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RESEARCH ARTICLE
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ZINC AND COPPER INFUSED BIOACTIVE GLASS FOR REGENERATIVE APPLIANCES

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ABSTRACT:
Bioactive glasses are a group of surface reactive glass-ceramic biomaterials and include the original bioactive glass, Bioglass. The biocompatibility and bioactivity of these glasses has led them to be used as implant devices in the human body to repair and replace diseased or damaged bones.
AIM: The purpose of this study is the establish how bio active glasses are used for regenerative materials.Bioactive materials are well known regenerative materials. Here, we introduced zinc and copper to enhance the antimicrobial activity of the materials, since they have prompt activity to inhibit microbial growth over the regenerative area.
MATERIALS AND METHOD: Bioglass was prepared using tetra ethyl ortho silicate (45 %), ortho phosphoric acid (6 %), calcium nitrate (24.5 %), and Sodium nitrate (24.5 %). Similarly, we prepared ZnO-CuO bi metallic components and that was introduced into the host bioglass structure to formulate a composite.

INTRODUCTION:

Modified phosphosilicate glasses, phenomenally discovered by Larry Hench in the year 1971, introduced an entirely new field in biomedicine as they have the capability to form stable chemical bonds with bone and tissues . Focus of this study is on bone-tissue regeneration, drug de-livery, hemostatic, and dental applications. In the aspect of biological applications' numerous resorbable polymeric materials were
used .However, major drawbacks of polymers are, they do not bond with bone and are not capable of stimulating genes in bone cells. Then, the research moved towards hydroxyapatite, due to its similarity with human bone, biocompatibility, and bioactivity. Nevertheless, hydroxyapatite reab- sorption is very slow, and dissolved ionic components do not stimulate genes of osteogenic cells, and it is week osteoconductive .Interest- ingly, bioactive glasses entered into the field of tissue engineering with incredible properties than polymers and hydroxyapatite. Bioactive ma- terials are bioactive, biocompatible, biodegradable, osteoconductive as well as osteo-productive, also these materials bond with soft and hard tissue.

These bioactive materials control osteoblastic cells and induce gene expression, which regulates osteogenesis and growth factor pro- duction. Amorphous silica network offers a vital contribution to bone mineralization and activation of genes.
Most bioactive glasses are silicate based glasses that are degradable in body fluids and can act as a vehicle for delivering ions beneficial for healing. Bioactive glass is differentiated from other synthetic bone grafting biomaterials (eg. hydroxyapatite, biphasic calcium phosphate, calcium sulfate), in that it is the only one with anti-infective and angiogenic properties.
Solid state NMR spectroscopy has been very useful in determining the structure of amorphous solids. Bioactive glasses have been studied by 29Si and 31P solid state MAS NMR spectroscopy. The chemical shift from MAS NMR is indicative of the type of chemical species present in the glass. The 29Si MAS NMR spectroscopy showed that Bioglass 45S5 was a Q2 type-structure with a small amount of Q3; i.e., silicate chains with a few crosslinks. The 31P MAS NMR revealed predominantly Q0 species; i.ePO43−; subsequent MAS NMR spectroscopy measurements have shown that Si-O-P bonds are below detectable levels.
While metals are not necessarily inherently bioactive, bioactive glass coatings which are applied to metal substrates via laser-cladding introduce the bioactivity that the glass would express, but have the added benefits of having a metal base.
Laser Cladding is a method by which bioactive glass microparticles are thrust in a stream at the bulk material, and introduced to a high enough heat that they melt into a coating of material.S53P4 bioactive glass was first used in a clinical setting as an alternative to bone or cartilage grafts in facial reconstruction surgery.The use of artificial materials as bone prosthesis had the advantage of being much more versatile than traditional autotransplants, as well as having fewer postoperative side effects.

MATERIALS AND METHOD:

Bioglass was prepared using tetra ethyl ortho silicate (45 %), ortho phosphoric acid (6 %), calcium nitrate (24.5 %), and Sodium nitrate (24.5 %).
Similarly, we prepared ZnO-CuO bi metallic components and that was introduced into the host bioglass structure to formulate a composite.
Preparation of Bioactive Glass SiO2 (45%), P2O5 (6%), CaO (24.5%) and Na2O (24.5%) sources were utilized to synthesize bioactive glass by sol-gel method. Synthesis protocol was briefly reported in our earlier report 18. Further to that, the prepared bioactive glasses were annealed at various temperatures such 600 oC for 3 h heat treated bioactive material designated as 600-3h, similarly
 600 oC for 2 h named as 600-2h, step-annealing was carried out to decompose the nitrate content while extending the heat treatment at 550 oC for 3 h along with 600 oC for 3 h and likewise 550 oC for 2 h with 600 oC for 3 h, the corresponding materials were designated as 550-3h and 550-2h respectively. Finally, the material was sintered at 800 oC for 3 h, and was designated as 800-3h.

Hemocompatibility Assay

Hemocompatibility is considered as one of the primary biocompatibility assay to assess the compatibility of biomaterials. Especially, for all the biomaterials at the point of contact at in vivo, foremost interaction will be with blood cells. Particles of bioactive glasses enter into the blood and get into contact with erythrocytes. Hence, it is essential to explore the compatibility of bioactive glasses in the bloodstream. In order to evaluate such response with red blood cells (RBCs), in vitro hemolytic activity was carried out. Hemolytic activity was performed to assess the biocompatibility of the bioactive materials. Protocol was followed from our previously reported work 19 and the percentage of lysis was determined through Eq (1).

$$Hemolysis\ (\%) = \frac{Sample\ absorbance - Negative\ control}{Positive\ control - Negative\ control}\ X\ 100 \ldots\ldots\ldots\ldots (Eq.\ 1)$$

Subsequently, clot lysis was carried out with fresh blood (without anticoagulant). For that, 100 µL of the blood was dropped on the bioactive materials in watch glasses. After 10 min, the clotted blood was washed with 10 mL of double distilled water. The corresponding solution was used to found the rupture rate of hemoglobin, which was obtained by the absorption at 540 nm 20. All the materials were tested with their triplicates.

Blood and Phosphate Buffer Saline Absorption Efficiency of Bioactive Materials

The absorption capability of bioactive materials in phosphate buffer saline (PBS - pH 7.4) and citrated whole blood was evaluated. (500 µL) blood either PBS was placed in well plates (tissue culture plates), pre weighed dry pellets (Pelletsdry) were dipped into a blood pool to absorb blood and PBS adequately. Corresponding plates were sealed and incubated at 37 °C, for an hour. After incubation, the pellets (pelletswet) were removed and gently wiped using absorbent paper to suck excess moisture and freely draining liquid on the surface of material. To estimate the absorption capability of bioactive materials, they were weighed twice before and after dipping into blood pool as well as in PBS. The fluid uptake of bioactive materials (%) was calculated using Eq.

$$Absorption\ Efficiency(\%) = \frac{Pellets_{(wet)} - Pellets_{(dry)}}{Pellets_{(dry)}} \ldots\ldots\ldots\ldots\ldots (Eq.\ 2)$$

In-Vitro Thrombus Formation on Bioactive Materials

Thrombogenic effect induced by bioactive materials was evaluated according to Sara et al. 21 Blood was collected from healthy volunteers and subsequently mixed with sodium citrate (9:1 ratio (whole blood to 3.2% sodium citrate)). Pre-weight bioactive materials powder (P0) was placed at cell culture plates (24 well plates) at 37 °C incubation. Further thrombus formation was estimated by adding citrated blood (500 µL) into bioactive materials. After (30 min) incubation, double distilled water (10 mL) was cautiously dropped into culture plates without disturbing the blood clot. Subsequently, formaldehyde (37%) solution was used to fix the formed thrombus, for 10 min. Further, the corresponding samples were dried in hot air oven

 at 50 °C, after that their weights were calculated as (Pt). The thrombogenicity formation with the influence of bioactive materials was calculated using Eq.3.

$$Thrombogenicity(\%) = \frac{P_t - P_0}{P_0} X\ 100 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (Eq.\ 3)$$

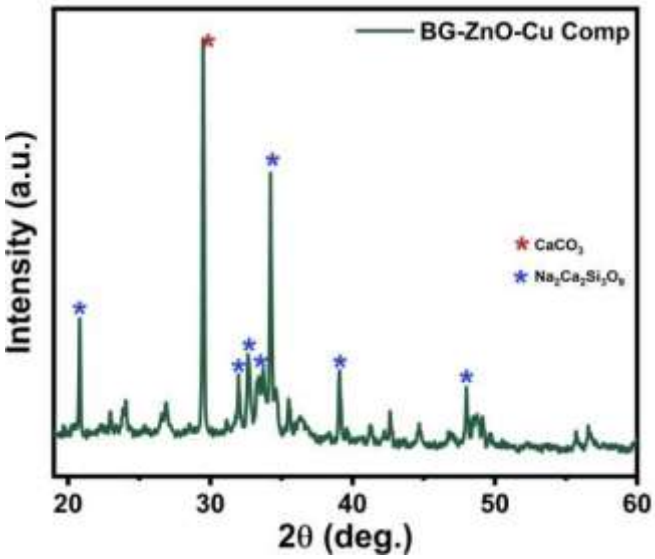
Materials Characterization

Thermal stability of bioactive glass was studied using Universal TA instrument (model SDT Q600 V8.0 Build 95) under nitrogen atmosphere. Nitrate quantification measured using 883 Basic IC plus. Bioactive materials were characterized to study their crystalline phases by X-ray diffraction (XRD - PANalytical Instruments, The Netherlands). Morphology of synthesized bioactive materials was imaged using scanning electron microscopy (SEM - Hitachi N3400, Singapore). Vibrational modes are recorded using Micro Raman spectra by confocal Raman microscope (RAMAN 11i - Nanophoton). UV-VIS spectrophotometer (LMSP-UV1200, LABMAN) was used to investigate the blood sample measurements.

RESULT AND DISCUSSION:

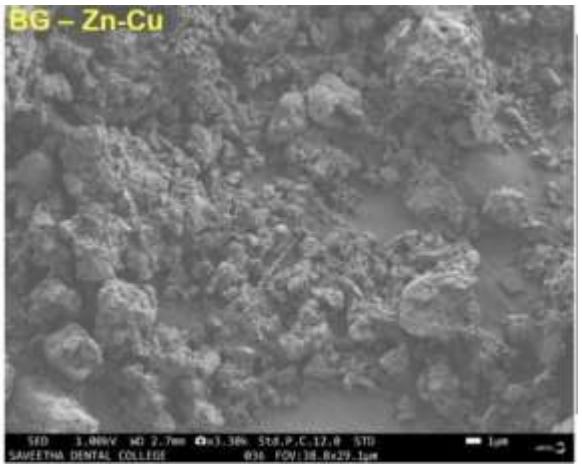
X-ray Diffraction:

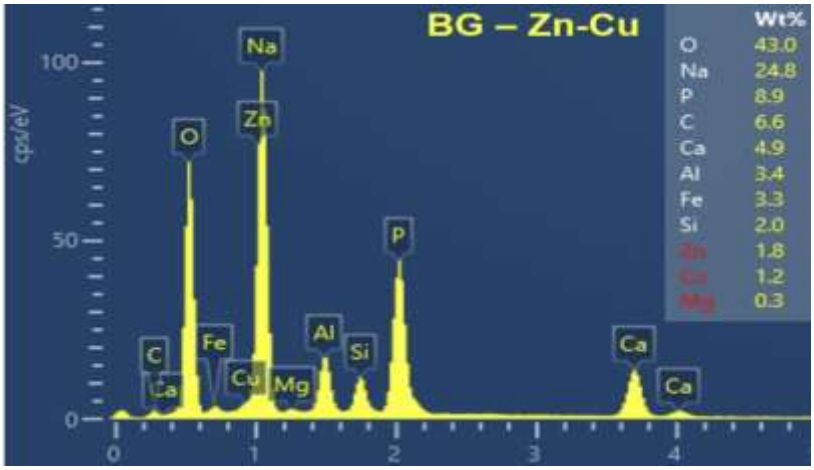
To analyze the crystal structure of the fabricated material



XRD results indicate that the diffracted peaks confirm the presence of calcium, phosphosilicate based mineral structure, which authenticate the material formation.

Field Emission Scanning Electron Microscopy & Energy Dispersive Spectroscopy: To analyze the morphological as well as elemental properties of the fabricated material



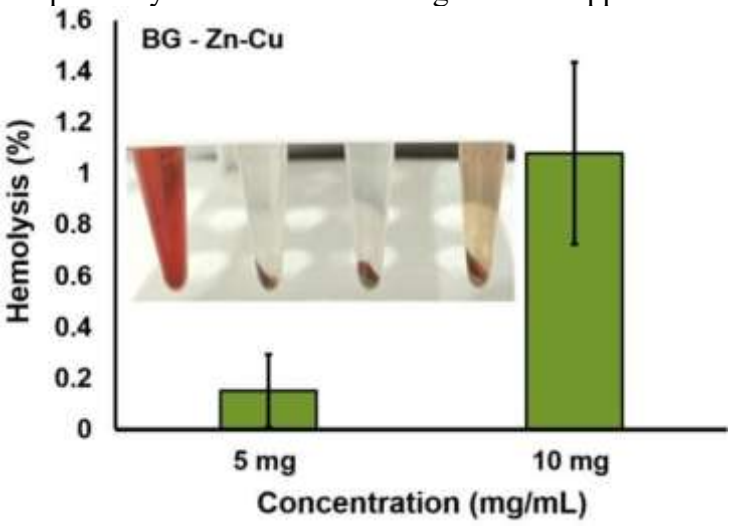


Morphological features showed the spherical and plate like morphology with non- homogeneous nature. Bulk silica ball and the crystal particles over the silica balls confirms the bioceramics nature.

Relevant Si, Ca, P, Na, Zn, Cu, C, and O elements were confirmed from the EDS spectra.

Hemocompatibility Assessment:

To analyse the blood compatibility of the material for regenerative applications.



According to ASTM standard upto 5% lysis is acceptable in this case we observed a maximum of 1.2 % lysis at the concentration of 10 mg/mL/. Hence, it is more compatible for regenerative applications.

Dynamic Whole Blood Clotting Assay

Optical density (OD) values of clot lysis exhibits the active haemoglobin rupture, which emphasize the rate of clot induced by the materials. Hence, in bioactive material treated blood, the hemolyzed hemoglobin rate was minimal compared to controls. Therefore, it can be understood that, materials induce the clot at the time of contact with RBCs. If the blood completely clotted means the OD will be zero. Compared to all the treated bioactive materials, lowest absorption and highest rate of clot was noted in 800-3h. The OD values displayed the clotting rate of blood with the presence of bioactive materials. Relatively optimal blood compatibility was displayed by 600-2h and 550-3h and 800-3h displayed highest clotting rate.Phosphate Buffer Saline and Blood Absorption Capability of Bioactive materials

Absorption ability of 800-3h and 550-3h was evaluated using blood as well as PBS to investigate the hemostatic responses. The 550-3h pellets showed 48.9% absorption in PBS alternatively the absorption rate decreased as 32.1% in blood. Similarly, 800-3h revealed 17.1% absorption in PBS and 12.4% in the case of blood.

Absorption capability of 550-3h is higher than 800-3h, this may be due to the morphology, porosity inclusive with the crystal structure. Among blood and PBS, dominant PBS absorption rate was visible as per the estimations, which might be attributable to clotting behavior of blood. Hence overall results exhibited that, highest absorption rate around 50% was acquired in 550-3h (pellets) soaked in PBS.

In-Vitro Thrombus formation and Clot initiation influence of Bioactive Materials Thrombogenic behavior of blood at the time of interaction with bioactive materials was evaluated in-vitro. Clot initiation time was investigated for bioactive materials and compared with control. Whole blood clot started at 8.32 min and 550-3h initiated the clot at 6.25 min, on the other hand earlier clot generation was noted within 1.29 min while treating with 800-3h (Fig.12 (b)). Complete blood clot (500 µL) was influenced by 800-3h within 3 min due to the influence of calcium release. Thrombogenicity estimation was followed according to Yohji et al. and Sara et al. 45, 46 Previous reports elucidated the considerable hemostatic behavior of bioactive glass 46, 47.

In this work, we found that bioactive glass sintered at 800 oC has tremendous ability to accelerate blood clot. Thrombogenic effect of 800-3h (50 mg/500 μL blood) is around 1100%, it is highest compared to 550-3h and control (natural clot without the influence of material.
We fabricated the materials with relevant Ca, Si, P, Na, Zn, and Cu sources . Then we make the bioglass and ZnO-CuO composite by solid state reaction.
Further we analyzed the structural, morphological and biocompatible properties of the material.
Relevant mineral phases ($Na_2Ca_2Si_3O_9$ and $CaCO_3$) was observed through X-ray diffraction.
Spherical and flake-like morphology and elemental compositions were obtained through FE-SEM & EDS.
Optimal blood compatibility was procured from Hemocompatibility assessment. Similarly, around 800 oC this silicorhenanite crystalline phase was reported by several authors 53, 54. Aina et al. 55 specified $Na_2CaSi_2O_6$ crystalline phase at 600 oC and also reported the presence of $NaCaPO_4$. In this investigation, sol-gel prepared bioglass underwent thermal treatment with various parameters (different temperature and time). It is evident that, crystallization noted from 550 oC onwards and phase transition was found in 800 oC. Transition of $NaCaPO_4$ crystalline phase (600-3h) into $Na_3CaPSiO_7$ (800-3h), exhibits the crystallization of phosphate ions in the bioactive glass network. All together it is concluded that crystallinity mainly depends on sintering parameters. Raman spectra revealed that, at 800 oC, the amorphous network turns into crystalline material and also dominates the intense phosphate vibrational modes owing to the phase transition of bioactive glasses. The 800-3h spectra sparsely deviated from the vibration modes of other bioactive materials due to the crystallization. Raman spectra interrelated with FT-IR vibration modes were displayed in Fig. 5. In the circumstance of morphology, sintering parameters stimulate the morphology and changed the appearances from spherical (600BG) to rod-like (550-3h) manifestations. Ciobanu et al. 56 stated that heat treatment influences the morphology, and explained that hydroxyapatite small flakes turned to bigger spherical shapes via annealing from 80 oC to 1000 oC. Similarly, by raising temperature from 600 oC to 800 oC, the particles are congregated and their appearance was changed from spherical to flake-like. Ninja et al. 57 suspected that bioactive glasses calcined at above 800 oC could not have negative influence in the presence of biological environment. Several authors 58, 59 described that, bioactive glass crystallization leads to decrease in the bioactivity and probably formulates, like inert materials.

CONCLUSION:

From this study, it is proposed that, highest compatibility was acquired from step annealed (550-3h) material, which could be ascribed to the less nitrate content and poor crystalline nature. Hence, step annealed bioactive materials improve the biocompatibility and generate a positive environment for the biological functions. Hence, we propose that bioactive material sintered at higher temperature (800-3h) can be a suitable hemostat at injured site, that can act as a hemostat as well as remodulate the wound by healing.

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