Development, characterization, and performance analysis of eggshell derived calcium phosphate

Summary of the Thesis

Submitted

By

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Doctor of Philosophy (Engineering)

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1. Title of the thesis:

"Development, characterization and performance analysis of eggshell derived calcium phosphate"

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INTRODUCTION:

Egg shell, a natural biogenic resource contains large amount of calcium ion. The hydroxyapatite ceramics have been prepared from egg shell waste. Hydroxyapatite being a bioactive biomaterials find wide applications in the biomedical field. The bone defects caused by wound or osteoporosis conventionally treated with hydroxyapatite as a bone grafting materials. In this present work, different doped variants (3% and 5% of Zinc, Magnesium and Titanium) of hydroxyapatite from laboratory grade calcium hydroxide (synthetic source) and egg shell (biogenic source) have been prepared. For the systematic study, six variants of doped Pure HAp (synthetic source) and Egg shell HAp (biogenic source) were used. A total of fourteen variant samples were prepared and the comparative assessments were done. Investigation have been carried out to find out some important characteristics which are essential for medical applications. These include green density, sintered density, lattice parameter, mechanical properties, contact behaviour in Stimulated Body Fluid (SBF), haemolytic properties, and cytotoxicity.

The tendency of new apatite formation on the surface of Pure HAp, Egg shell HAp and their doped variants were also studied and compared with Simulated Body Fluid (SBF) to evaluate their interaction with human body fluid. The main focus of this work is to study the performances of eggshell sourced hydroxyapatite and their doped variants over pure HAp and their dopants on bone regeneration. The assessment of bone regeneration for pure and doped (5% Ti-Egg shell HAp) samples were evaluation through implantation in femoral bone defect model (New Zealand white rabbits). The bone regeneration after 1- and 2-months post implantation were done through histological, clinical radiological, fluorochrome labelling and scanning electron microscopy (SEM) study. The fluorochrome labelling and clinical radiological study revealed in reduced size for 5% Ti-Egg shell HAp implant indicating accelerated new bone formation in comparison to other groups. The SEM study observed a less interfacial gap between implant and bone.

EXPERIMENTAL DETAILS

Preparation of Synthetic and Biogenic HAp:

The bio-sourced HAp power was prepared from calcined egg shell (termed as Egg Shell HAp) and synthetic HAp powder (termed as Pure HAp) from laboratory grade calcium hydroxide following wet chemical precipitation process. Egg shells after collecting from the canteen, was washed with boiled water several times to remove the contaminants. In a crucible, the dried egg shells were crushed into small pieces and then calcined at 800^0C for 2 hrs in a muffle furnace. The required amount of dried calcined egg shell powder was added in steps with a spatula to distilled water in a beaker. The mixing was done through magnetic stirrer with hot plate (REMI India) at 80^0C for about 30 min. The stoichiometric amount of orthophosphoric acid (0.6M) was added to the beaker very slowly from a stop cock fitted flask with a duration of about 45 min. Continuous stirring by magnetic stirrer was done to get a homogeneous mixer. After completion of the mixing, it was kept aside in cool and dark place for 24 hrs to settle down the precipitate and then filtered. The filtered precipitate was taken in Petri dishes and dried in hot air oven at about 40^0C for 48 hrs. The dried precipitate was calcined at 800^0C for 2 hrs. The obtained Egg shell HAp was ball milled to get fine particles. Similarly, Pure HAp was prepared from laboratory grade calcium hydroxide.

Preparation of Doped Hydroxyapatite:

For preparing the doped variants (zinc, titanium, and magnesium) of both Egg shell HAp and Pure HAp, required quantities of zinc oxide (ZnO, 99% pure), titanium oxide (TiO_2,99% pure) and magnesium oxide (MgO, $6H_2O$, 98% pure) was mixed to the calcined egg shell powder or

calcium hydroxide powder before addition to the acid solution. Here 3% and 5% doped variants of both Egg shell HAp and Pure HAp had been prepared. After forming the doped samples in solution, it was kept aside for ageing and then filtrate the precipitated using Buckner funnel. The filtrate cake was placed in Petri dishes and dried in hot-air oven at about 80^0C. The resultant dried samples were calcined at 800^0C in muffle furnace with a heating rate of 5^0C/min.

Powder Compaction:
The developed pure HAp, Egg shell HAp and their doped samples were hard pressed uniaxially in a steel mould having 12.5 mm internal diameter. A pressure of 2-ton for 2 min was applied through PEECO hydraulic pressing machine (PEECO Pvt Ltd, M/C NO.-3/PR-2/HP-1/07-08). The green density of the pellet samples were measured and then sintered at 950^0C and 1050^0C in furnace for 2 hrs.

Average Grain Size and Pore Size:
Perfect Screen Ruler Software was used to measure the pore size and grain size of the samples. For this measurement, five random SEM pictures of each sample at same magnification were captured.

Porosity Measurement:
Porosity plays a crucial role in implantable bio ceramic fields as tissue is regenerated throughout it. It is either interconnected or closed. Apparent porosity was measured by weighing the dry compact sample (Wd), then reweighting both the compact sample when it is immersed in water (W_S), and after it is removed from the water, i.e., saturated compact sample (W_W).

Apparent Porosity, $\phi = \frac{W_w - W_d}{W_w - W_s} X100$

Where, Wd = weight of dry sample.
Ws = weight of sample immersed in water.
W_W = weight of the saturated sample after removal from the water

XRD, Lattice Parameter and FTIR analysis:
To determine the phase identification in several crystalline samples, XRD measurements have to be compared with the standard data base of International Centre for Diffraction Data (ICDD). In XRD analysis, the peak plays a crucial role for phase identification and material characterization. The width of the peak indicates average crystallite size. The sharp peak indicates larger crystallite whereas broader peak indicates smaller crystallites.

SEM and EDX Study:
The pore dimension of the sintered HAp and its dopant samples was measured by breaking the sintered pellets and placed the broken surfaces under Scanning Electron Microscope and studied it. The pore diameters were calculated using perfect screen ruler software. The average value from the five different SEM images of each sample was taken for reporting.

Hardness:
Vickers hardness tester was used for the measurement of hardness. In this method constant load is applied on the sample's surface making an indentation on the surface by using a diamond indenter and the depth is measured optically. A load of 1-100Kgf was applied for 10-15 seconds and an angle of 136^0 was formed between two opposite surfaces. The diagonals marked on the material surfaces after removing the loads were measured through microscope and calculated the average value. The area of indentation was calculated from the sloping surfaces. The

hardness value was measured by dividing the load applied on the materials by indenting area over the surfaces.

SBF Study:
Bio resorption study was done to check the dissolution performance of the prepared sintering samples in laboratory prepared (Kokubo method) biological solution maintaining at a normal physiological pH level 7.4. The prepared samples immersed in SBF solution which was changed after every three days. The experiment was continued for one month. Apatite layer formation on the pellet surface was analysed by taking SEM pictures.

Haemolysis Study:
This study was performed by following the ASTM guidelines. Human blood sample was taken for this analysis. 3.8% sodium citrate solution (used as anti-coagulant) was mixed with collected blood samples in the ratio of 10:1. To make this anticoagulant solution more dilute, normal saline solution was added in 4:5 ratio. In test tubes, sample pellets were dropped in 10 ml of normal saline solution and then incubated at 37^0C for 30 min. 0.2 ml of the prepared diluted blood samples were added in each test tubes and incubated again for 1 hr. For preparing a positive control solution, 0.2ml of diluted blood solution was added in 10 ml of sodium carbonate solution (0.1%) and then incubated for 1 hr. For a negative control solution, 0.2ml of diluted blood was added in 10 ml normal solution and incubated for 1 hr. All the test tubes were centrifuged at 500g for 5 min. The optical density (OD) of the supernatant liquids were measured at 545 nm in a UV-Vis spectrophotometer (Elico India, SL-177). The percentage of haemolysis was calculated from the following equation:

$$\% \text{ Haemolysis} = \frac{\text{O.D.(Test)} - \text{O.D.(Negative)}}{\text{O.D.(Positive)} - \text{O.D.(Negative)}} \times 100$$

MTT Assay:
The assay was conducted using Murine Osteoblastic MC3T3-E1 Cells (NCCS, Pune). The cells were cultured in αMEM (Gibco) supplemented with 10% heat-inactivated fetal bovine serum and 1% Penicillin Streptomycin solution at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The Cells were grown in a 25 mm standard flask and on confluence, it was detached using 0.5% trypsin and was seeded onto a 24-well plate at a density of 5 x 10^4 cells/cm^2. The cells were treated with prepared Pure HAp, Egg shell HAp and their dopant variants and incubated for 24 hrs. Then added MTT (3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and shaken for 15 min, and again incubated for 4 hrs. The viable cells convert the MTT into formazan. After removing the media, DMSO (dimethyl sulfoxide) was added for dissolving the crystals. The intensity of purple-coloured solution depends on the amount of crystal formed. The optical density (OD) of the solution was measured at 545nn against a blank. The experiment was performed in triplicate and the mean data was reported.

Bactericidal Study:
Bactericidal study was performed to detect any bacterial colonies grow on the samples that causes implant failure. In a conical flask diluted solution of nutrient agar media was taken and autoclave at121^0C for 20 min. After cooling at 40-45^0C, culture Staphylococcus aureus was added to the media and mixed thoroughly and then poured it in Petri dishes and solidify it. Two porcelain bits containing the sample solution having 2mg/10ml (2mg sample powder in 10ml solution) and 1mg/10ml (1mg sample powder in 10ml solution) were taken and placed in the Petri dishes and incubated at 35^0C for 24 hrs.

In-vivo Study:
Animal experiment was carried out in compliance with the guidelines of the Institutions Animal Ethical Committee, West Bengal University of Animal and Fishery Sciences, India. This study was evaluated for early-stage osteogenesis and bone remodelling in-vivo through a critical size defect model in the femoral condyle of New Zealand white rabbits over an 8-week period

Implant Preparation:
After preparing the Egg shell HAp, Pure HAp and their dopant powders, the samples were pressed at 150 MPa pressure in hydraulic press machine. The prepared green specimens were dried at 80^0C for three days. The cylindrical shaped samples (dia. 5 mm and height 6 mm) were sintered at 950^0C for densification (holding time in furnace is 2 hours). Before implanting, the sintered specimens were autoclaved at 121^0C for 30 min.

Surgery and Implantation Procedure:
In this study, 42 New Zealand white rabbits (2-2.5 kg) of either sex randomly distributed into two groups: A. Control Group I (only defect was done and no implant was given: 6 animals were taken, three animals at each time point) and B. Test Group II. In group II category: Pure HAp (06 sample), Egg shell HAp (06 sample), Pure HAp with 5% titanium dopant sample (06 sample), Egg shell HAp with 5% titanium dopant (06 sample), Pure HAp with 5% zinc dopant (06 sample), Egg shell HAp with 5% zinc dopant (06 sample) were implanted in the created defect sites of the animals and rate of bone healing with osteogenic activity was observed. Before surgery and sample implantation, rabbits were housed in individual cages in a humidity-controlled rooms with alternating 12-hr cycles of light and dark condition. Rabbits were kept without restriction of movement and provided with adequate water and libitum. After implantation, assessment carried out over a duration of 30 days and 60 days.

Local Inflammatory Reaction and Healing of Wound:
The local inflammatory reactions and associated signs viz. Lameness, weight bearing, capability fracture repair in terms of palpable callus, swelling, sarcoma formation, hematoma, and oedema were observed over 2-months postoperatively. All changes were evaluated by visual and manual examinations

Radiological Examination:
The status of implant, host-bone reaction and new bone formation were observed and recorded through Radiographs.

Histological Study:
The implant along with the surrounding bones were collected from the animals at 1-and 2-months postoperatively. The collected specimens both from normal and implanted area were cut into 3-4 mm thin slices using hacksaw. All slices washed thoroughly with normal saline and then fixed in 10% formalin for 7 days. Later, decalcification of bones were done by Gooding and Stewart's fluid containing formic acid 15 ml, formalin 5 ml and distilled water 80 ml solution. The decalcified tissues were cut into 4 µm sections and stain with haematoxylin and eosin solution. The status of the bone implants and cellular response of host bone to the implants were observed under microscope.

SEM of the Implants:
For SEM analysis, samples were fixed with 5% glutaraldehyde phosphate solution and washed twice for 30 min with phosphate-buffered saline (pH 7.4) and distilled water. The dehydration were done using graded alcohol solution and then dried by hexamethyldisilazane. All samples

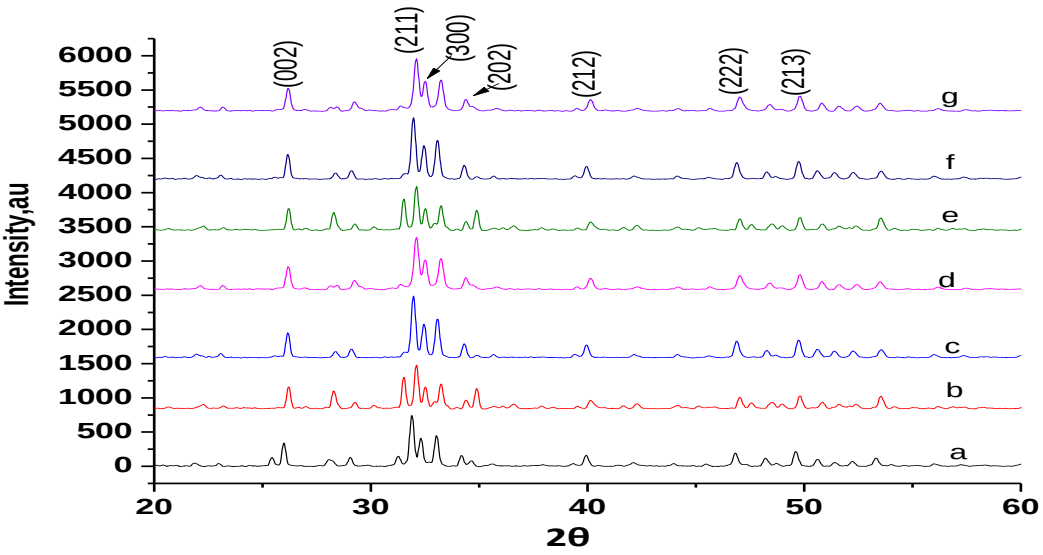
were coated with gold conductive coating by ion sputtering (JEOL ion sputter, model JFC 1100, Japan) at 7-10 mA and 1-2 kV for 5 min. SEM (JEOL JSM 5200 model, Japan) examinations after proper alignment were carried out for the resin-mounted sample surfaces.

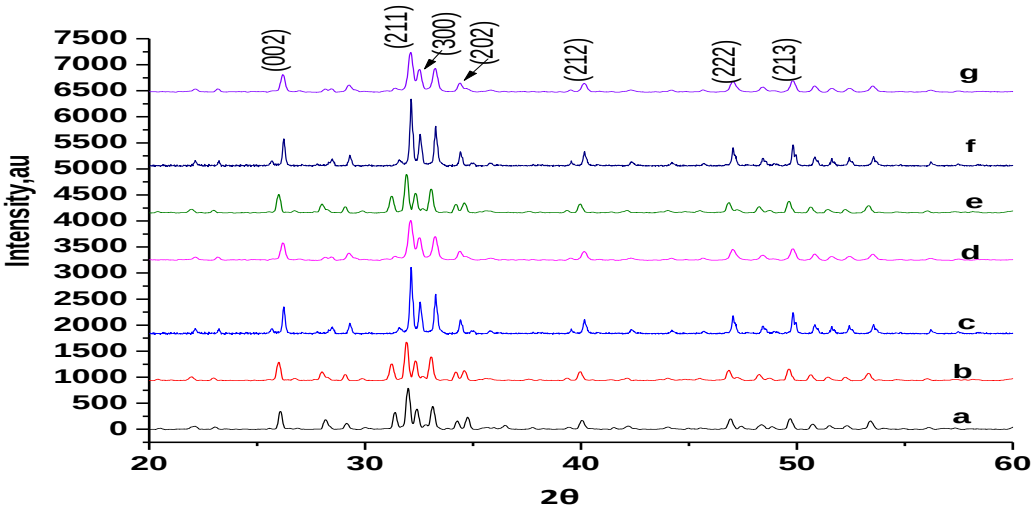
Oxytetracycline Labelling Study:

Oxytetracycline dihydrate, a fluorochrome at a dose rate 50 mg/kg body weight was introduced into the implant sides. The implanted portions of bones were taken and cut transversely into 2-3 mm thickness with a hacksaw. The un-decalcified ground sections were grounded to 20 µm thickness using different grade sand papers and finally grinding the bone in slow circular motions under moderate pressure. The formed ground sections were taken and observed repeatedly through microscope for noting the structural details of the bone. The samples were dipped into the water during total processing time. The ground un-decalcified sections were examined under ultraviolet incidental light by a Leitz Orthoplan Universal Widefield Microscope, USA (Excitation filter, BP-400 range) and observed the amount of tetracycline labelling and source of new bone formation.

RESULT & DISCUSSION

XRD pattern of Egg shell HAp and its Dopants:





XRD pattern of Pure HAp and its dopants

The obtained powder samples of Pure HAp, Egg shell HAp and their 3% and 5% dopant variants (Zn, Ti, Mg) were calcined at 800^0C temperature and the phase analysis of these calcined powder were examined through X-ray diffractometry in 2θ range. The calcined Pure HAp and Egg shell HAp samples showed several high intensity peaks conforming to various planes i.e. (0 0 2), (2 1 1), (3 0 0), (2 0 2), (2 1 2), (2 2 2), (2 1 3) which matches with the standard card number JCPD (09-0432) for hydroxyapatite. A sharp peak at 2θ range of ~32.07 corresponding to (2 1 1) plane clearly proved the presence of hydroxyapatite crystals. Presence of this peak advocates that crystallites of hydroxyapatite were formed due to calcination at 800^0C.

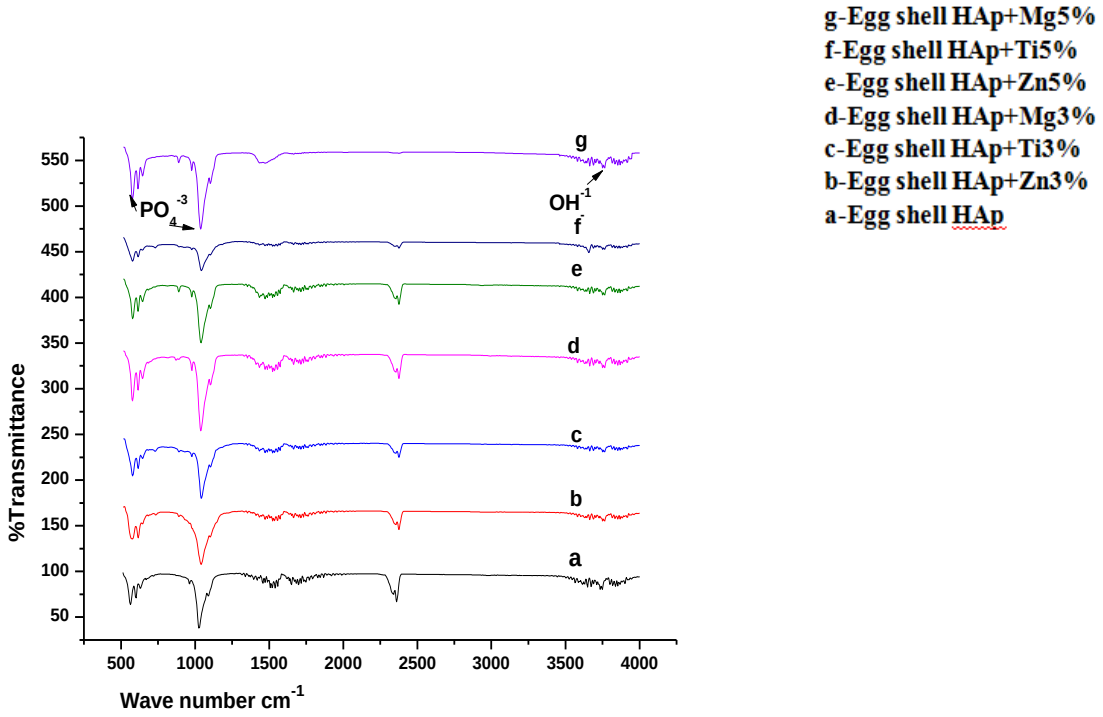
The HAp prepared from egg cell powder produced the peaks those were matched with the standard card no JCPD (09-0432). The 3% and 5% Zn, Ti and Mg verities had the same intensity peaks with a little bit of shifting due to the calcium ion substitution by zinc, titanium and magnesium ion. A comparative analysis with laboratory grade hydroxyapatite (Pure HAp) and its dopants signifies that the experimentally prepared Egg shell HAp and their derivatives carries almost same crystalline structure reflected in the picture

Lattice parameter study

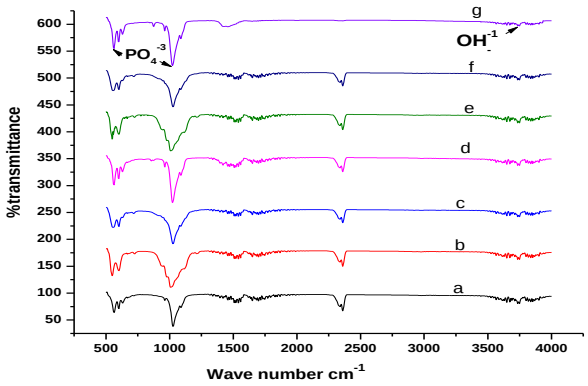
Sl. No	Composition	'a' axis (Å)	'c' axis (Å)	Unit cell volume (Å3)	Crystallite size (Å)	% Crystallinity
1	Pure HAp	9.4230±.0.097	6.841 ±0.120	533.93±22.02	3.6286±12.67	93.81± 3.82
2	Egg shell HAp	9.416±0.11	6.787±0.17	521.05±18.29	3.5556±9.84	84.81±5.76
3	Pure HAp + Zn 5%	9.221±.0.077	6.693 ±0.142	508.78±22.54	3.3681±11.57	82.41± 4.12

4	Egg shell HAp + Zn 5%	9.215±0.31	6.671 ±0.320	487.05±18.29	3.3291±14. 77	84.01±5.76
5	Pure HAp + Ti 5%	9.28±0.34	6.731±0.21	501.91±16.29	3.4121±8.0 2	82.47±6.82
6	Egg Shell HAp + Ti 5%	9.22±0.23	6.71±0.37	493.97±19.16	3.3912±11. 12	88.46±4.02
7	Pure HAp + Mg 5%	9.383±.0.08 6	6.791 ±0.110	517.83±26.12	3.5475±22. 61	80.54± 3.94
8	Egg Shell HAp + Mg 5%	9.326±0.19	6.741 ±0.020	509.05±16.19	3.4581±10. 42	81.61±5.06

The lattice parameters suffered minor changes as a results of incorporation of different dopant ions. The values of both ‘a’ and ‘c’ axis diminished systematically in all the cases, shown in below Table 1. This may be credited to smaller atomic radii of the dopants e.g., Zn (134 pm), Mg (160 pm), & Ti (147 pm) in comparison with Ca (197 pm). The results revealed the substitution of calcium ion by the dopants and consequent reduction in unit cell dimensions. In lattice parameter study, percentage of crystallinity is decreasing in doped variants compared to Pure HAp and Egg shell HAp. In Egg shell HAp, % crystallinity is less due to the presence of a number of trace elements



FTIR spectra of Egg shell HAp along with its dopants



g-Pure HAp+Mg5%
f-Pure HAp+Ti5%
e-Pure HAp+Zn5%
d-PureHAp+Mg3%
c-Pure HAp+Ti3%
b-Pure HAp+Zn3%
a-Pure HAp

FTIR spectra of Pure HAp along with its Dopants:
At 570 &1023 the peak indicates the presence of phosphate ion whereas at 3752, a stretching band of hydroxyl ion is present. A sharp peak at 2357 is due to the presence of some unreacted carbonate ion.

Green density and Sintered Density:
After the prepared HAp and their dopants, it is pressed to form pellets and green density is measured. The diameters of the pellets are 10±0.03 mm and thickness are 6±0.03 mm. After sintering at 950^0C &1050^0C, there was shrinkage of diameter, length. It revealed that on increasing the sintering temperature from 950^0C to 1050^0C, the density was increased but there were no major changes happened.

Apparent Porosity at Different Temperatures:
Porosity plays a significant role in scaffold engineering. A minimum percentage of porosity is needed for cell growth during tissue regeneration. The properties of the scaffold would be such that it can withhold the body pressure along with its inherent characteristics. It is reported that pore size greater than 150µm in scaffold is essential for osteoconduction. Bone tissues passes through the pores causes the mechanical fixation of implant to the body.

Apparent porosity of Egg shell HAp and their dopants at different temperatures

Sl. No.	Compositions	At 950^0C (%)	At 1050^0C (%)
1	Egg shell HAp	30.48 ± 1.72	19.41 ± 1.21
2	Egg shell HAp + Zn 3%	37.13 ± 0.96	27.34 ± 1.12
3	Egg shell HAp + Ti 3%	34.90 ± 1.06	24.81 ± 0.85
4	Egg shell HAp + Mg 3%	31.90 ± 1.20	23.87 ± 0.95
5	Egg shell HAp + Zn 5%	34.95 ± 0.94	24.55 ± 0.68
6	Egg shell HAp + Ti 5%	32.57 ± 1.02	23.05 ± 0.52
7	Egg shell HAp + Mg 5%	32.64 ± 0.65	22.54 ± 0.61

Apparent porosity of Pure HAp and their dopants at different temperature

Sl. No.	Compositions	At 950^0C (%)	At 1050^0C (%)
1	Pure HAp	22.02 ± 1.66	17.52 ± 0.75
2	Pure HAp + Zn 3%	25.91 ± 1.94	20.93 ± 1.89

3	Pure HAp +Ti 3%	24.31 ± 2.48	18.27 ± 0.83
4	Pure HAp + Mg 3%	23.22 ± 0.73	18.01 ± 0.92
5	Pure HAp + Zn 5%	23.36 ± 2.13	20.42 ± 0.78
6	Pure HAp + Ti 5%	21.05 ± 2.94	17.57 ± 1.01
7	Pure HAp + Mg5%	21.67 ± 1.18	16.53 ± 1.28

It is observed that porosity is lesser at 1050°C than 950°C. All the doped samples are showing higher porosity than Pure HAp and Egg shell HAp. Further it can be said that all the 5% doped variants are showing lesser porosity of its own 3% doped variants. Egg cell HAp has more porosity than pure HAp for all its varieties owing to presence of a number of trace elements.

Grain Size and Pore Size:

The measurement of grain size and pore size is an important parameter in porous scaffold materials as this is responsible for osteoconduction, cell migration and cell-cell interaction.

Grain Size, pore size of Egg shell HAp and their dopants at different temperatures

Sl. No.	Compositions	Grain Size (µm)	Pore Size (µm)
1	Egg shell HAp	569 ± 3.05	158 ± 1.15
2	Egg shell HAp + Zn 3%	539 ± 2.52	157 ± 3.0
3	Egg shell HAp + Ti 3%	552 ± 5.0	169 ± 2.08
4	Egg shell HAp + Mg 3%	555 ± 4.04	180 ± 1.52
5	Egg shell HAp + Zn 5%	547 ± 6.0	170 ± 4.04
6	Egg shell HAp + Ti 5%	565 ± 4.0	168 ± 3.51
7	Egg shell HAp + Mg 5%	568 ± 3.51	192 ± 2.08

Grain size, pore size of Pure HAp and their dopants at different temperatures

Sl. No.	Compositions	Grain Size (µm)	Pore Size (µm)
1	Pure HAp	572 ± 5.51	152 ± 3.6
2	Pure HAp + Zn 3%	545 ± 4.51	154 ± 2.0
3	Pure HAp + Ti 3%	555 ± 3.61	164 ± 3.51
4	Pure HAp + Mg 3%	558 ± 2.52	174 ± 4.16
5	Pure HAp + Zn 5%	549 ± 2.0	175 ± 2.64
6	Pure HAp + Ti 5%	565 ± 3.51	158 ± 3.6
7	Pure HAp + Mg 5%	569 ± 2.51	187± 2.51

In our study it is observed that Pure HAp and Egg shell HAp have the largest grain size 572 µm .and 569 µm. The 3 % Zn doped pure HAp and Egg shell HAp sample are showing the smallest grain size 545 µm and539 µm.

SEM-EDX Study:

The SEM images of samples support enough nodular shaped pores presence in the sample which are essential for bony ingrowth throughout it. The presence of pores gives the structural integrity of the scaffolds. Elemental analysis through E-DAX study provides the presence of trace elements zinc, titanium, and magnesium ion.

Hardness

Hardness value of Egg shell HAp and their dopants at different temperatures

Sl. No.	Composition	Hardness (GPa)
1	Egg shell HAp	3.71 ± 0.020
2	Egg shell HAp + Zn 3%	3.92 ± 0.031

3	Egg shell HAp + Ti 3%	3.98 ± 0.036
4	Egg shell HAp + Mg 3%	3.84 ± 0.021
5	Egg shell HAp + Zn 5%	3.97 ± 0.010
6	Egg shell HAp + Ti 5%	4.0 5± 0.040
7	Egg shell HAp + Mg 5%	3.91 ± 0.066

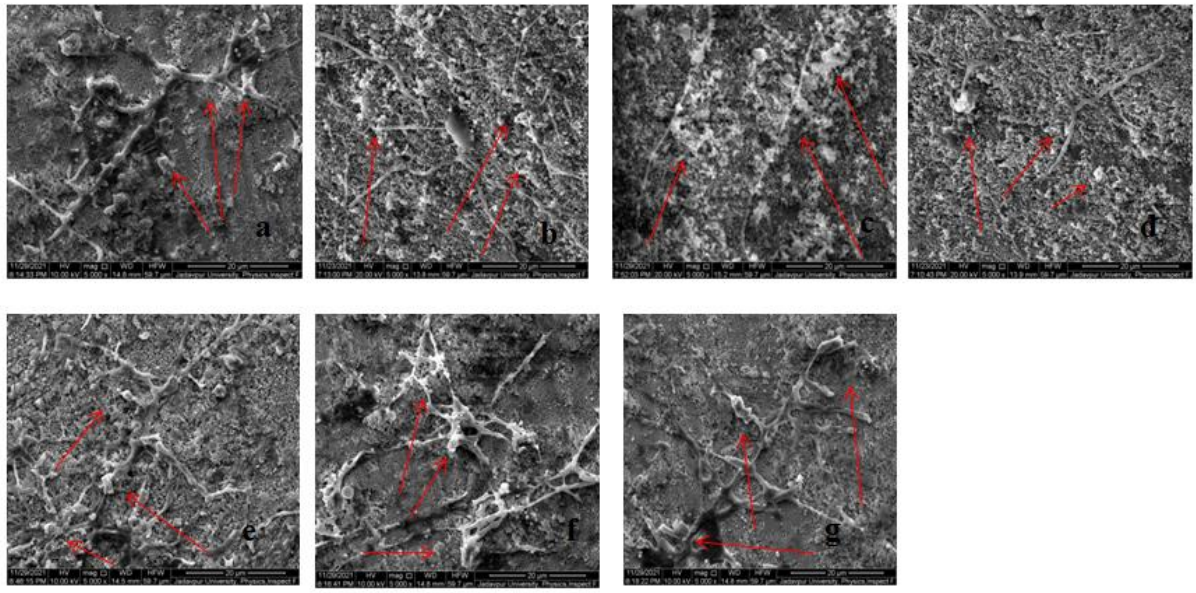
Hardness value of Pure HAp and their dopants at different temperatures

Sl. No.	Composition	Hardness (GPa)
1	Pure HAp	3.64 ± 0.030
2	Pure HAp + Zn 3%	3.78 ± 0.031
3	Pure HAp + Ti 3%	3.83 ± 0.015
4	Pure HAp + Mg 3%	3.67 ± 0.017
5	Pure HAp + Zn 5%	3.86 ± 0.025
6	Pure HAp + Ti 5%	3.94 ± 0.03

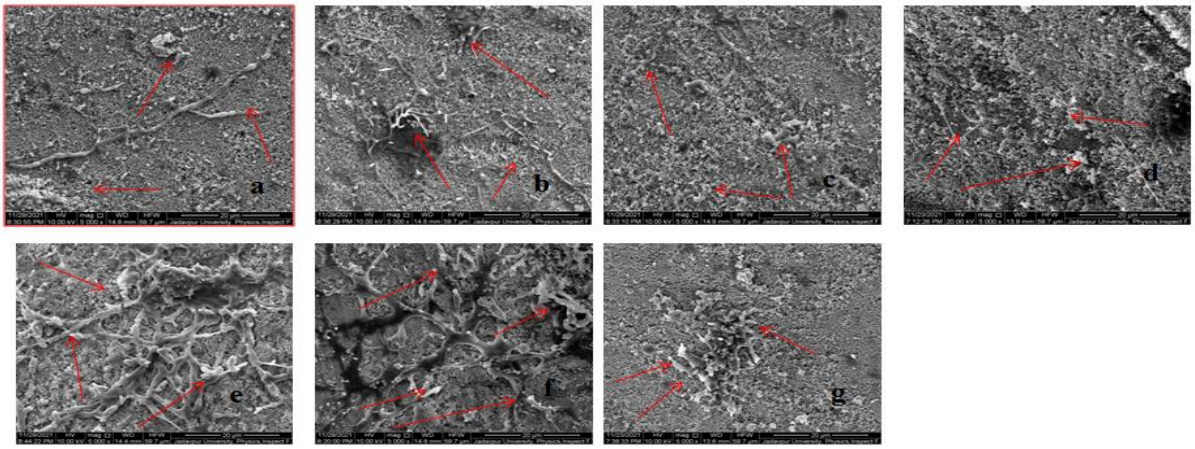
The hardness value is increasing with addition of dopant element due to the densification properties. This increment in hardness value of egg shell component may be due the transformation of calcium carbonate into calcium oxide and presence of trace elements

SBF Study:

Apatite layer formation above the sintered pellets after 7 days were noticed through scanning electron microscopy. At the 30th day, the apatite was denser as compared to the previous one. The size and thickness of the apatite layer were increased with increasing the immersion times.



SBF apatite images of Egg shell HAp and their dopants (a) Egg shell HAp (b) Egg shell HAp + Zn 3% (c) Egg shell HAp + Ti 3% d) Egg shell HAp + Mg 3% (e) Egg shell HAp + Zn 5% (f) Egg shell HAp + Ti 5% (g) Egg shell HAp + Mg 5%.



SBF apatite images of pure HAp and their dopants (a) Pure HAp (b) Pure HAp + Zn 3% (c) Pure HAp + Ti 3% d) Pure HAp + Mg3% (e) Pure HAp + Zn 5% (f) Pure HAp + Ti 5% (g) Pure HAp + Mg 5%.

Hemolysis Study:

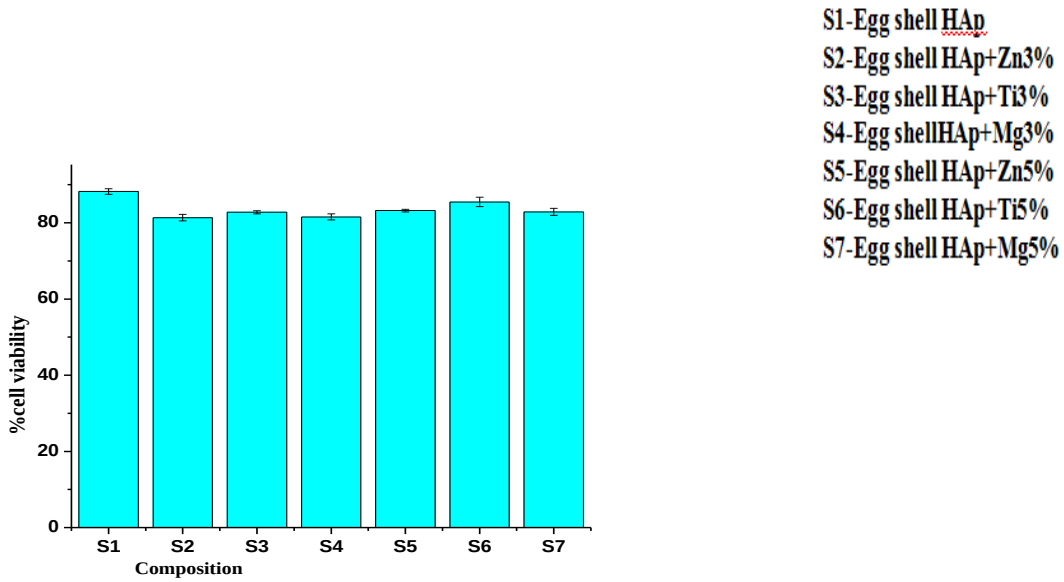
Data for hemolysis analysis of Egg shell HAp and their dopants

Sl. No.	Composition	% Hemolysis
1	Egg shell HAp	1.98 ± 0.036
2	Egg shell HAp + Zn 3%	2.90 ± 0.030
3	Egg shell HAp + Ti 3%	3.17 ± 0.050
4	Egg shell HAp + Mg 3%	3.68 ± 0.040
5	Egg shell HAp + Zn 5%	2.49 ± 0.055
6	Egg shell HAp + Ti 5%	2.19 ± 0.041
7	Egg shell HAp + Mg 5%	3.42 ± 0.026

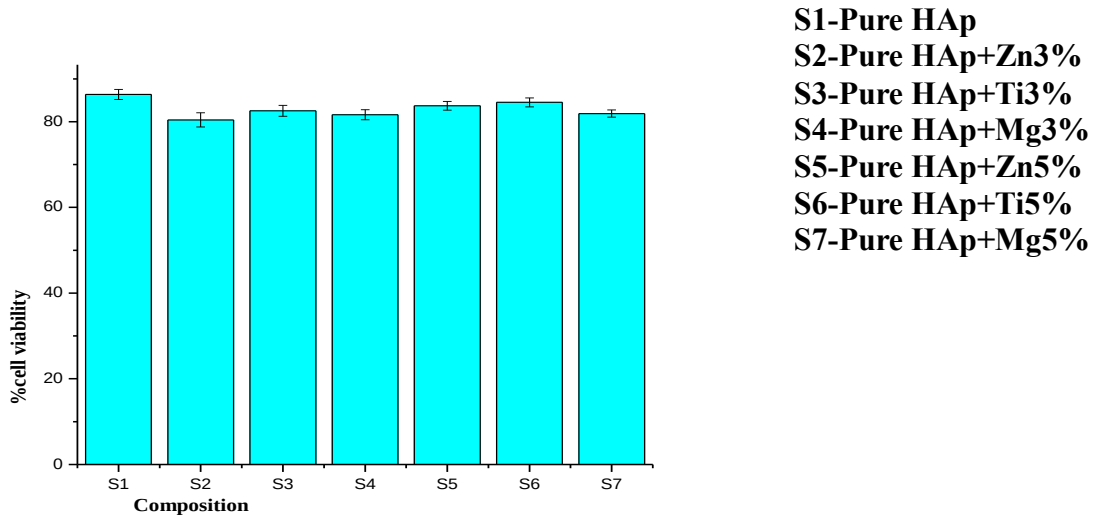
Data for hemolysis analysis of Pure HAp and their dopants

Sl. No.	Composition	% Hemolysis
1	Pure HAp	2.82 ± 0.20
2	Pure HAp + Zn 3%	3.43 ± 0.34
3	Pure HAp + Ti 3%	2.97 ± 0.21
4	Pure HAp + Mg 3%	4.10 ± 0.43
5	Pure HAp + Zn 5%	3.50 ± 0.46
6	Pure HAp + Ti 5%	3.15 ± 0.20
7	Pure HAp + Mg 5%	3.74 ± 0.38

All the samples have percentage of hemocompatibility less than 5%. So according to the ASTM guidelines all samples are highly hemocompatible. Among the different compositions Pure HAp and Egg shell HAp have shown the maximum hemocompatibility



% cell viability of Egg shell HAp and their dopants as observed in MTT assay



% cell viability of Pure HAp and their dopants as observed in MTT assay

The cell viability for all the samples more than 80% indicated that all samples are non-cytotoxic.

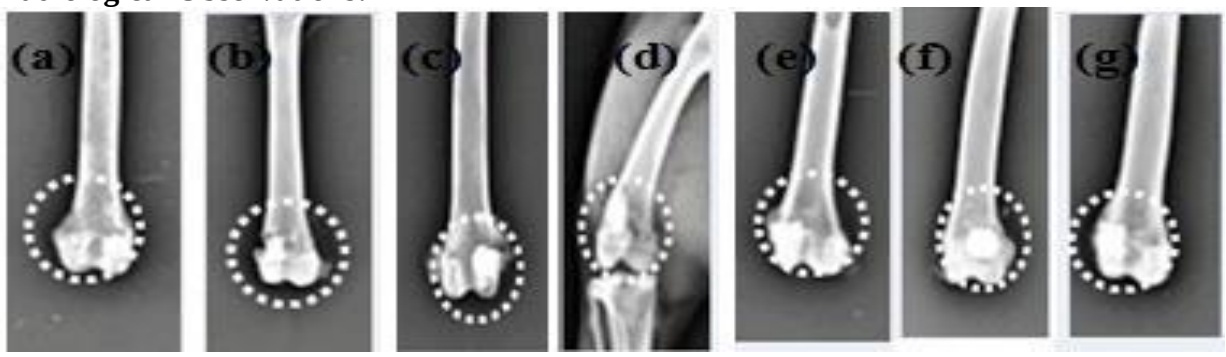
Bactericidal Study:

It is observed and noted that there are no zone of inhibition for all concentration after 24 hour. This suggests that at 1mg/10ml &2 mg/10 ml, Pure HAp and Egg shell HAp and its dopants elicits no bactericidal effect.

In Vivo Analysis of Pure Hydroxyapatite and Egg Shell HAp:
For group I (control) defect was created, not placing any sample and for group II (test), the created defected portion placing with pure HAp, Egg shell HAp and 5% zinc and titanium composite of both verities. The healing effect was studied at starting day (0 day), 30th days post operatively and 60th days post operatively.

Local Inflammatory Reactions and Healing of Wound:
It is observed that there is no marked hematoma or oedema as a result of adverse side effects, so the implants were clinically stable. Similarly, no foreign body response or toxicity was elicited. It confirmed that the implant is biocompatible and can be acceptable as a suitable alternative bone graft material to fill bone defect.

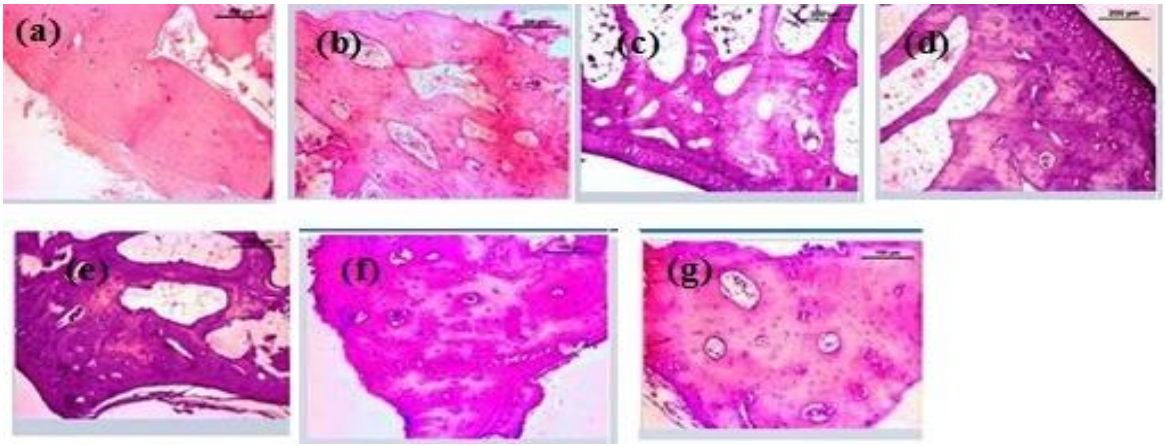
Radiological Observations:



Radiographs of the '60' day (a) Control (b) Pure HAp (c) Egg shell HAp (d) Zn doped Pure HAp (e) Zn doped Egg shell HAp (f) Ti doped Pure HAp (g) Ti doped Egg Shell HAp

After two months, the group I implants showed that the gap was reduced but not totally filled up. But in group II implants- in Pure HAp and Egg shell HAp group, the cortical discontinuity is radiographically evident even on 2 months along with visible implant. But in Zn doped Egg shell HAp, the bony defect is healed completely along with further degradation of implanted materials. In this group there is no gap between host tissue and bone itself. Implant-bone interface is not clearly evident indicating good acceptance of the implant material by bone tissue. In Ti doped Egg shell HAp, the implant to some extent degraded with establishment of cortical continuity but no visible defect was seen radiographically.

Histological Study:
Group I, control sections showed a greater number of haversian canals with presence of osteoblast at 2-month time point. The medullary cavity had few RBCs with mononuclear cells. It contained less amount of fibrin and osteocyte.



Histological section of the bone of control & Gr. II animal after 2 M: (a) Control (b) Pure HAp (c) Egg shell HAp (d) Zn doped Pure HAp (e) Zn doped Egg shell HAp (f) Ti doped Pure HAp (g) Ti doped Egg Shell HAp

The control sections showed a greater number of haversian canals with presence of osteoblast at 2-month time point, whereas Pure HAp sections revealed the presence of bigger size of haversian canals with few osteoclasts and abundant of osteoblasts. The Zn-doped and Ti-doped Pure HAp sections showed a bony structure involving of haversian canal and few sinusoidal spaces along with penetrated RBCs, osteoblasts, and abundant mononuclear cells. The Ti-doped Egg shell HAp microphotograph showed copious osteoblasts, osteoclasts with haversian canals and angiogenesis. The Zn-doped Egg shell HAp showed presence of abundant osteoblast and osteoclasts cells.

Oxytetracycline Labelling Study:

Sl. No.	Composition	1 month	SD (±)	2 months	SD (±)
1	Control	15.5	1.88	26.47	1.98
2	Pure HAp	21.41	2.1	31.47	1.86
3	Egg shell HAp	25.6	1.99	43.3	1.84
4	Zn doped Pure HAp	32.5	1.67	53.34	2.09
5	Zn doped Egg shell HAp	36.35	1.85	58.52	2.06
6	Ti- doped Pure HAp	33.69	2.06	54.41	1.93
7	Ti-doped Egg Shell HAp	37.5	2.17	59.31	1.89

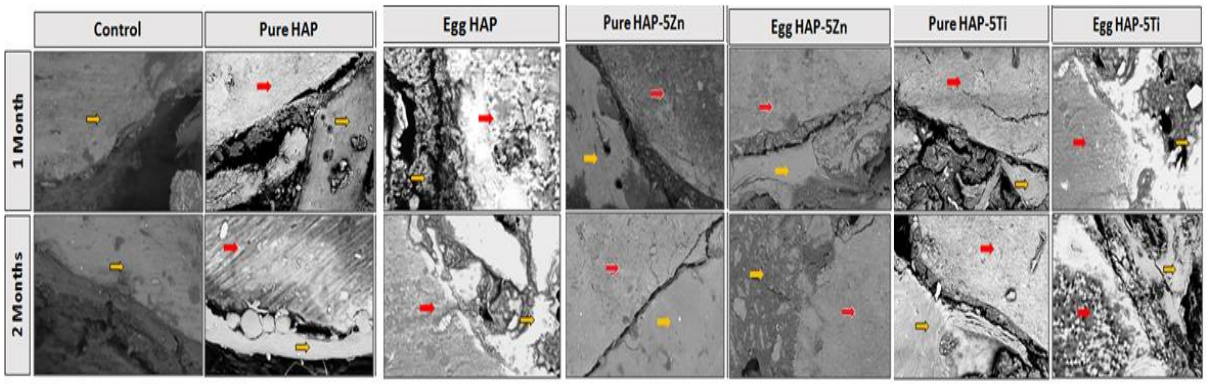
Group I (Control): Control sample showed mild bone formation (15.50 ± 1.88) at 1 month and increased with the passage of time at 2 months (26.47 ± 1.98).

Group II: The image of the control group showed minute golden yellow fluorescence at few area of central part (15.50 ± 1.88%) in the field of deep-sea green coloured backdrop at 1 month. The Pure HAp image revealed minute area of golden yellow fluorescence at the central zone representing to some extent newer bone formation (21.41 ± 2.10%). The Egg shell HAp image indicated a compressed area of golden yellow fluorescence depicting superior new osseous tissue formation (25.60 ± 1.99%), whereas Zn-doped Egg shell HAp exhibited (36.35 ± 1.85%) new bone formation. Comparatively more and abundance of bright golden yellow fluorescence colour was noted in Ti-doped pure HAp and Ti-doped Egg shell HAp samples nearly (33.69 ± 2.06%) and (37.50 ± 2.17%) in the background by sea green coloured host tissue at the same time point.

Significant improvement of new osseous bone formation was seen in all the groups at 2 months as compared previous time point. The image of control sample showed more golden yellow

fluorescence of newly formed osseous tissue (26.47 ± 1.98%) at 2 months. The pure HAp group exhibited intense golden yellow fluorescence at the central zone (31.47 ± 1.86%) demonstrating better bone formation as compared to base values. The image of Egg shell HAp indicated the presence of golden yellow coloured fluorescence newly formed osseous tissue (43.30 ± .84%) in more areas representing active state of bone regeneration. The Ti-doped Pure HAp bone section indicated the presence of golden yellow fluorescence in majority of the section representing more osseous activity (54.41 ± 1.93 %). The Zn-doped Egg shell HAp shows more intense golden yellow fluorescence showing new osseous tissue formation (58.52 ± 2.06%) and nearly approaching for Zn-doped Pure HAP samples. In Ti-doped Egg shell HAp samples, abundant plaques of golden yellow was seen representing more formation of newly formed osseous tissue in the background of deep green sea coloured host tissue (59.31 ± 1.89%).

SEM images of Bone Implant Interface Study:



Control: The SEM images of the defect site show that the gap is quite visible in 1 month and after 2 months, a minor portion has been covered.

Group II: After 1 month of implantation, the Pure HAp and Egg shell HAp samples showed some irregular bone tissue arrangement. The samples exhibit binding abilities to the old bone with the implant. The bridging gap was found less in 5% Zn doped and 5%Ti doped varieties on comparison to pure sample. At the end of 2 months interval, Ti doped Egg shell HAp and Zn doped Egg shell HAp implant showed firmly bonded to the host tissue with filling of entire gap.

CONCLUSION

In this present study waste egg shell collected from different house hold kitchen or canteen has been successfully converted to hydroxyapatite. Doped variants (3% and 5%) of Zinc, Titanium, and magnesium from both sources have been successfully prepared through wet precipitation method. XRD study confirms the presence of pure hydroxyapatite phase whereas the doped variant causes minor shifting due to doping effect. The lattice parameter study showed that on doping with Zn, Ti, Mg ions, the percentage of crystallinity is decreasing. A uniform pattern of shrinkages was observed of all kind of samples upon increasing the temperature of green specimens. Enhancing sintering temperature from 950^0C-1050^0C minor changes were noticed. The porosity is greatly diminished with the increase of temperature. The grain size, porosity and crystal structure does not alter any kind of significant changes with the concentration variation of dopants. A substantial amount of porosity in all compositions signifies that the developed materials can promote anchorage of bone cells and bony in-growth. The hardness of the materials clearly indicate that there is little bit change due the addition of dopants, but no direct proportionate relation establish between the hardness and dopants amount. Hemolysis

and histological studies confirmed that the samples biocompatible as well as nontoxic in nature. SBF study showed that 5% Ti and 5% Zn doped Egg shell HAp are better than other varieties as it exhibited maximum apatite layer formation. A comparative assessment of 5% zinc and 5%titanium of both sourced samples were done in-vivo and observed the differences on their impact on living body. It was noted that 5% doped variants of egg shell HAp exhibited faster healing rate (radiography study), more number of osteoblasts, osteoclasts with haversian canal (histological analysis), more percentage of new bone formation (oxytetracycline labelling study).

Scope of the Future Work:

1) Calcined egg shell powder may be chosen instead of synthetic calcium source powder.
2) Alternative bone substitute may be taken into consideration
3) Time span of application can be altered.
4) In- vivo study in another model can be tried.
5) Clinical study can be done if ethical committee permits