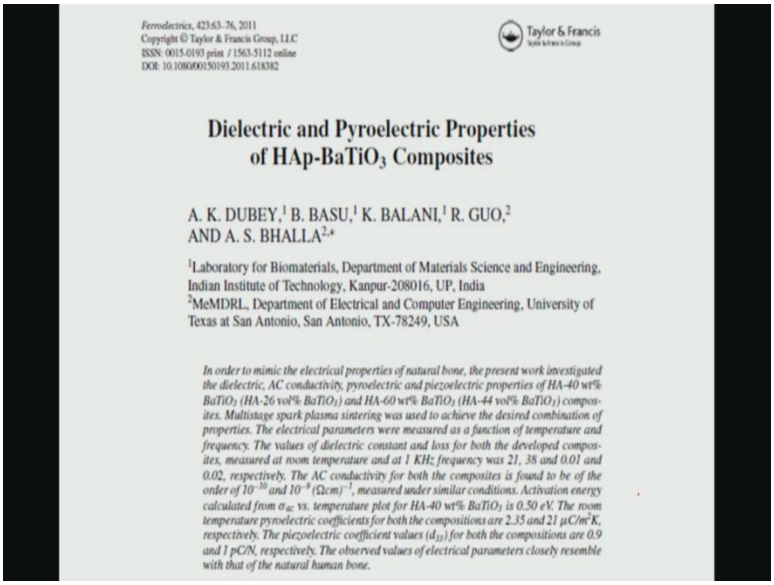

Now dielectric and pyroelectric properties are also published here in another journal of which is ferroelectrics and this is again in collaboration with some US group.

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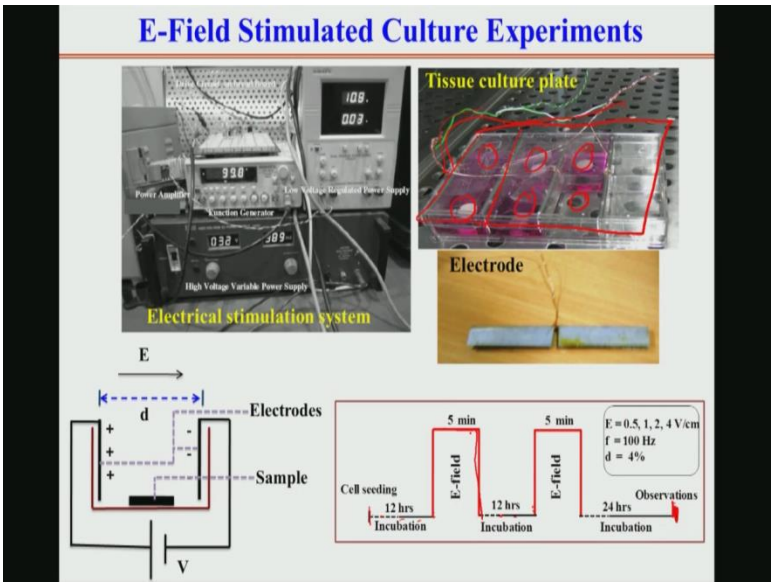

Ok. Now one of the things that we have been pursuing in our group for quite sometime is electrical field stimulated culture experiments. Now what we notice here that this materials which are piezoelectric in nature here you can apply this you can grow the cells and you can apply electric field stimulation for a certain amount of time 5 minutes or so and up to 48 hours in culture foundations and then you can see that what is the difference in the cell

proliferation or cell viability or cell growth pattern with respect to unstimulated cells like those which do not receive this electrical stimulation.

Typical experimental set up you can see that is the (())(12:33) plate and in this (())(12:35) plate Hydroxyapatite materials are placed in individual culture plate and then you can put the culture medium and then you can put some you know you can you can put some wire and through this wire then you can give this electrical stimulation.

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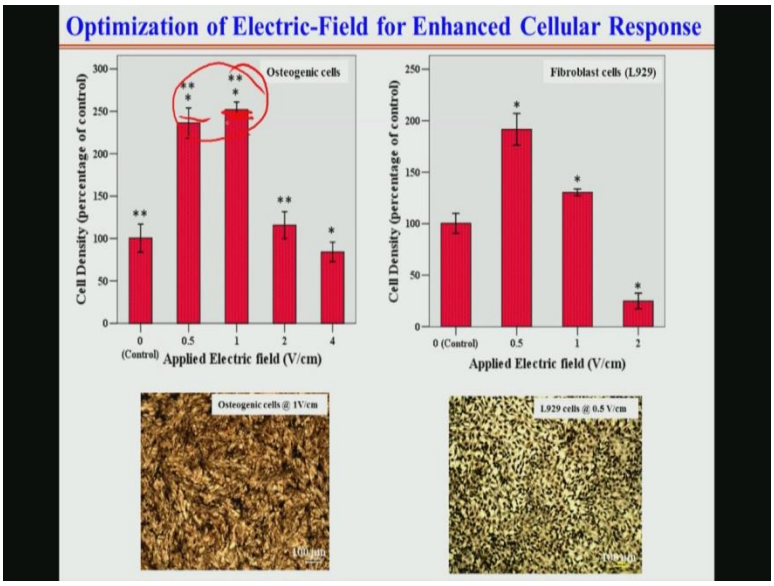

Now when it is when it is kind of when the cells have been cultured up to they are different field strain conditions like 0.5 and 1 hole per centimeter and you can see at 0.5 and 1 hole per centimeter it shows the statistically significantly higher number of cells so, essentially it shows that the cell growth goes to a maximum at a very narrow window 0.5 to 1 hole both for osteogenic cells and fibroblast cells that is L9 to 9 cells.

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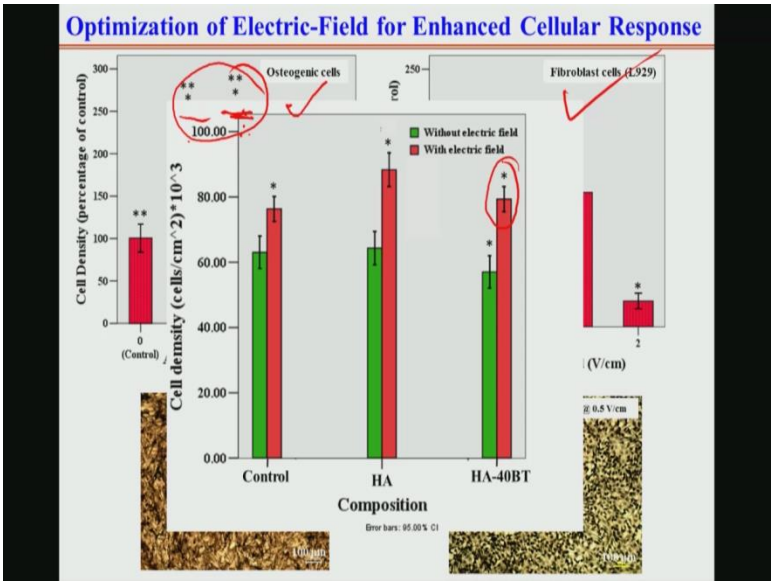

And then when it is grown without electric field and with electric field it also shows that Hydroxyl Barium Titanate it shows that higher density with respect to unstimulated cells like which do not receive any electrical stimulation.

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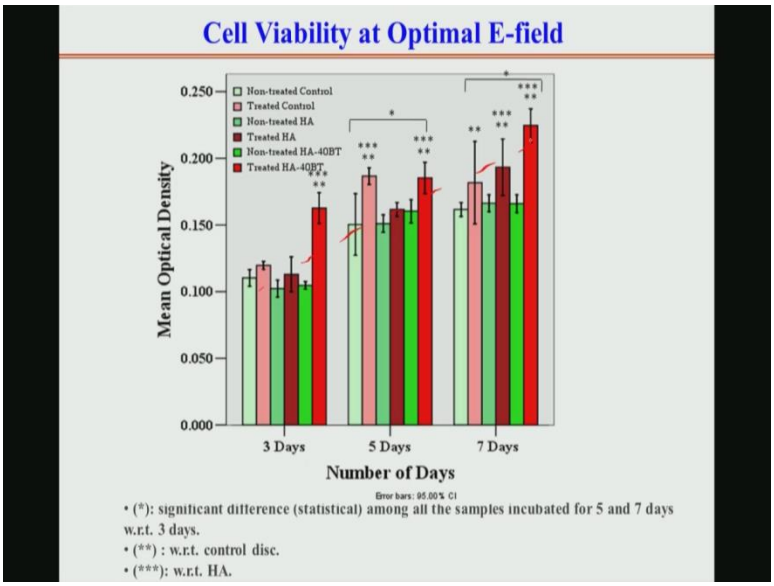

Ok. Now, one of the things that is that is a valid question in the cell biology field that when you give this electrical stimulation and when you grow the cells for different time point in culture like 3 days, 5 days and 7 days so, whether these after that cells receive the stimulation their growth pattern changes because they they are given certain stim biophysical stimulation

so, to address this we have grown this cells after they receive the electric stimulation for different time point in culture and we have seen that there is they show kind of a standard linear increase in the entity optical density value essentially showing that mitochondrially active cells or cell viability is essentially increases with increases time point in culture so there is no alteration in the growth pattern because of this electrical field stimulation.

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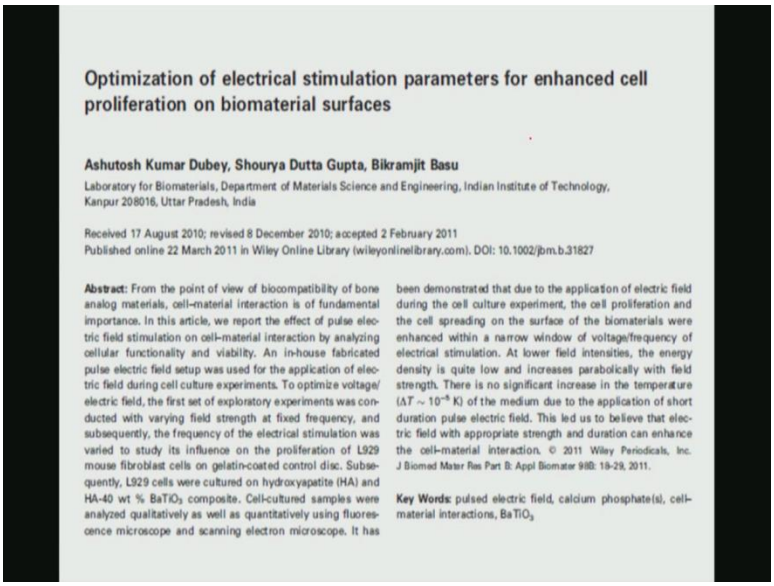

Optimization of electrical stimulation parameters for enhanced cell proliferation on biomaterial surfaces

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Abstract: From the point of view of biocompatibility of bone analog materials, cell–material interaction is of fundamental importance. In this article, we report the effect of pulse electric field stimulation on cell–material interaction by analyzing cellular functionality and viability. An in-house fabricated pulse electric field setup was used for the application of electric field during cell culture experiments. To optimize voltage/electric field, the first set of exploratory experiments was conducted with varying field strength at fixed frequency, and subsequently, the frequency of the electrical stimulation was varied to study its influence on the proliferation of L929 mouse fibroblast cells on gelatin-coated control disc. Subsequently, L929 cells were cultured on hydroxyapatite (HA) and HA-40 wt % $BaTiO_3$ composite. Cell-cultured samples were analyzed qualitatively as well as quantitatively using fluorescence microscope and scanning electron microscope. It has been demonstrated that due to the application of electric field during the cell culture experiment, the cell proliferation and the cell spreading on the surface of the biomaterials were enhanced within a narrow window of voltage/frequency of electrical stimulation. At lower field intensities, the energy density is quite low and increases parabolically with field strength. There is no significant increase in the temperature ($\Delta T \sim 10^{-6}$ K) of the medium due to the application of short duration pulse electric field. This led us to believe that electric field with appropriate strength and duration can enhance the cell–material interaction. © 2011 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater 98B: 19–29, 2011.

Key Words: pulsed electric field, calcium phosphate(s), cell–material interactions, $BaTiO_3$

Well, again this is published in JBMRB in 2011.

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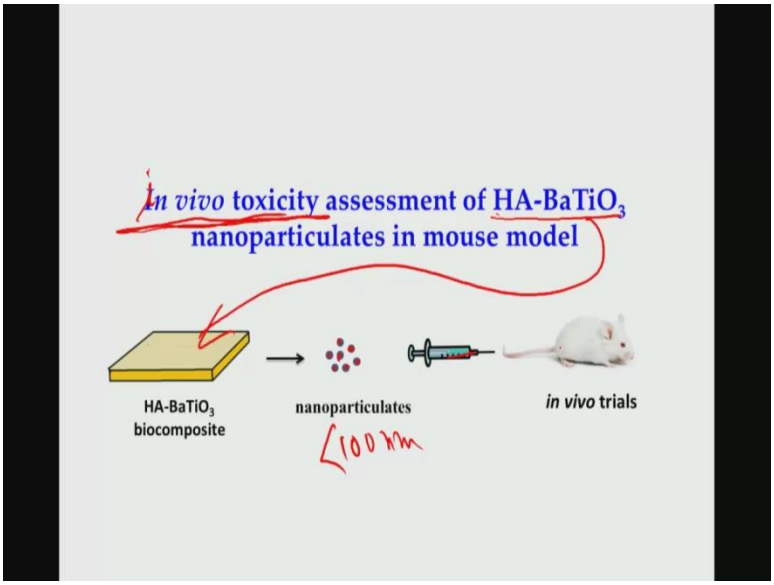

Ok. Now, one of the things that is again a kind of a from bio medical point of view again quite important to be addressed is In-vivo toxicity. Now to show this In-vivo toxicity of this Hydroxyl Barium Titanate we have not only done In-vitro studies but also we have done In-vivo In-vivo mouse model study. So, what we have done we have taken this Hydroxyl Barium Titanate pallets now this is your Spark Plasma Sinter pallets, we have done some mechanical crushing and grinding and in a very tailored manner using ball milling and so on.

Then we get this small small particles nano particles like and this particles is less than 100 nano meter in size. Then disperse them in Phosphate Buffered Saline solution like PBS solution and after that we have injected in the intra-articular region of the mouse model.

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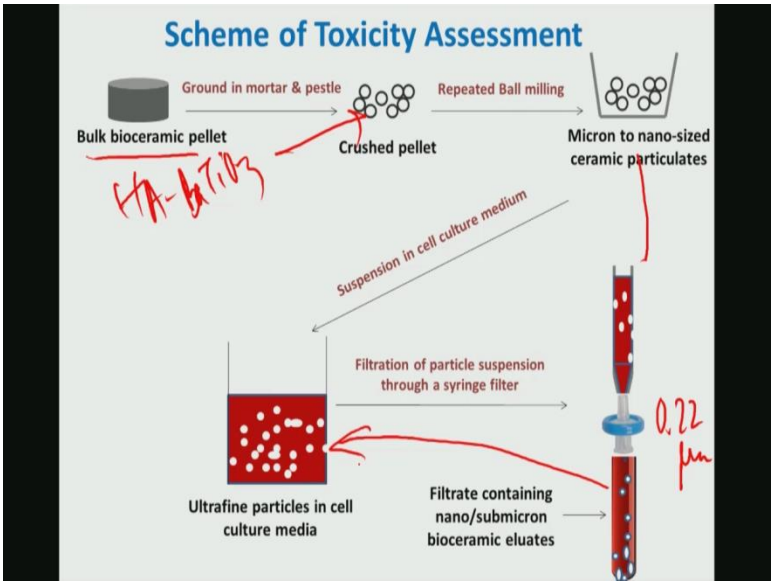

And this is the scheme of the things that I have shared that first is this Hydroxyl Barium Titanate pallet and then from there you get this finer particles crushed pallets and then after that micro sized ceramic particles, you filter it using this 0.22 micron filter and so that you get a very fine particles which are dispersed in the solution and then you can do you can grow the cells or you can inject them into the animal model.

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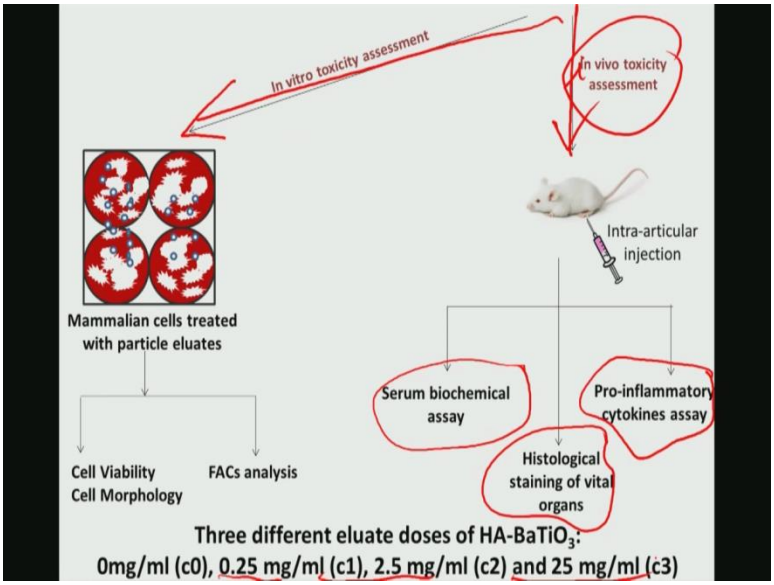

So, this is exactly what I said that you first grow them and grow the cells mammalian cells treated with particle eluates or you can do this In-vivo toxicity assessment by intra-articular injection and after that intra-articular injection you can do cerum biochemical asses histological staining of the pro vital organs and pro-inflammatory cytokines assay. In this particular case, we have used 3 different kind of a eluate concentration in they are like 0.25 miligram per mili litre, 2.5 mili gram per mili litre, 25 mili gram per mili litre essentially showing that 10 out of 100 some magnesium should higher and higher concentration of the cell waste and then injected.

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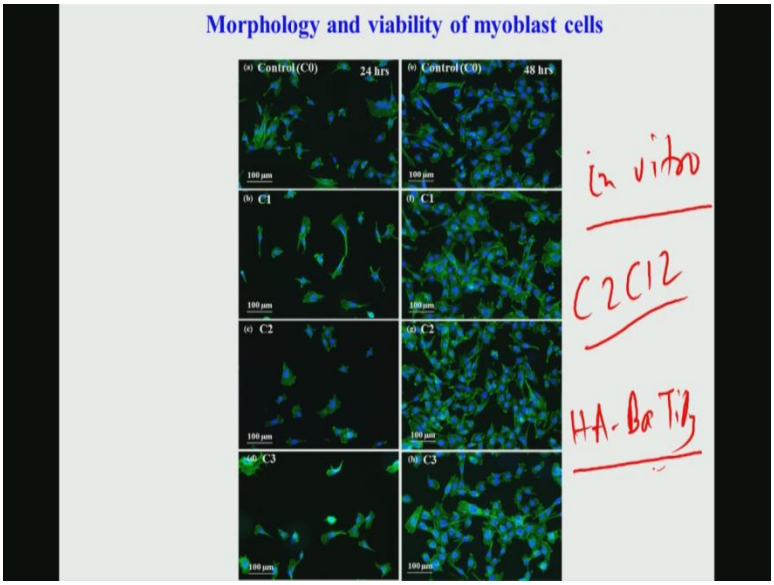

First is the cell viability this is the In-vitro study so, we have taken that you know Osteogenic cells and then we have grown particular this is I think C2C C12 Myoblast cells from the murrain type so, from the mouse Myoblast cells we have grown them and when the particles have suspended Hydroxyl Barium Titanate particles are suspended in a culture medium and what we have seen that they do not show any adsorbable toxicity reduction in the cell viability in this kind of materials.

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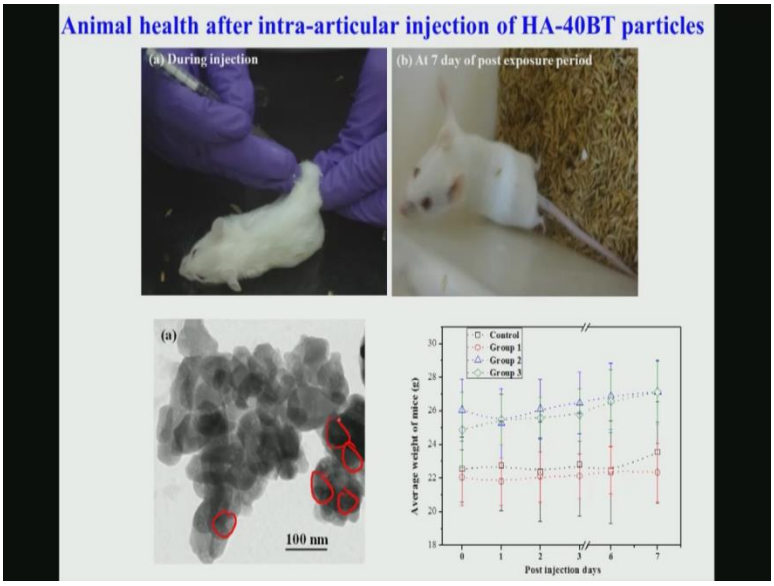

Now, when these materials are injected into the mouse model and this is just showing that you know veterinary surgeon is giving this intra-articular injection in the mouse and this is the particles that we have done TM so, essentially all the particles is less than 100 nano meter. Then we have taken each day after the post injection post exposure period each day taken the weight systematically just to show that weight does not decrease or the animals actually maintain their standard health conditions.

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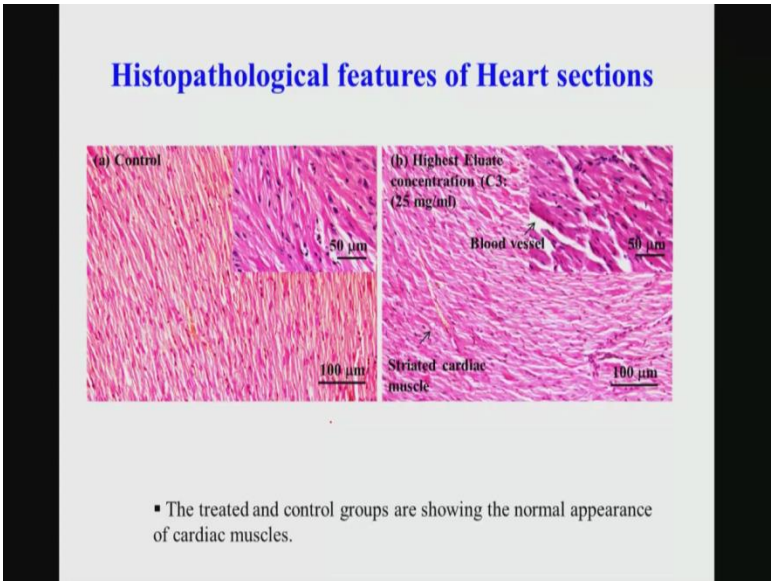

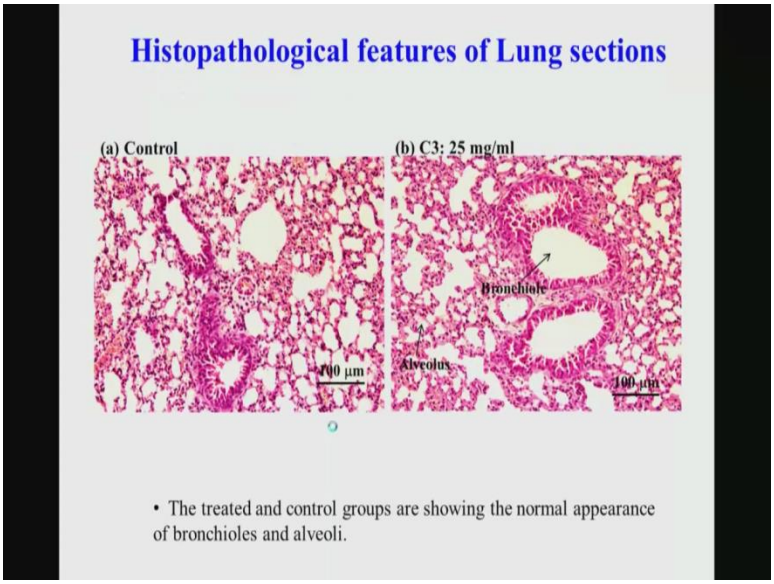

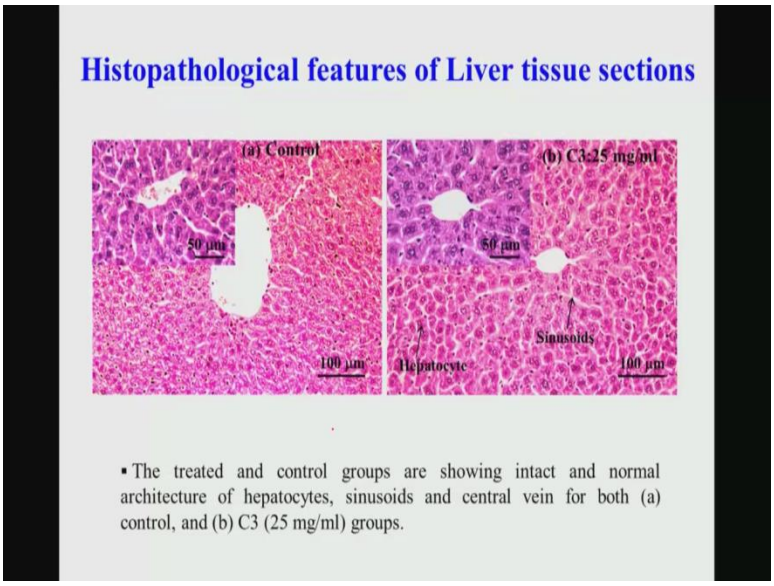

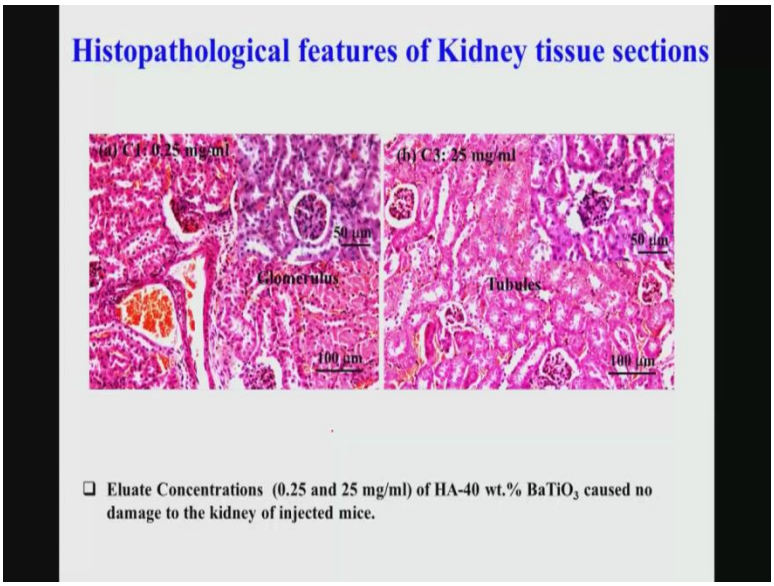

After that we have taken the vital organs from each of this animal model and then we have done the theme sections of the vital organs like Heart, Liver, Lung and Kidney and also Spleen. So, in all these histology sections if you see they show a normal tissue architecture and then suddenly this particular case it shows the normal appearance of the cardiac muscles and we did not see any which you can clearly see blood vessels and striated cardiac muscles and so on but we did not see that particles are distributed and causing any inflammation.

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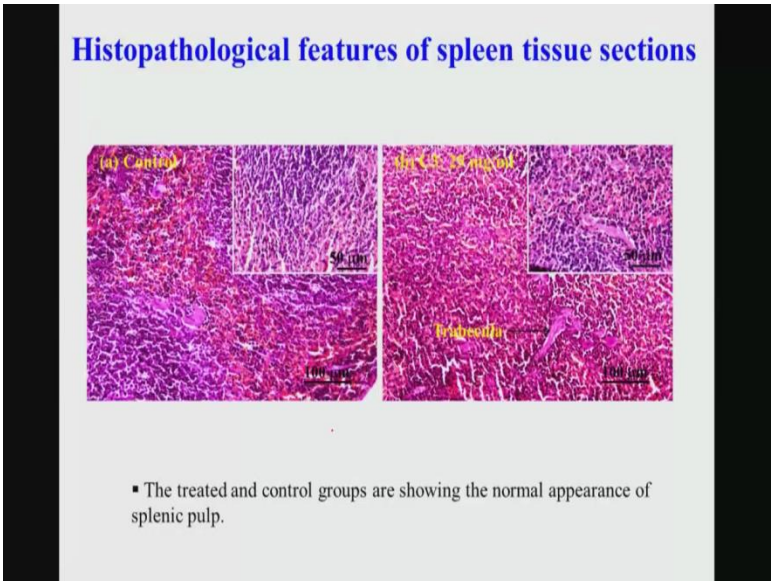

The same is true for long sections, so, what we have compared we have compared the control like which did not receive any Hydroxyl Barium Titanate particulates and which we which we receive that Hydroxyl Barium Titanate particulates and then for the comparison I am showing you the largest amount of the Hydroxyl Barium Titanate which is received like 25 mili gram per mili litre. So, in all these things if you see that it shows that normal appearance of Broncholi and Alveoli in this histology thin sections. The same is true for Liver so, there is no abnormal histological features or irregular histological features or inflammation which one can immediately notice.

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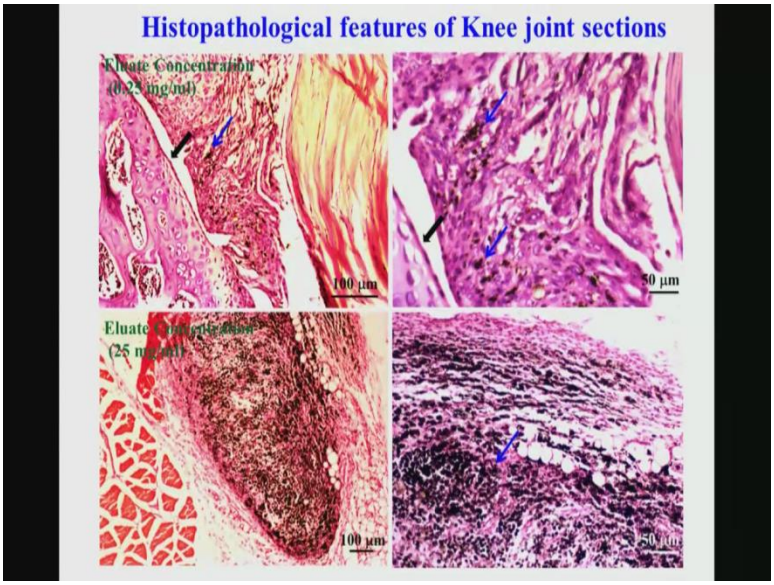

In the Spleen sections also similar things. Now, when it comes to the Knee joint sections where this Hydroxyl Titanium Barium particulates are injected I have put some arrow here and what this arrow essentially indicate, this indicates the accumulation of the Hydroxyl Barium Titanate particulates and these are like distributed in large regions and this Barium plated particulates are distributed particularly along the particular region. We did does not show very altered cell morphological features in this particular tissue sections but certainly it shows that this particulates are not uniformly bio distributed in different organs instead they are accumulated in the knee joint sections.

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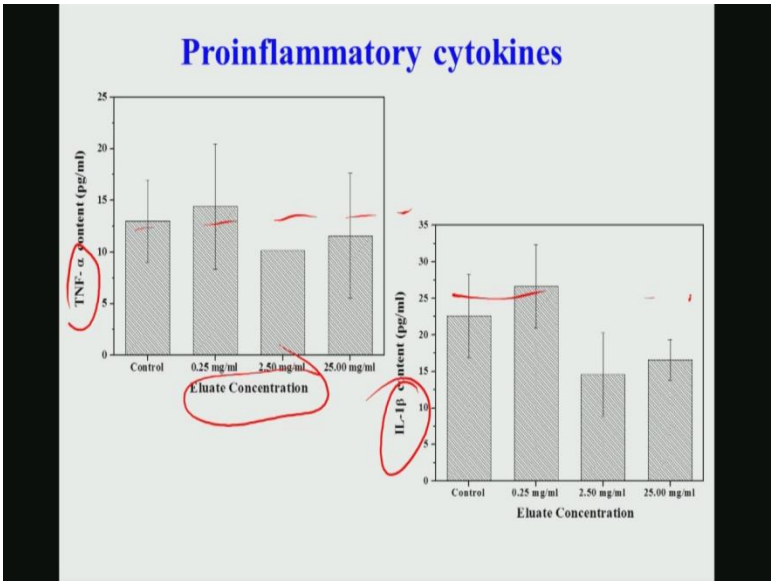

Now in terms of the pro-inflammatory cytokines expression we have done for the TNF Alpha as well as that IL 1 Beta expression and again we did not see any eluate concentration depended any statistically significant difference in terms of that that pro-inflammatory cytokine expression.

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BIOCOMPATIBILITY STUDIES

Absence of systemic toxicity in mouse model towards $BaTiO_3$ nanoparticulate based eluate treatment

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Abstract One of the existing issues in implant failure of orthopedic biomaterials is the toxicity induced by the fine particles released during long term use in vivo, leading to acute inflammatory response. In developing a new class of piezobiocomposite to mimic the integrated electrical and mechanical properties of bone, bone-mimicking physical properties as well as in vitro cytocompatibility properties have been achieved with spark plasma sintered hydroxyapatite (HA)-barium titanate ($BaTiO_3$) composites. However, the presence of $BaTiO_3$ remains a concern towards the potential toxicity effect. To address this issue, present work reports the first result to conclusively confirm the non-toxic effect of HA–$BaTiO_3$ piezobiocomposite nanoparticulates, in vivo. Twenty BALB/c mice were intra-articularly injected at their right knee joints with different concentrations of HA–$BaTiO_3$ composite of up to 25 mg/ml. The histopathological examination confirmed the absence of any trace of injected particles or any sign of inflammatory reaction in the vital organs, such as heart, spleen, kidney and liver at 7 days post-exposure period. Rather, the injected nanoparticulates were found to be agglomerated in the vicinity of the knee joint, surrounded by macrophages. Importantly, the absence of any systemic toxicity response in any of the vital organs in the treated mouse model, other than a mild local response at the site of delivery, was recorded. The serum biochemical analyses using proinflammatory cytokines (TNF-α and IL-1β) also complimented to the non-immunogenic response to injected particulates. Altogether, the absence of any inflammatory/adverse reaction will open up myriad of opportunities for $BaTiO_3$ based piezoelectric implantable devices in biomedical applications.

1 Introduction

The adverse biological/clinical implications of wear debris particles, such as toxicity, early/late aseptic loosening at the prosthetic implant joints have become a prime concern for the stability of orthopedic implants, as this often results in osteolysis due to the activation of macrophages [1–4]. The particles released from a biocompatible bulk material due to friction/wear/corrosion/in vivo degradation can cause acute or chronic inflammation that depends upon the physical properties and chemistry of the particles as well as the immunological response of the host cells [5, 6]. The fine (<2 μm) wear particles can be translocated to the other organs via systemic circulation, which can penetrate the plasma membrane and lead to cell toxicity [7–9]. Wang et al. [10] demonstrated that intra-articular injection of TiO_2 nanoparticles with the concentrations of 2 and 20 mg/ml in the knee joints of rats has toxic effect in the vicinity of the injected region. In addition, the injected TiO_2

So, this work is published in (())(20:40) in medicine journal.

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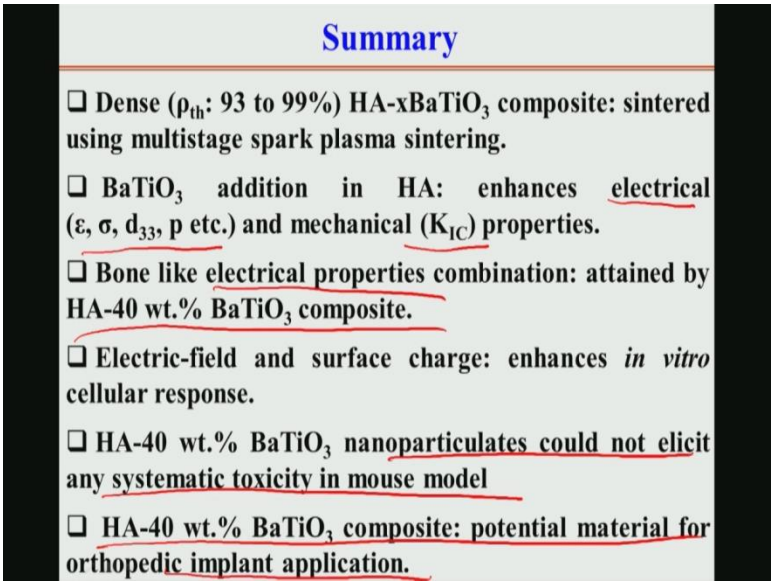

So, in summary Barium Titanate additional hydroxyapatite moderately enhances the electrical properties that is the dielectric constant, piezoelectric strain coefficient as well as the conductivity property and to moderate extent it increases a reliable fracture toughness property like 0.86 or 0.9 mpa square root meter. And this bone like electrical properties were particularly obtained in case of the hydroxyl 40% penetrated composite and this nano particles did not elicit any systemic toxicity in the mouse model and therefore this materials can be potentially used as an orthopedic implant applications.

So, what we need to do is that we need to see that how Barium Titanate materials which is electrically conductive they can be implanted in the mouse model and in that particular bone model and then we can see we can keep some electrical stimulation and we can see that without this Barium Titanate part materials Barium Titanate containing materials can induce very oddly Osteo integration. Oddly Osteo integration means normal bone healing process takes place in human beings is certainly around 10 to 12 weeks.

So, whether we can shorten this bone healing time to 3 to 4 weeks using this electro conductive materials so, those study and experimental related pre-clinical trials are currently under way in our laboratory at in a subside and certainly all these studies will point towards the fact that relevance of the electrically conducted materials together with electrical stimulation indeed is helpful for the oddly bone oddly Osteo integration in animal model.

And finally this materials are bio-medically safe and that is very clear and then this does not show any toxicity in the animal model experiments but certainly some long term animal model experiments is necessary and that can be conducted in the rabbit model.

Thank you.