

Bioconversion of zinc added fluorophosphate glasses and method of making thereof

**The following specification particularly describes the invention
and the manner in which it is to be made**

DESCRIPTION**Field of invention**

The present invention describes the composition of zinc added fluorophosphate glasses by melt quenching technique for different bio-medical applications.

Background of invention with regard to the drawback of associated known art

A number of materials have been examined for their ability to regenerate new bone. Currently, autologous bone remains the preferred material in bone graft and regenerative procedures. This bone, taken from a secondary site within the body is often excised and reimplanted. It contains both the inorganic mineral hydroxyapatite as well as the cell characteristics of bone. Even this is not a living material, but can remodel into new and functional bone. Autografts may be combined with supplementary agents such as growth factors or synthetic bone replacement materials. The disadvantage of autologous bone usage is the creation of a secondary trauma site that has to heal. Further, its usage is limited by availability. The materials taken from cadavers (allograft) are not constrained by supply. The above material is often demineralized, leaving behind a collagenous scaffold for the growth of a new bone. Further, it fails to function as an osteoinductive but only as osteoconductive material. These materials always carry the risk of disease transmission with them.

The third generation of materials used for the regenerating new bone is bioactive glass. These materials have the unique ability to form a chemical bond with the host tissue when implanted. Thus, it allows to retain the structural integrity and to resist movement at the implantation site. The chemical bond that occurs through the formation of hydroxyapatite on the glass surface converts the glass into bone rich material. The beneficial effects of bioactive glasses on cellular growth also substantiate to their use.

Attempts were made to synthesis artificial bone graft material which is suitable for different biomedical applications. L.L.Hench in Imperial college, London succeeded in developing silica based material, named Bioglass, in the year of 1960. But, it was hard to get good compatibility with biological tissues because of their relative insoluble nature. Silica-free phosphate glasses, namely bioactive glasses, were made in the year 1996. These glasses show better bioactivity due to their chemical composition, which is compatible with that of natural bone. But still the slow rate of biological conversion and poor mechanical strength of bioactive glasses hampers its clinical application. To circumvent these pitfalls a better composition of bioactive glass with higher porosity, elastic moduli, mechanical strength, bioconversion and controlled rate of dissolution near that of natural bone is continuously searched.

Object of invention

Fluorine when added in low non-toxic concentration to phosphate based glasses produce fluorophosphate glasses which have a higher bioconversion rate than phosphate glasses. But to increase the mechanical strength of the fluorophosphate glasses addition of various metal oxides are tried. Of all the metal oxides zinc oxide has the unique property of increasing both bioconversion rate as well as mechanical strength.

Zinc is an essential trace element that has an important role in bone formation. It is fundamental for bone cell growth and its development and differentiation. Zinc is a cofactor for many enzymes. It stimulates protein synthesis and it is essential for DNA replication. Zinc deficiency is associated with skeletal growth retardation and alterations in bone tissue calcification. Zinc-modified calcium silicate ceramics ($\text{Ca}_2\text{ZnSi}_2\text{O}_7$) were shown to support human osteoblast-like cells to attach and form a well-organized cytoskeleton structure. They also effect an increased cellular proliferation and differentiation expressed by the increased levels of osteoblast-related mRNAs (alkaline phosphatase, collagen type I, osteocalcin) as compared to calcium silicate ceramics (CaSiO_3). Further, ZnO is a network modifier in phosphate based glasses and is able to modify the P-O-P glass network into P-O-Zn⁺⁺ which results in altered dissolution rate of the glass in SBF solution.

The present invention discloses the different composition of zinc oxide added fluorophosphate glasses $\text{P}_2\text{O}_5\text{--CaO--Na}_2\text{O--CaF}_2\text{--ZnO}$ (hereafter termed, respectively, as ZnFP1, ZnFP2, ZnFP3, ZnFP4 and ZnFP5) are prepared by keeping the ratio of P/Ca as a constant. The addition of ZnO to fluorophosphate glass system modifies surface nucleation. It is known that the degradation of glass alone is not an important factor for bone-bonding ability of glass, but the composition of glass also plays an important role. The present invention discloses the improved bioactivity by adding fluorine and zinc molecules and enhanced mechanical strength, load bearing capability, elastic moduli etc. by adding zinc oxide.

Statement of invention

1. The protocol for melt quenching method to produce zinc added fluorophosphate glasses.
2. The addition of zinc with fluorophosphate yield better mechanical strength.
3. Physico-chemical properties of zinc added fluorophosphate glasses are accessed.
4. The bioconversion of zinc added fluorophosphate glass is ascertained.
5. No cytotoxicity is observed in zinc added fluorophosphate glasses up to the addition of 8 mol% of zinc content.

A summary of invention

The present invention discloses the composition with different contents of P_2O_5 —CaO— Na_2O — CaF_2 —ZnO (ZnFP1, ZnFP2, ZnFP3, ZnFP4 and ZnFP5) glass systems are prepared using normal melt-quench method by keeping the ratio of P/Ca is constant. The physico-chemical and bioactivity of the zinc added fluorophosphate were assessed using density measurements, ultrasonic velocity measurements, x-ray diffraction (XRD) patterns, Fourier transform infrared spectra (FTIR), pH variation of the sample glasses soaked 21 days in the laboratory prepared simulated body fluid (SBF) solution, scanning electron microscope (SEM) images, energy dispersive x-ray spectra (EDS) and x-ray photo electron spectrograph (XPS). Toxic nature of the selected glass sample was assessed using cytotoxicity study in cell culture lines. Bioconversion was also accessed by *in vivo* studies of implantation of the glass into animal bone followed by SEM, EDS and confocal laser scanning micrograph (CLSM) images. The results obtained before, after *in vitro* and *in vivo* studies are discussed in terms of change in structure, stability, mechanical properties, bone-bonding ability and bio conversion of the prepared glass samples.

Detailed Description

ZnO added fluorophosphate glass composition P_2O_5 —CaO— Na_2O — CaF_2 —ZnO, methods of preparation and use thereof are disclosed. The glass can be used for different bio-medical applications such as prosthetic implants, stents, screws, plates, tubes, controlled drug delivery etc. The addition of fluorine and zinc is made at the expense of Na_2O content in the glass composition and keeping the P/Ca ratio as constant. The glasses were prepared in the mentioned compositions include various salts in mol% with the following ranges:

S. No.	Salt	Ratio in mol%
1.	P_2O_5	35-65
2.	CaO	22-36
3.	Na_2O	11-32
4.	CaF_2	0.01-20
5.	ZnO	0.1-15

To access the variation if any, the following chemicals were alternatively used as per its proportionate molecular weight:

S.No.	Required salt	Alternatively used
1.	P_2O_5	Ammonium di hydrogen phosphate, Phosphorous chloride, ammonium phosphate, calcium phosphate, sodium phosphate, silver phosphate etc.
2.	CaO	Calcium sulphate, calcium carbonate, calcium fluoride, calcium fluorophosphate, calcium chloride, calcium caseinate, calcium bicarbonate etc.
3.	Na_2O	Sodium carbonate, sodium citrate etc.
4.	CaF_2	Calcium di fluoride, calcium tri fluoride, calcium fluorophosphate, sodium fluoride etc.
4.	ZnO	zinc, zinc chloride, zinc iodide, zinc fluoride, zinc sulphate etc.

The influence of zinc added fluorophosphate glass system is studied in terms of pH variations during *in vitro* studies, FTIR spectra, XRD etc. The structural role of zinc in packing of glass network is noticed. The hydroxyapatite (HAp) forming ability of prepared glasses was carried out through *in vitro* studies in simulated body fluid (SBF). The scanning electron microscopy (SEM) images before and after *in vitro* studies show the formation of HAp in all glass surfaces, while a higher rate of formation of HAp is evidenced on zinc added fluorophosphate glasses rather than zinc free fluorophosphate glass. FTIR spectra and XRD patterns that are observed support its higher bioactivity. Further, the cell viability test showed no cytotoxicity on MIT assays and hence these glasses are suitable for synthetic bone graft material for human use. The *in vivo* studies were done on rabbit by implanting the optimised sample ZnFP4 into the femoral condyle. SEM and CLSM studies on the un-decalcified sectioning of the implant after 10 weeks confirm the bioconversion of glass into bone.

Materials and method

ZnO added fluorophosphate glasses were prepared by melting the homogeneous mixture of phosphate, calcium, sodium, zinc and fluoride salts followed by sudden quenching of the melt. The derivatives of phosphate, calcium, sodium, zinc and fluoride salts were weighed accurately and ground using mortar and pestle/ planetary ball mill. The mixture was fed into alumina crucible and then preheated at a temperature ranging from 140 °C to 190 °C for 1 h in a closed furnace and cooled to room temperature at a rate of 1 °C per minute. The preheated mixture was ground using mortar and pestle/ planetary ball mill. The mixture was taken in a platinum crucible and melted at the temperature ranging from 1050 °C to 1400 °C for 1 h. The temperature in the furnace was kept constant throughout the process. The melt was poured in a preheated graphite/ steel mould and cooled to room temperature. In this method, the entire synthesis protocol is successfully done without the usage of any toxic chemicals.

The method for preparing zinc added fluorophosphate glasses compare the following steps:

- a) The calculated quantities of the chemicals were weighed accurately using an electronic balance.
- b) The weighed chemicals were ground using mortar and pestle/ planetary ball mill for 1 h to obtain a homogeneous mixture.
- c) The mixture was preheated at a temperature ranging from 140 °C to 190 °C for 1 h in a closed furnace and cooled to room temperature at a rate of 1 °C per minute.
- d) The preheated mixture was ground using mortar and pestle/ planetary ball mill to obtain a homogeneous powder.
- e) The obtained homogeneous powder was taken in a platinum crucible (10% Rhodium doped) and melted at the temperature ranging from 1050 °C to 1400 °C for 1 h in electric furnace.
- f) The melt was suddenly quenched in a preheated steel/ graphite mould of temperature 300 °C – 600 °C and then cooled to room temperature.
- g) The obtained solid glass sample is annealed at 300 °C – 500 °C for 1 h and cooled at the rate of 0.5 °C - 2 °C per minute.
- h) The prepared glass sample was cut into required shape and size using a diamond cutter for different characterisation studies.

Example 1:

21.3221 g of P_2O_5 , 4.7899 g of CaO, 2.7383 g of Na_2O and 1.1497 g of CaF_2 pure chemicals were taken in agate mortar/ planetary ball mill. Ethanol was added with the mixture and ground for 1 h to obtain a homogeneous mixture. The mixture was dried at 100 °C -200 °C for 1 h and ground using agate mortar/ planetary ball mill to obtain a fine powder. The powdered mixture was melted in platinum doped with 10% Rhodium crucible and heated at the temperature of 1050 °C – 1400 °C for 1–4 h and then quenched in a preheated stainless steel/ graphite mould at a temperature of 300 °C – 600 °C and then cooled. The prepared glass sample was annealed at 300 °C– 500 °C for 1 h then cooled at the rate of 0.5 °C – 2 °C per minute to release the stress in the glass sample. The prepared glass sample was cut into required size and shape using diamond cutter. The code for the present sample is called as ZnFP1.

Example 2:

20.4877 g of P_2O_5 , 4.5528 g of CaO, 3.5410 g of Na_2O , 1.1738 g of CaF_2 and 0.2447 g of ZnO pure chemicals were taken in agate mortar/ planetary ball mill. Ethanol was added with the mixture and ground for 1 h to obtain a homogeneous mixture. The mixture was dried at 100 °C -200 °C for 1 h and ground using agate mortar/ planetary ball mill to obtain a fine powder. The powdered mixture was melted in platinum doped with 10% Rhodium crucible and heated at the temperature of 1050 °C – 1400 °C for 1–4 h and then quenched in a preheated stainless steel/ graphite mould at a temperature of 300 °C – 600 °C and then cooled. The prepared glass sample was annealed at 300 °C– 500 °C for 1 h and then cooled at the rate of 0.5 °C – 2 °C per minute to release the stress in the glass sample. The prepared glass sample was cut into required size and shape using diamond cutter. The code for the present sample is called as ZnFP2.

Example 3:

19.5013 g of P_2O_5 , 4.2801 g of CaO, 3.7845 g of Na_2O , 1.1918 g of CaF_2 and 1.2424 g of ZnO pure chemicals were taken in agate mortar/ planetary ball mill. Ethanol was added with the mixture and ground for 1 h to obtain the homogeneous mixture. The mixture was dried at 100 °C – 200 °C for 1 h and ground using agate mortar/ planetary ball mill to obtain a fine powder. The powdered mixture was melted in platinum doped with 10% Rhodium crucible and heated at the temperature of 1050 °C – 1400 °C for 1 – 4 h and then quenched in a preheated stainless steel/ graphite mould at a temperature of 300 °C – 600 °C then cooled. The prepared glass sample was annealed at 300 °C– 500 °C for 1 h and then cooled at the rate of 0.5 °C – 2 °C per minute to release the stress in the glass sample. The prepared glass sample was cut into required size and shape using diamond cutter. The code for the present sample is called as ZnFP3.

Example 4:

20.0138 g of P_2O_5 , 4.3926 g of CaO, 2.3304 g of Na_2O , 1.2231 g of CaF_2 and 2.0401 g of ZnO pure chemicals were taken in agate mortar/ planetary ball mill. Ethanol was added with the mixture and ground for 1 h to obtain the homogeneous mixture. The mixture was dried at $100\text{ }^{\circ}\text{C} - 200\text{ }^{\circ}\text{C}$ for 1 h and ground using agate mortar/ planetary ball mill to obtain the fine powder. The powdered mixture was melted in platinum doped with 10% Rhodium crucible and heated at the temperature $1050\text{ }^{\circ}\text{C} - 1400\text{ }^{\circ}\text{C}$ for 1 – 4 h then quenched in a preheated stainless steel/ graphite mould of temperature $300\text{ }^{\circ}\text{C} - 600\text{ }^{\circ}\text{C}$ then cooled. The prepared glass sample was annealed at $300\text{ }^{\circ}\text{C} - 500\text{ }^{\circ}\text{C}$ for 1 h then cooled at the rate of $0.5\text{ }^{\circ}\text{C} - 2\text{ }^{\circ}\text{C}$ per minute to release the stress in the glass sample. The prepared glass sample was cut into required size and shape using diamond cutter. The code for the present sample is called as ZnFP4.

Example 5:

18.4472 g of P_2O_5 , 3.9909 g of CaO, 3.8356 g of Na_2O , 1.2079 g of CaF_2 and 2.5184 g of ZnO pure chemicals were taken in agate mortar/ planetary ball mill. Ethanol was added with the mixture and ground for 1 h to obtain a homogeneous mixture. The mixture was dried at $100\text{ }^{\circ}\text{C} - 200\text{ }^{\circ}\text{C}$ for 1 h and ground powder using agate mortar/ planetary ball mill to obtain a fine powder. The powdered mixture was melted in platinum doped with 10% Rhodium crucible and heated at the temperature ranging from $1050\text{ }^{\circ}\text{C}$ to $1400\text{ }^{\circ}\text{C}$ for 1– 4 h and then quenched in a preheated stainless steel/ graphite mould at a temperature ranging from $300\text{ }^{\circ}\text{C}$ to $600\text{ }^{\circ}\text{C}$ and then cooled. The prepared glass sample was annealed at $300\text{ }^{\circ}\text{C} - 500\text{ }^{\circ}\text{C}$ for 1 h then cooled at the rate of $0.5\text{ }^{\circ}\text{C} - 2\text{ }^{\circ}\text{C}$ per minute to release the stress in the glass sample. The prepared glass sample was cut into required size and shape using diamond cutter. The code for the present sample is called as ZnFP5.

Physico-chemical and *in vitro* studies

1. Density Measurements

The density of the prepared glass samples was measured using Archimedes' principle with water as a buoyant and the relation, $\rho = \frac{W_a}{W_a - W_b} \times \rho_b$, where W_a is weight in air, W_b is the weight in water, and ρ_b is the density of water. A digital balance (model BSA224S-CW; Sartorius, Goettingen, Germany) with an accuracy of ± 0.0001 g was used for weight measurements. The measurements were repeated five times to find an average and an accurate value. The overall accuracy in density measurement is ± 0.5 kgm⁻³. The percentage error in the measurement of density is ± 0.05 .

2. Ultrasonic Measurements

Ultrasonic velocities (U_L , longitudinal and U_s , shear) and attenuations (U_L , longitudinal and U_s , shear) measurements were carried out using pulse echo method and cross-correlation technique. The measurement system consists of an ultrasonic process control system (model FUII050; Fallon Ultrasonics Inc. Ltd., ON, Canada), a 100-MHz digital storage oscilloscope (model 54600B; Hewlett Packard, Palo Alto, CA), and a computer. The measurements were carried out by generating longitudinal and shear waves using X- and Y- cut transducers operated at a fundamental frequency of 5 MHz.

3. X-ray Photoelectron Spectroscopy

Elemental analysis of the prepared glass sample was done using X-ray photo electron spectroscopy (model AXIS Ultra DLD; Kratos, Kyoto, Japan) with Al $K\alpha$ source operating at 210 W. The glass sample was ground using planetary ball mill (model PM 100; Retsch, Haan, Germany) and the powdered glass sample was used for XPS analysis. A survey spectrum (0–1200 eV) was recorded and high-resolution spectra for C1s and N1s band were obtained. X-ray as the excitation radiation was used for the XPS measurements. The spectra were collected in a fixed retarding ratio mode with bandpass energy of about 10 eV.

4. Fourier Transform Infrared Analysis

Infrared absorption of powdered glass samples after *in vitro* studies were analyzed from FTIR spectra. The FTIR absorption spectra were recorded at room temperature using an FTIR from 4000 to 400 cm⁻¹, (model 8700; Shimadzu, Tokyo, Japan) spectrometer. A 2.0 mg sample was mixed with 200 mg KBr in an agate mortar and then pressed under a pressure of 100 kg/cm⁻¹. It gave a pellet of 13 mm diameter. For each sample, FTIR spectrum was normalized with blank KBr pellet.

5. X-ray Diffraction Analysis

To confirm the amorphous nature of prepared glasses and the presence of HAp layer on the surface of glass samples, XRD studies were carried out on each glass sample. An X-ray diffractometer (model PW 1700; Philips, Eindhoven, The Netherlands) was used with $CuK\alpha$ as a radiation source to obtain the XRD pattern in the range of a scanning angle between 20° and 80° . The glass samples were removed from SBF solution after 21 days *in vitro* studies and then washed gently with double distilled water. The washed glass samples were dried at room temperature. The dried glass was subjected to obtain the XRD pattern as discussed above.

6. pH Measurements

The prepared glass samples were soaked for 21 days in laboratory prepared SBF solution and kept in CO_2 incubator at the temperature of $37^\circ C$ in 6% CO_2 . The variation in pH values of simulated body fluid (SBF) solutions was measured on all the 21 days using a pH meter (model 3-star; Thermo Orion, Beverly, MA) for all glasses under identical conditions. The pH electrode was calibrated using the standard buffer solution with a pH value of 4.01, 7.00, and 10.01 before taking the measurements. The percentage error in the measurement of pH is ± 0.005 .

7. Scanning Electron Microscopy

The surface morphology of the prepared glass sample was analysed using SEM studies. The glass samples were gently washed with double-distilled water and dried at room temperature. A thin layer of a gold film was coated on the surface of glass sample using sputtering technique. The SEM (model Ultra 55; Zeiss, Oberkochen, Germany) was used to obtain a surface image of all glass samples before and after *in vitro* studies to analyse their surface morphology.

8. Energy Dispersive X-ray spectroscopy

Energy dispersive X-ray spectrograph (EDS) was taken for all the prepared glass samples before and after *in vitro* studies using EDS (model X-max 50 mm²; Oxford, Abingdon, England) for obtaining semi quantitative elemental information of the surface of samples. The percentage of error associated with the elemental composition analysis is ± 0.1 .

9. Cell culture and cytotoxicity assay

Nontoxic nature of the selected glass sample was assessed using cytotoxicity study in cell culture lines. Human gastric adenocarcinoma (AGS) cell line (ATCC-1739) was obtained from the National Centre for Cell Science, Pune, India. The cells were grown and maintained in Dulbecco's modified Eagle's medium (DMEM)/nutrient mixture F-12 HAM (1:1) with 2 mM L⁻¹ glutamine supplemented with 10% fetal bovine serum, 45 IU ml⁻¹ penicillin and 45 IU ml⁻¹ streptomycin. Growth ingredients were also added and incubated in a humidified atmosphere at $37^\circ C$ in 5% CO_2 . After a number of passaging, the pure

confluent AGS cell lines were obtained and cells at a density of 10^3 were used to evaluate the cytotoxicity at a concentration of 100 mg ml^{-1} for the selected bioactive glass samples. The morphology of AGS cell lines was observed regularly under binocular inverted microscope. After 48 h of incubation, MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay was performed to evaluate the viability of the bioactive glass treated AGS cells. The percentage of cell viability from triplicates of the bioactive glass treated and non-treated cells was calculated using optical density (OD 590 nm) as follows:

$$\text{Cell viability \%} = \frac{\text{OD of the glass particles treated cells}}{\text{OD of the cells}} \times 100$$

***In vivo* animal studies**

Animal studies were done after getting the approval from animal ethical committee (Approval number IAEC-LDC/8/14/1 dated 14th February 2014). Young rabbits of weight about 1.600 Kg were purchased from King Institute, Chennai as per CPCSEA guidelines. Under ketamine anaesthesia, one centimetre incision was made over the medical epicondyle of femur under image control. Under microscopic magnification, Periosteum was opened and a 2 mm drill hole was made. The selected sample of fluorophosphate glass rod of diameter 2 mm was pegged into the hole. After saline wash, wound was closed using single layer 3-0 ethilon sutures and then 250 mg of ceftriaxone was given intra-muscularly.

Rabbit was allowed to move freely immediately after recovery from anaesthesia. Fluorescent calcein (Sigma Aldrich, Japan) (10 mg kg^{-1}) was administered intramuscularly on the day of surgery and then every week until 2 days before euthanising to label newly formed bone continuously. After 10 weeks, the animals were euthanised using a lethal dose of ketamine and the lower end of femur harvested and preserved in 10% formalin and used for SEM, EDS, CLSM studies. Un-decalcified sectioning was done on the harvested femur perpendicular to the implant rod using microtome (Model SP1600; Leica, Nussloch, Germany) to get slices of 1 mm thickness.

10. Histomorphological studies

The un-decalcified section of the implant was dried at room temperature using desiccator. A thin layer of a gold film was coated on the surface of glass sample using sputtering technique. SEM and EDS studies were done on the dried slice to find the formation of HAP at the glass-bone interface.

11. Histophysiological analysis

Calcein fluorescence was used to examine the newly formed bone using a confocal laser scanning microscope (model: LSM 510 META; Zeiss, Gottingen, Germany). The excitation wavelength was set at 488 nm (Ar laser). Calcein fluorescence was detected through a BP 515 -565 nm bandpass filter.

Details obtained from the Figures:

Fig. 1 illustrates the protocol used for the synthesis of zinc added fluorophosphate glass samples with different contents of zinc.

The density variation of the prepared fluorophosphate glass samples as a function of added ZnO content is shown in **Fig. 2**. A gradual increase in the density of fluorophosphate glasses from 2628.3 kgm^{-3} to 2771.2 kgm^{-3} due to the addition of ZnO is noted. The increase in density value is due to the tight packing of glass network when the addition of ZnO content. The observed behaviour explains the alteration of glass network by zinc atom. **Fig. 3a and 3b** shows the variation of elastic moduli of all the prepared glass samples as a function of ZnO content. Elastic moduli such as shear and young's modulus shows the same trend with the density variations during the addition of ZnO content. But the bulk modulus and longitudinal modulus value is decreased during the initial addition of ZnO content. Further addition of ZnO increase the bulk and longitudinal modulus value up to the addition of 6 mol% of ZnO content.

Fig. 4 shows the elemental composition of the glass sample ZnFP4 using XPS. The observed intensity at the binding energies at 31.8 eV, 89.2, 135.5 eV, 193.1 eV, 349.8 eV, 437.6 eV, 533.1 eV, 1021.9 eV, 1072.5 eV, shows respectively the presence of CaF_2 , ZnO, P_2O_5 , P_4O_{10} , CaO, Ca, O, ZnO, NaPO_3 the glass sample ZnFP4. SEM image of the prepared glass sample ZnFP4 is showed in **Fig. 5**. The smooth surface of the glass sample ZnFP4 obtained from SEM image exhibits the amorphous nature of the sample. **Fig. 6** shows EDS spectrograph of the glass sample ZnFP4. A close agreement is noted between experimental and nominal composition of the glass sample ZnFP4. Fig. 5 and Fig. 6 confirm the presence of zinc oxide and fluorine atoms in the glass network.

21 days of *in vitro* studies were made on all the prepared glass samples. The observed pH variations during 21 days *in vitro* studies help to assess the bioactivity of the prepared glasses. The pH variations of all the prepared glasses during *in vitro* studies are given in **Fig. 7**. The initial release of phosphate ions lead to form phosphoric acid resulting in sudden decrease in pH value of all the glass samples. At the end of second day of immersion, sodium ions are released and hence the increase in pH value. At the end of 21 days *in vitro* studies, the sample ZnFP4 shows higher pH value than all the other samples.

FTIR spectrograph of all the prepared glass samples after *in vitro* studies is shown in the **Fig. 8**. The FTIR absorption assignment band at 530 cm^{-1} , 720 cm^{-1} , 887 cm^{-1} , 1100 cm^{-1} , 1275 cm^{-1} , 1630 cm^{-1} , 3430 cm^{-1} are respectively of vibration bands of HAp, P—O—P (symmetric mode), C—O, $\text{Ca}_2\text{P}_2\text{O}_7$, PO_2 (asymmetric mode), bending mode of OH^- , and water associated in HAp. The presence of HAp confirms the bone bonding ability of all the glass samples.

XRD patterns of all the glass after *in vitro* studies are shown in **Fig. 9**. In the obtained XRD shoulder peak at 31.92° and a weak one at 45.39° in the sample ZnFP4 shows respectively the rich concentration of HAp and Weak concentration of FAp.

Fig. 10 shows the SEM image of the glass sample ZnFP4 after *in vitro* studies. The SEM image confirms the rich deposit of Ca-P layer on ZnFP4 glass surface. The size of the deposited particles is in the order of 20-100 nm. **Fig. 11** shows the elemental composition of the deposited precipitate using EDS. The Ca/P ratio of the deposit is about 1.6 indicating the deposit is HAp. But the presence of fluorine molecules in the surface precipitate confirms the existence of fluoroapatite. The EDS spectrograph clearly indicates the presence of hydroxyapatite and fluoroapatite on the surface of the glass sample ZnFP4.

Fig. 12 shows the optical microscope image of cell viability test for the sample ZnFP4. The image clearly shows there is no cytotoxicity observed in sample the glass ZnFP4.

Fig. 13a shows the SEM image at 100X magnification of un decalcified section of the femoral condyle of the rabbit after 10 weeks of implanting. Extreme fragmentation of the glass is noted and bioactivity is seen not only around the periphery of the glass but also in the cleaved segments. **Fig. 13b** shows SEM image at 1000X magnification and it exhibits well the binding of glass to the bone by interface which is wavy in nature. **Fig. 14** shows the EDS spectrograph of the interface which shows it is transforming HAp and this proves the glass has made a bond with the bone.

Fig. 15 shows the CLSM of the glass implanted into the rabbit femoral condyle at the end of 10 weeks. The morphological image shows a wide inter zone developed in the glass close to the bone. On Ar laser scanning there is a layer of dense intense fluorescence which becomes wavy as it proceeds in to the deeper layers of glass. Together there is a total depth of 485 μm of brilliant fluorescence indicating very high degree of bioconversion.

We claim:

1. A fluorophosphate glass having the composition of (35-65)phosphorus pentoxide — (22-36)calcium oxide — (11-32)sodium oxide — (0.01–20)calcium fluoride — (0.01-15)zinc oxide, said percentages being molar percentages.
2. The composition for prosthetic device or fluorophosphate glass or coating as claimed in claim 1, wherein phosphorus pentoxide is substituted by any of the following:
Ammonium di hydrogen phosphate, Phosphorus chloride, ammonium phosphate, calcium phosphate, sodium phosphate, and silver phosphate.
3. The composition for prosthetic device or fluorophosphate glass or coating as claimed in claim 1, wherein calcium oxide is substituted by any of the following:
Calcium sulphate, calcium carbonate, calcium fluoride, calcium fluorophosphate, calcium chloride, calcium caseinate, and calcium bicarbonate.
4. The composition for prosthetic device or fluorophosphate glass or coating as claimed in claim 1, wherein sodium oxide is substituted by any of the following:
Sodium carbonate, and sodium citrate.
5. The composition for prosthetic device or fluorophosphate glass or coating as claimed in claim 1, wherein calcium fluoride is substituted by any of the following:
Calcium di fluoride, calcium tri fluoride, calcium fluorophosphate, and sodium fluoride.
6. The composition for prosthetic device or fluorophosphate glass or coating as claimed in claim 1, wherein zinc oxide is substituted by any of the following:
Zinc, zinc chloride, zinc iodide, zinc fluoride, zinc sulphate.
7. The composition of claim 1, wherein the bioactive glass has a composition by either molar percentage or weight percentage:

Compound	Mol%	Wt.%
P ₂ O ₅	35–65	60–70
CaO	22–36	12–20
Na ₂ O	11–32	6–20
CaF ₂	0.01–20	0.3–20
ZnO	0.01–15	0.01–15

8. Any prosthetic device or implant or bone substitute containing the composition of claim 1 wherein said device is made essentially of said fluorophosphate glass or coated with said fluorophosphate glass.

Signed on dated the 9th December 2014