Development of Cobalt-Magnesium doped hydroxyapatite for bone tissue engineering application

A Project report submitted to National Institute of Technology, Rourkela in partial fulfillment of the requirements for the award of the degree of

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By

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CERTIFICATE

This is to certify that the Project Report entitled **"*Development of Cobalt Magnesium doped hydroxyapatite for bone tissue engineering application* "** submitted by Ms Upasana Mishra in partial fulfillment of the requirements for the award of Bachelor's of Technology in Biotechnology and Medical engineering with specialization in Biotechnology at the National Institute of Technology, Rourkela is an authentic work carried out by her under my supervision and guidance.

To the best of my knowledge, the matter embodied in the Report has not been submitted to any other University/Institute for the award of any Degree or Diploma.

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Abstract

This thesis delineates the development of doped hydroxyapatite and their different characterizations. (5% $CoCl_2$ - 5% $MgCl_2$ w/w)-HAp and (5%$Co(NO_3)_2$-$Mg(NO_3)_2$ w/w)HAp were synthesized by slight modifications of the conventional ammoniacal precipitation method. Then their physico-chemical and biological characterizations were carried out along with the standard Hydroxyapatite. Phase content was studied by XRD analysis, different functional groups of the samples were characterized using FTIR. TGA and DSC was carried out to analyze the thermal stability of the sample. Stability studies by zeta potential measurement was also carried out. SEM was used to observe the morphology and EDAX was done to find the extent of actual dopings in the hydroxyapatite samples. The biological performance of the samples was evaluated by hemolysis test to check the haemocompatibilty of the samples, protein adsorption tests, antimicrobial study, and cytocompatibility study using MG-63 cell. Data showed that replacement of both cation and anion from hydroxyl apatite have more profound effect on biological performance of the samples.

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Introduction:

Improvement of osteoconductive properties of synthetic hydroxy apatite by cobalt and magnesium doping

Introduction: Effective replacement/restoration of traumatized, damaged or lost bone is a major clinical and socioeconomic need. Since last three decades, numerous natural and synthetic materials have been explored alone or in combination for orthopedic application. Among them, synthetic hydroxyapatite (HAp) has been used extensively as a substitute in bone grafts because of its close chemical resemblance with the inorganic phase of the bone. Although, application of HAp based artificial bone construct have been successful in certain cases, they often fail to serve as efficiently as natural bone because of the their (i) low mechanical strength (brittleness), (ii) inappropriate degradation rate (failure to complement *in vivo* bone regeneration) and most importantly (iii) comparatively poor biological properties.

A close inspection of the chemical structure of synthetic HAp has revealed that it differs slightly from natural bone apatite with respect to its chemical composition, percentage crystallinity and crystal structure. Chemical formula of pure synthetic hydroxyapatite is $[Ca_{10}(PO_4)_6(OH)_2]$ where as that of bone apatite could be represented as

$[Ca_{10-(x-u)}(PO_4)_{6-x}(HPO_4 \text{ or } CO_3)_x (OH, F..)_{\{2-(x-2u)\}}]$ where $0 \leq x \leq 2$, $0 \leq 2u \leq x$.

It has been discovered that in *in vivo* condition, bone apatite efficiently exchange its constitute ions (Ca^{+2} & PO_4^{-3}) with different ions present in physiological fluids (CO_3^{-2}, F^-, Cl^-, Mg^{+2}, Na^+, K^+) that resulting in different ions substituted apatite. These substitutions are believed to be one of the probable reasons for the changes in properties such as crystallinity, solubility, and

ultimately biological response of bone apatite. These set of findings have led researchers to think of a strategy for improvising the physico-chemical and biological response of synthetic HAp through different ion substitution. Experimental ventures have shown that it is also possible to substitute constituent ions of synthetic HAp like in case of natural bone apatite. Utilizing different techniques, people have successfully synthesized different element doped/substituted HAp. These include the synthesis of (Na), potassium (K), barium (Ba), magnesium (Mg), strontium (Sr), manganese (Mn), Zinc (Zn), Titanium (Ti), Copper (Cu), Chromium (Cr) Silicon (Si), fluorine (F), chlorine (Cl) doped HAp. Experiments have further shown that doped elements significantly change the lattice parameters and cell volume of HAp crystal in native and sintered state, resulting in different types of crystal defect and surface charge distribution, and consequently change the original degradability, mineralization and most importantly biological response of HAp. It is also observed that certain doping have profound effect on the absorption and release of different bioactive molecules (proteins) from the materials. So far therapeutic potential is concerned, orthopedic application of synthetic hydroxy apatite generally faces two major challenges, the first one is definitely its poor osteogenic properties with respect natural bone apatite, the second one is associated with limited angiogenesis at the site of application of biomaterials (in forms of bone graft, bone cements, or bone fillers). Consequence of the later is the low viability of tissue at the site of grafting.

Angiogenesis is very important while using artificial grafts. Present ventures of angiogenesis induction relies upon the use of growth factors like VEGF. However it’s cost limits its commercial application. In this regard cobalt has recently drawn attention of the scientific community. Cobalt chloride has long been used to upregulate HIF which in turn promotes angiogenesis creating a hypoxia mimicking condition by three mechanisms- it replaces Fe

cofactor diminishing the availability of Fe2+ for the enzyme PHD[1]; it causes down-regulation of the expression of FIH-1 and ARD1; it causes depletion of intracellular ascorbate which acts as a cofactor for the enzyme PHD [2](where all these enzymes help in the ubiquitinisation of HIF-1α)(Ke et al., 2005)[3,4].So the HIF-1α remains stabilized and binds to the HIF-1β subunit in the nucleus and helps in transcription of genes, some of which are proangiogenic factors like VEGF[5,6].So cobalt might be used as a angiogenesis inducer and could be incorporated into biomaterials for use in tissue engineering. Recently Chengtie et al has reported the development ionic Co^{2+}incorporated Mesoporous bioactive glass (MBG) mimicking Hypoxia conditions and subsequently showing osteogenic properties.[7]

Magnesium is the most abundant minor element found in biological apatites. It has been seen that presence of magnesium significantly reduces the particle-size of the powder compared to normal HAp and improves it's mechanical strength[9].In in-vitro conditions it has been seen to be mitogenic to growth of osteoblast cells and its absence can be inhibitory to the same[8]. It has been observed to influence the process of bone mineralization and it increases osteoblast adhesion compared to normal Hydroxyapatite

So, in the present study we synthesized hydroxyapatite and Cobalt and Magnesium doped hydroxyapatite (one containing 5% $CoCl_2$ + 5% $MgCl_2$ and the other with 5 % $Co(NO_3)_2$ + 5% M $g(NO_3)_2$ and have carried out their physico-chemical and biological characterization as a comparative study with normal Hydroxyapatite

Literature Review:

Bone Tissue Engineerng:

There are many reasons to go for Bone Tissue Engineering, one of the reasons being the need for filler materials especially in case of large bone defects and the need of material with proper mechanical properties which show appropriate functionality in their biological microenvironment. The traditional methods of bone defect management of using autografts and allografts are long being used but they have many drawbacks associated with them. Though autografting provides osteoinductive, osteoconductive and osteogenic properties these are used preferably in case of small and non-load bearing bone defects. It also leads to donor site morbidity and lack of structural integrity. It might also lead to problems like chronic residual pain at the donor site and blood borne infections at the harvesting site. Even though allografts can be used for large bone defects but they don't fully revascularize and remodel. Use of biomaterials coated with osteogenic cells or osteoinductive genes might serve the purpose. Another problem, especially in case of large bone defects is that the bone graft may be completely resorbed before the process of osteogenesis has been completed. The use of orthopaedic implants was tried instead of natural grafts due to lack of donors and to reduce the problem of donor site morbidities. But the wearing of the implant material at times leads to the osteolysis of the surrounding tissues and the lack of bonding between the implant's chemically inert material and the biological tissue might lead to failure due to instability caused by their relative motion.

For the bone tissue engineering approach few things have to be kept in mind. The material should be biocompatible i.e. it should not elicit any immune response against the host. It should

have a critical biodegradability time. The rate of formation of the new bone should be almost same to the rate of degradation of the scaffold. The mechanism of biodegradation may lead to change in pH of the surrounding and subsequent response. It should have mechanical properties enough to withstand the processes of sterilization, packaging, transport to place of surgery and finally to withstand in vivo load bearings. It should be osteoinductive i.e. it should have the ability to recruit osteoprogenitor cells to the site of defect and also should be able to induce their differentiation. The porosity should normally be 70-80 % as it requires tissue in-growth, neovascularisation, mass transport and osteogenesis.Appropriate surface properties are required for cell adhesion, proliferation and their differentiation. Gogolewski's laboratory reported that polyester membranes with pore sizes up to 200 µm diameter promoted bone growth within a 1-cm defect of the radii [10]. Tsurga and coworkers have suggested that theoptimal pore size of ceramics that supports ectopic bone formation is 300-400 µm [11]. Materials are being used in this regard for basically three different purposes. Materials used in acellular systems are t he ones on which no additional cellular component has been cultured prior to it's use.These are generally used as bone filler materials. In case of materials used in cellular systems cellular components are added prior to implantation. The third kind is u used for drug delivery systems.

In case of acellular systems the most widely used natural polymer is demineralized bone matrix (DBM). Recent research has proven that cortical bone is the preferred choice for DBM synthesis as it is more osteoinductive with a lower antigenic potential than cancellous bone [12]. In case of usage of synthetic polymers for acellular systems, Elisseeff and coworkersdeveloped photopolymerizable materials that can be in-jected as liquids and photopolymerized to localize the material which they have tested for transdermal applications but it may be used for orthopaedic applications as well. Use of poly-L-lactide (PLLA) membrane [13], PLLA or a poly-

L-co -D,L-lactide (PLDL) membrane or with a PLLA or PLDL membrane synthesized with calcium carbonate in order to decrease the relative amount of polymer in the membrane [14], poly- e-caprolac-tone-co –lactide [15] and Photocrosslinkable polyanhydrides also have been used in acellular systems of bone tissue engineering applications.. Polymer composites with ceramic"llers have also been investigated for acellular systems. Investigation of poly(lactide-co -glycolide) constructs formed bysolvent casting/particulate leaching were crushed and then compression molded with hydroxyapatite (HA) in order to improve compressive yield strength [16], poly(propylene fumarate) (PPF) biodegradable bone cement that can be combined with a leachablecomponent and injected into osseous defects [17], poly-dioxanone- co -glycolide based composite reinforced with HA or TCP in the form of an injectable or moldable putty [18], PLLA/HA constructs formed through a standard polymer processing technique, thermally induced phase separation (TIPS) have been reported [19]. In case of ceramics, BoneSource^{TM} is a hydroxyapatite that in a powdered form, when mixed with sterile water,it is gives a conformable, paste-like consistency and it rapidly adheres to bones and has the unique capability of osteoconversion [20]. Stereolithography technology to fabricate ceramic constructs using a concentrated colloidal dispersion in an aqueous photocurable polymer solution has also been reported [21].

The collagen materials have also been applied as cellular scaffolding systems. It’s engineering modification can help it gain the required amount of mechanical strength. It has been demonstrated that porous collagen foams could be treated with calcium solution to allow the deposition of calcium phosphate and improvement of mechanical integrity [22]. Similarly many other modifications have been proposed taking collagen as the base material. PL-stabilized PG mesh have been used as the Polyglycolide mesh demonstrates relatively low mechanical integrity

in vitro [23]. Use of poly-anhydride- co -imides as bone tissue-engineering scaffold alternatives have been reported [24]. HA-based constructs with a well-characterized porous calcium phosphate ceramics have been reported [25].

Use of natural polymers can serve as a matrix in bone tissue engineering is popular mainly because of it's biodegradability and biocompatibility. Natural polymers such as collagen, chitosan, hyaluronic acid have a low immunogenic potential but show high bioactivity. They often contain extracellular substance called ligand that guide cellular response in them . Synthetic polymers have the advantage of their tailorable biodegradability and high predictibilty of their properties. Ceramics are good insulators of heat and electricity and they are more resistant to harsh environment and temperatures compared to polymers and metal.

Calcium Phosphate Ceramics are bioactive ceramics. They show osteoconduction and osteointegration. They produce minimal foreign body reaction or immunogenic response. They show high biocompatibility and low systemic toxicity. They are light in weight and it is chemically and morphologically similar to inorganic part of natural bone. At body temperature only two calcium phosphates are stable in contact with aqueous media, such as body fluids; at pH <4.2 the stable phase is $CaHPO_4$. $2H_20$ (dicalcium phosphaten or brushite, C_2P), whereas at pH 24.2 the stable phase is $Ca_{10}(P0_4)_6(OH)_2$(HA). At higher temperatures other phases, such as $Ca_3(P0_4)_2$ (p-tricalcium phosphate, C_3P, or TCP) and $Ca_4(P0_4)_2O$, (tetracalcium phosphate, C,P) are present. $Ca_{10}(PO_4)_6(OH)_2$

Hydroxyapatite

Hydroxyapatite ,with the chemical formulae $Ca_{10}(PO_4)_6(OH)_2$ is a poly-crystalline ceramic which is chemically similar to inorganic phase of natural bone. It is chemically stable at pH < 4.3, i.e acidity levels of blood and bioactive. Bioactivity is related to a modification of the surface of the material with formation of naturally induced; biologically equivalent HAp as a result of dissolution, precipitation and ion exchange reactions with the physiological environment. It's bioactivity has been seen to be affected by structural crystallinity. The theortical mole ratio of Ca and P in it is 1.67. However, this is not the value observed in the organism because small amounts of carbon, nitrogen, iron and another elements are also incorporated. Thus, the general chemical formula is $Ca_{10-x}(HPO_4)_x(PO_4)_{6-x}(OH)_{2-x}$ where $0<x<2$. HA displays two types of crystalline systems hexagonal (present in teeth, bones and)and monoclinic.(as found in dental enamel). In the hexagonal systems, the hydroxyl ions (OH^-) are located at the centre of $Ca^{2}+$ triangles along the c-axes of the hexagonal unit cell. The OH^- ions are aligned in columns parallel to the c-axis along with Ca^{2+} and (PO_4^{3-}) ions.There are various parameters are to be considered for usage of hydroxyapatite such as size, morphology, appropriate stoichiometry, phase composition and crystallinity.

Hydroxypatite have been prepared by many different techniques. Precipitation techniques have been carried out using Calcium hydroxide [$Ca(OH)_2$] and orthophosphoric acid [H_3PO_4] [26]were starting materials of this reaction. Precipitation reactions can also be carried out using ammonium phosphate [$(NH_4)_2.HPO_4$] and $Ca(OH)_2$ [27]were starting materials Whereas in another reaction approaches, calcium hydrogenphosphate [$Ca(H_2PO_4)_2.H_2O$] and $Ca(OH)_2$ were used as starting materials. Another possibility of precipitation reaction was proposed i.e. the wet chemical reaction of calcium nitrate [$Ca(NO_3)_2.4H_2O$], with $(NH_4)_2.HPO_4$. Many other methods

for synthesis of hydroxyapatite have been reported like the Sol-Gel Approach, Hydrothermal Technique, Multiple Emulsion Technique, Biomimetic deposition technique, Electrodeposition technique etc.

Doped Hydroxyapatite:

The hexagonal structure of hydroxyapatite contains two different cation sites,Ca(I) and Ca(II), and only one phosphate environment (Ca(I), Ca(II) are used for stoichiometric apatite; M(I), M(II) are the general symbols for substituted apatites). Among the 10 cations, the 4 Ca(I)s are tightly bonded to 6 oxygens and less strongly to the other 3 oxygens (mean Ca(I)–O distance 0.255 nm), whereas the 6 Ca(II) atoms are surrounded by 7 oxygens (mean Ca(II)–O distance 0.245 nm). Ca(I) atoms are strictly aligned in columns and any small change in the metal–oxygen interactions affects the entire lattice. However, the Ca(II) atoms belonging to consecutive layers are staggered due to which it allows random local misplacements without affecting the whole structure. As a consequence, cations smaller than Ca or also low concentrations of slightly larger cations are preferably accommodated in site Ca(I) where stronger interactions are present, while larger cations should be accommodated in position Ca(II), even at high concentrations.

In particular, the high stability and flexibility of the apatite structure accounts for the great variety of possible cationic and anionic substitutions. HA is a member of a family of inorganic crystalline compounds, of general formula $M_{10}(XO_4)_6Y_2$. M is usually a bivalent cation, such as Ca^{2+},Sr^{2+},Ba^{2+},Cd^{2+},Pb^{2+}, but monovalent and trivalent cations, such as Na^{+},$K^{+}$and Al^{3+}can be replaced as well; XO_4 is usually PO_4^{3-}; VO_4^{3-}or AsO_4^{3-}but possible replacements include SiO_4^{4-}; $CO_3^{2-}$and SO_4^{2-}; Y is a monovalent anion like OH-.F-,Cl-,Br-[28,29,30].Cationic substitutions can occur for the whole range of composition, as happens for Sr^{2+},$Cd^{2+}$and Pb^{2+}, or to just a

limited extent as in the case of smaller ions which inhibit the crystallization of HA, such as $Zn^{2+}$and Mg^{2+} {31,32,33].

Hydroxyapatite has been doped by different ions to tailor it's properties according to our need. It has been doped by Ce^{3+} which increases it's antibacterial activity and also improves it's biodegradability[34]. The doping of HAp with silicon has shown improved bioactivity which is due to release of Si^{2+} ions rather than physicochemical modifications HAp due to increase of it's surface defects as reported earlier[35]. Doping HAp with F^- it increases it increases it's dissolution resistant property in biological microenvironment and hence increases it's stability and leads to long term performance compared to the pure HAp[36]. Doping of HAp with Ga^{2+} have been reported to increase calcium and phosphorus content of the bone and is found to be effective against bone resorption. It has been reported that HAp doped with Ag in small percentages can have an antibacterial effect, larger amounts can be toxic[37]. By doping of HAp with suitable concentration it has shown that the grain size decreased with increasing amount of Ti, and thus increasing the number of surface grain boundaries per unit length and increasing it's biological activity[38]. Zinc ions in the small quantities are essential for various metabolic processes in most of the living organisms and also helps in bone formation indirectly. Zinc doped hydroxyapatite has been reported to show antimicrobial activity[39]. The beneficial effect of low doses of Sr in the treatment of osteoporosis has long been known. So Sr doping can be beneficial against bone defects.

Due to large size difference between Mg^{2+} and Ca^{2+} (-0.28 Å difference in radius according to the Pauling scale), which leads to strong distortions of the HA lattice and reduces its crystallinity. Magnesium is one of the essential elements for all living organisms. Over 100 enzymes require the presence of magnesium ions for their catalytic action, including all enzymes

utilizing or synthesizing adenosine-5-triphosphate (ATP). Mg deficiency affects all skeletal metabolism stages causing cessation of bone growth, decrease of osteoblastic and osteoclastic activities, generation of osteopenia and bone fragility. Doping HA with magnesium directly influences the mineral metabolism, modifies the dissolution rate of crystals and the biodegradation of the corresponding materials. It has been reported, that doping HA with 2 mol% $Mg^{2+}$and Zn^{2+} significantly enhanced osteoblast adhesion as compared to pure HA, however higher doses of Mg^{2+} caused the opposite effect. Mg doped HAp has shown to reduce the deterioration rate of surface hardness, improve the surface hardness and the mechanical strength compared to normal HAp.

Requirement of Angiogenesis in Bone tissue Engineering:

Bone is highly vascularized. So, the performance of a bone scaffold is governed by its ability to induce new blood vessel formation which are important for the transport of oxygen, nutrients, soluble factors. These are essential for growth of the cells and tissues within the scaffold. Improper and insufficient vascularization leads to oxygen and nutrient deficiency, which may result in non-uniform cell differentiation and cell death. The primary aim of bone tissue engineering is to construct substitutes for bone grafts. It involves the preparation of a supporting matrix and ehich supports osteogenic cells and bioactive substances. One very critical problem in using of bone tissue engineering approach for large bone defects is the cell viability and nutrient supply at around the central region of scaffold is hampered as it is deprived of sufficient nutrient and oxygen supply as the diffusion distance is limited to 150-200 μm**.** A plethora of mediators associated with foetal and postnatal bone development play a prominent role in the cascade response in bone fracture repair like VEGF, pro-angiogenic factor.

Recently VEGF has been tried to be delivered to through nanoparticles to the zone of bone defect or have been directly incorporated along with the bone graft. But these procedures are afr too costly. Moreover VEGF binds to fibronectin domains in our body which are chopped off by MMPs (Metallo-Matrix Proteins) and hence their local availability gets minimized. Apart from that the molecular weight of VEGF is 43 kDa which makes it's transportation and availability difficult. This has led researches to think of alternative ways to induce angiogenesis in bone tissue engineering applications.

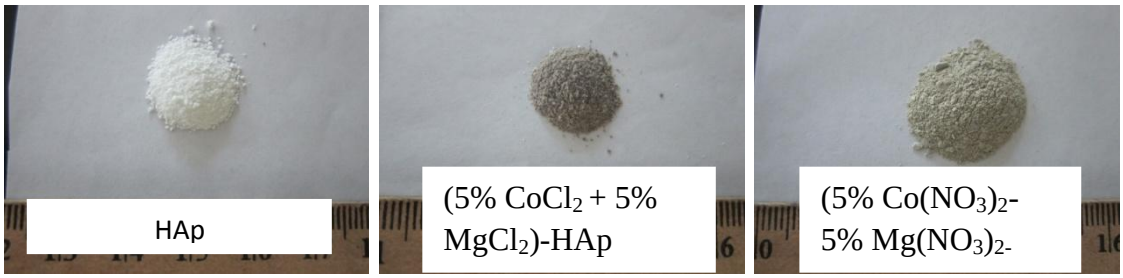
Under Hypoxia conditions it has been seen the HIF-1 (Hypoxia Inducible Factor) induces transcription of pro-angiogenic factors like VEGF for adaptation and survival of the cells. The HIF-1 factor consists of a constitutively expressed subunit- HIF-1β and an oxygen sensitive subunit- HIF-1α. HIF-1α has two transactivation domains i.e. N-Terminal Domain and C-Terminal domain. C-TAD as generally been seen to be interacting with the CBP/p300 that act as transcriptional coactivators. It also contains an oxygen-dependent degradation domain. Under normoxia conditions , the hydroxylation of 2 Proline residues (present in it's ODDD) occurs in presence of the enzyme PHD (in presence of oxygen, 2-OG, and Fe^{2+} and ascorbate) and acetylation of it's Lysine residues (in presence ARD1),promotes it's interaction with von Hippel-Lindau (pVHL) ubiquitin E3 Ligase complex leading to it's ubiquitinisation[19]. The hydroxylation of 1 Aspargine residue (present in it's C-TAD) blocks the recruitment of the transcriptional coactivator CBP/p300 (in presence of the the enzyme FIH). Under Hypoxia conditions the activity of these enzymes are disrupted and hence keeping the HIF-1α stable, which binds to Hypoxia Regulatory Elements of the target genes (eg- VEGF) and binds the coactivators to induce their gene expression. It has been seen that replacing Fe^{2+} ions with Co^{2+} ions stabilizes HIF-1α, by creating or mimicking a condition similar to hypoxia condition. This

idea can be implemented in preparing scaffolds by doping them with Co^{2+} ions hence increasing angiogenesis and formation of better vasculature at the site.

Materials method:

Synthesis:

Hydroxyapatite and Cobalt-Magnesium doped Hydroxyapatite are produced using Hayeck And Stadlman's Ammoniacal Precipitation Method as mentioned by V´azquez et al , with some modifications made[40]. The molar ratio of Ca/P is kept 1.67 as found in natural bone and teeth. For doped HAp the mole ratio of (Ca+Co+Mg)/P was set to 1.67 where mole ratio of (Ca: Co: Mg) was 20:1:1. Reagents which were used as starting material for divalent ions doped HAp synthesis are $Ca(NO_3)_2 .4H_2O$, $(NH_4)_2HPO_4$, $Co(NO_3)_2.6H_2O$, $Mg(NO_3)_2.6H_2O$ from HiMedia(Mumbai, India) , $MgCl_2.6H_2O$, $CoCl_2.6H_2O$, NH_4OH from Merck (Mumbai, India). Glasswares from Tarsons were used. Two different sets of doped HAp has been prepared one with chlorides of divalent metal ions noted as (5% $CoCl_2$ + 5% $MgCl_2$)-HAp and another with nitrates of divalent metal ions noted as (5% $Co(NO_3)_2$-5% $Mg(NO_3)_2$-HAp. In a 500ml glass beaker, 200 mL of 0.5 M of $Ca(NO_3)_2 .4H_2O$ solution containing Co and Mg ions in the respective ratio were taken and the corresponding required volume of 0.3 M of $(NH_4)_2HPO_4$ solution was added drop-wise at a rate of ~2 mL/min from a burette under sonication at 80°*C* (using apl Digital Ultrasonic Cleaner). NH_4OH was added to maintain pH 10 -12 throughout the process. Then the mixture was aged for 1 day at room temperature. After aging period, the solution was centrifuged at 6000 rpm for 10 minutes (REMI C-24 BL)and the pellet was washed several times with distilled water to remove residual ammonia and other impurities. Then it was dried at 50±5 °*C* for 24 hours and they were crushed using mortar and pestle to get the hydroxyapatite powder.



Characterisation :

XRD

The phase content of the samples were characterized by X-Ray Diffraction(XRD) by using Philips XRD-PW1700 diffractometer producing a monochromatic CuKα radiation of wavelength(λ=1.514 Å). The scanning range of 2θ from 20-60° with rate of 2°/min. The step size was 0.02°/second[41].

FTIR

FTIR is the technique used to characterize and find out the functional groups present in the samples. FTIR spectrophotometer AKTR was used with scanning range of 400 – 4000 cm^{-1}. The samples were made into pellets using KBr pelleting technique with KBr as reference.[42]

SEM

The morphological properties of the samples were characterized by scanning electron microscopy (JOEL India JSM-6480Lv). The samples were sputter coated with platinum under vacuum for SEM studies and the scanning was done at 25 kV and 40 mA in vacuum. The

elemental analysis of the samples were done with energy dispersive X-Ray spectroscopy attached with the SEM[42].

Zeta

Zeta potential of the samples was determined using Malvern Instruments' Zetasizer with PBS as dispersion medium. The samples were dispersed in Milli-Q-water under sonication for 20 minutes.[43] 5 mg of samples were dissolved in 5 ml of PBS.

TGA and DSC

TGA was done to determine the thermal stability of the sample. It was done at a scanning rate of 10°C/min with scanning range from 25°C to 800°C. Sample amount of ~10 mg was used [44]. DSC of the moisture incubated samples was carried out at a scanning rate of 10°C/min with scanning range from 50°C to 600°C.

Biological Characterization

Hemolysis:

The biocompatibility of the sample was studied by carrying out hemolysis test using goat blood. The reagents used were Tricalcium Sodium Salt Hydrochloric Acid ,Sodium Chloride from Merck (Mumbai,India).Glasswares of Tarsons were used. 2.8g of the anticoagulant Trisodium Citrate was added for every 100 ml of blood and was stored at 4°C till use. This sample was diluted by adding 10 ml of saline to 8 ml of it before using it for the test. Saline solution was prepared by adding 0.9 g of NaCl to 100 ml water. The test was carried out for with 10 mg of hydroxyapatite, (5% $CoCl_2$ + 5% $MgCl_2$) Hydroxyapatite and 5 % $Co(NO_3)_2$ + 5% $Mg(NO_3)_2$ Hydroxyapatite each in triplicates.1 ml of saline was added to each test tube and it was incubated

for a day at 37°C for neutralization. After that 0.5 ml of blood saline solution is added to each sample. For positive control 0.5 ml of 0.01 N HCl is added and for negative control 0.5 ml PBS was added in triplicates. The volume of each test tube was made upto 10 ml by adding saline solution and it is incubated for an hour. Then the solutions were transferred to centrifuge tubes and centrifuged at 4000 rpm for 10 minutes (using REMI C-854/6) and then the absorbance of the supernatant was measured using a UV Spectrophotomometer (Systronicx Double Beam Spectrophotometer 2203).

Protein Adsorption:

In vivo cellular response on the implant after implantation depends on the initial amount of serum proteins that get adsorb to the implant. Hence the estimation of protein adsorption is important. From the previous experiment the effective saturation concentration of BSA(Bovine Serum Albumin) was found to be 1200 µg/ml which was used further in the following experiments. HAp samples of 10mg/ml were added to 1ml aqueous solution of BSA of concentration 1200 µg/ml in the respective test tubes [45]. Then the tubes were incubated at 37 °C for 24 hours. After 24 hours of incubation, the samples were centrifuged at 8000 RPM for 10 mins[46]. The residual protein concentration was found out from the supernatant using Bradford assay, measuring absorbance at 595 nm. Subtracting the residual protein amount from the total protein amount, the protein absorbed(µg) was determined.

Antimicrobial Test:

200 ml Nutrient Agar was made in 500 ml conical flask and it was autoclaved.The media is poured into 6 petridishes and allowed to solidify.100 µl of culture of Bacillus subtilis and 100µl

of culture of Escherechia coli were spread plated on 3 petridishes each. Three punctures were made on each solidified nutrient agar plate. In one puncture 0.1 g of the drug Metronidazole is added, 30 µl of blank (distilled water) was added in the 2^{nd} puncture and 30 µl (containing 1 mg of sample) of the three samples in the 3^{rd} punctures of the three different nutrient agar plates for each bacteria.

MG-63 Cell Culture:

The MG63 cell line was kindly provided by Prof. T.K.Maitin (IIT Kharagpur) . The cells were maintained in DMEM with 10% FBS at 37^{o}C, 5% CO_2 and 95% humidity. While passaging, the cells were washed and harvested using 1X Phosphate Buffer Saline and Trypsin solution respectively. The viable cell density in the harvested culture was accessed using Trypan Blue dye exclusion test and the cells were then seeded onto 96 well plate at a cell concentration of 5×10^4 cells/ml. The seeded plate was kept in incubator for next 12 hours for adherence. After that, cells were incubated with the samples for 24 hours and the cell viability was accessed using MTT Assay. To evaluate cell hydroxyapatite interactions, cells exposed to hap samples were fixed with 2.5% Gluteraldehyde then serially dehydrated using ethanol. The dehydrated samples were further sputter coated with palladium and visualized using SEM (JOEL JSM6480LV) at an accelerated voltage of 20kV.

Results and Discussions:

XRD Analysis:

XRD analysis of the samples (Fig.1) are very similar to the standard $Ca_{10}(PO_4)_6(OH)_2$ as mentioned in the standard data (JCPDS No. 09–0432)[47]. The XRD peaks of the samples are identical to the characterization peaks of hexagonal crystalline hydroxyapatite [48]. Both the doped HAp doesn't reveal any peaks which are corresponding to magnesium or cobalt indicating that the ions may be doped into the frame of HAp crystal structure perfectly [47]. The XRD pattern of 5%$Co(NO_3)$2-$Mg(NO_3)_2$-HAp is very similar to that of HAp compared to 5%($CoCl_2$-$MgCl_2$)-HAp. In the case of 5% ($CoCl_2$-$MgCl_2$)-HAp the peaks are broadened and merged together which is due to the replacement of high ionic radius Ca(0.099nm) by less ionic radii Co(0.070nm) and Mg(0.071nm) [49,50]. And also may be due to replacements of $(PO_4)^{-3}$ anion by (Cl^-) anion. Decrease in the intensity and broadening of peaks reveals that there is decrease in crystallinity and crystalline size as the chlorides of the divalent ions are incorporated into the crystal structure of HAp[49] But in case of 5%$Co(NO_3)$2-$Mg(NO_3)_2$ HAp the peaks are very similar to the HAp with slight decrease in intensity which is due to the cation replacement.

FTIR:

The FTIR analysis of all the three samples showed the characteristic bands that are corresponding to standard hydroxyapatite. In case of 5%$Co(NO_3)$2-$Mg(NO_3)_2$ the characteristic bands were present but they were divided and less intense. This may be due to the doping of Co and Mg. But in case of 5% ($CoCl_2$-$MgCl_2$)-HAp the bands were very similar to that of HAp. The bands about ~3450 cm^{-1} and 1640 cm^{-1} correspond to absorbed water [51] The band at about 3572cm^{-1} corresponds to the stretching vibration of the hydroxyl group (reference b). The band

at 1035 cm^{-1} and 1095cm^{-1}, in the samples were assigned to the P–O stretching vibration of the phosphate groups ($PO4^{3-}$)[51]. The band at 470 cm^{-1} corresponds to bending vibration of phosphate group. And also the bands around at 550 and 600 cm^{-1} correspond to the bening mode of $PO4^{3-}$[52]The band at 1470 cm^{-1} and 870 cm^{-1} were assigned to CO_3^{2-} ions [53]

Protein Adsorption:

The protein adsorption of HAp and 5%$Co(NO_3)_2$-$Mg(NO_3)_2$-HAp are more or less same with slight high of first over the second one. But the protein adsorption of 5% ($CoCl_2$-$MgCl_2$)-HAp was significantly less compared to the above two. The protein adsorption increases with increase in surface area of the samples where the crystal size is small [54]. 5% ($CoCl_2$-$MgCl_2$)-HAp are more denser with less surface area compared to that of less dense and high surface area 5%$Co(NO_3)_2$-$Mg(NO_3)_2$-HAp. And also protein adsorption is directly proportional to the distribution of Ca^{2+} and $PO4^{3-}$ ions on the crystal planes of the sample [55]. In case of 5% ($CoCl_2$-$MgCl_2$)-HAp as per the XRD results the pattern was little broadened indicating deformation or unevenness in the crystal structure and charge distribution over the surface which leads to less protein adsorption compared to non deformed 5%$Co(NO_3)_2$-$Mg(NO_3)_2$-HAp. The amino group of proteins have good affinity towards PO_4^{3-} ions(17). Therefore in case of 5% ($CoCl_2$-$MgCl_2$)-HAp part of the PO_4^{3-} ions by Cl^- ions hence there may be slight decrease in protein adsorption compared to HAp and 5%$Co(NO_3)_2$-$Mg(NO_3)_2$-HAp.

Hemolysis:

Hemolysis test is an important way for determining biocompatibility of biomaterial(Xiao et al, 2010). If the rate of Hemolysis is <5 % the sample is finely haemocompatible, 5% -10% then it is

haemocompatible and if it exceeds 10% it is non-haemocompatible.(Puvvada et al,2010).The rate of hemolysis for HAp was found to be ~1.4 %, in case of 5% ($CoCl_2$-$MgCl_2$)-Hap was found to be ~ 2.5 % and that of 5%$Co(NO_3)_2$-$Mg(NO_3)_2$-HAp was found to be ~ 0.8 %. So all the samples are found to be non-hemolytic.

SEM

Here we find the formation of agglomerations in presence of the doped hydroxyapatites which may be due to the presence of cobalt doping as they are having magnetic properties.

Zeta:

In case of zeta potential we find out the zeta potential of the particle with less negative zeta potetial has more protein adsorption as it has more surface area as matches with our protein adsortion data.

Sample Name	Zeta Potential in mV)
Control	-23.6
Hydroxyapatite	-16.4
5 % $CoCl_2$+5% $MgCl_2$ Hydroxyapatite	-22.5
5 % $Co(NO_3)_2$ +5 % $Mg(NO_3)_2$ Hydroxyapatite	-20.4

EDAX

The EDAX was carried out to estimate the exact proportion of Cobalt and Magnesium in the samples.The EDAX confirmed the presence of both cobalt and magnesium. The amount of cobalt was found to be 0.33 wt %,4.93 wt % and 2.92 wt % respectively for HAp, (5% $CoCl_2$ + 5% $MgCl_2$)-HAp and (5% $Co(NO_3)_2$-5% $Mg(NO_3)_2$)-HAp respectively. Similarly the amount of Mg was found to be 0.37%, 1.31 % and 1.79 % for HAp, (5% $CoCl_2$ + 5% $MgCl_2$)-HAp and (5% $Co(NO_3)_2$-5% $Mg(NO_3)_2$-HAp respectively. We can find some percentage by wt of Mg and Co in HAp due to their presence as impurity. This shows the doping has not occurred in the expected amount.

Element Doped	Pure HAp	$[Co^{+2}/Mg^{+2}/Cl^{-}]$doped Hap	$[Co^{+2}/Mg^{+2}/NO^{-}_3]$doped Hap
Mg	0.37	1.31	1.79
Co	0.33	4.93	2.92

TGA and DSC:

Here we see the samples show weight loss initially till around 180°C but after that it shows constancy or we can say inert behavior. In case of (5% $Co(NO_3)_2$-5% $Mg(NO_3)_2$)-HAp it shows again loss of weight between 500-600 °C and this might be due to loss of water from the lattice

crystals.In case of the DSC, we can see in case of (5% $Co(NO_3)_2$-5% $Mg(NO_3)_2$).HAp there is more water retention capacity as it shows an endothermic peak, the (5% $CoCl_2$ + 5% $MgCl_2$)-HAp behaves inertly and the standard HAp shows exothermic peak which might be due to decomposition of some carbonate or oxide present in

Antimicrobial Test:

In case of (5% $CoCl_2$ + 5% $MgCl_2$)-HAp and (5% $Co(NO_3)_2$-5% $Mg(NO_3)_2$).HAp in case of the E coli plates we found a concentric circle formed, which is the zone of inhibitio

of (5% $CoCl_2$ + 5% $MgCl_2$)-HAp a clearer and more prominent zone of inhibition is formed. So, it posseses good antimicrobial properties.

Cell Study and MTT Assay:

The MTT assay is a measure of activity of Mitochondrial Succinate Dehyrdogenase enzyme. It is generally observed that such activity often corresponds to the cell number present in a given sample. In this present study, it was found that none of the Hydroxyapatite was showing cytotoxicity on MG63 cells. Hydroxyapatite as a native component of bone mineral is a known osteoconductive agent that promotes osteoblast proliferation. Here, doping of Cobalt and Magnesium ions in the synthetic Hydroxyapatite donot imparts any toxicity. Rather, Mg/Co Chloride doped sample has shown more proliferative effect in comparison to control. From the SEM micrograph, it is evident that in all cases cells are remained in a healthy condition. It is

important to mention that in case of Mg/Co chloride doped samples cells have started forming nodules in a very short time span, which is a hallmark of osteogenic property of any materials.

Conclusion:

The physico-chemical characterizations and biological characterizations of the HAp and the doped HAp produced. The particle size of (5% $Co(NO_3)_2$-5% $Mg(NO_3)_2$).HAp was found to be smaller as compared to the standard HAp and (5% $CoCl_2$ + 5% $MgCl_2$)-HAp, so it has a smaller negative zeta potential and higher protein adsorption. The (5% $CoCl_2$ + 5% $MgCl_2$)-HAp shows nodule formation with MG-63 cell lines after 48 hours of incubation and it shows good haemocompatibilty and antibacterial properties. So, it can be preferably used for tissue engineering applications.

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