INTRODUCTION

Metals, ceramics, natural or synthetic polymers, and their composites are used as biomaterials aiding the clinician in the repair. Among these, biomaterials in orthopedics have very many demanding characteristics like tunable density, elasticity, and strength near to that of bone, bioreabsorbability and the ability to bond to live bone and not being toxic to the cells. Hench and Clark paved the way for preparing bioglasses and it is named as 45S5 bioglass. The 45S5 bioglass promotes osteogenesis by

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ORIGINAL ARTICLE

Preparation and physical-biological characterization on titanium doped fluorophosphate nanobioglass: Bone implants

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Abstract
In bone tissue repair, metallic implants like screws, pins, plates, stents are considered as boon for patients but it has the disadvantage of being a foreign body and can never get absorbed or get converted to bone. These metallic implants serve the purpose often temporarily, but it fails to attain the mechanical, elastic properties near to that of the normal human bones. To circumvent these drawbacks, the preparation of nanobioglasses having titanium and different proportions of fluoride using melt quenching method is reported. The physical, thermal, and elastic properties of the materials were characterized by their densities, thermal transitions, and elastic moduli by ultrasonic study. The in vitro degradation and bioactivity of these nanobioglasses were assessed by immersion in simulated body fluid (SBF). The hydroxyapatite(HA) formation was assessed by the variation in pH over 21 days and its presence was confirmed by FTIR, XRD, SEM, and EDS. Human osteo sarcoma MG-63(ATCC-1427), SAOS-2(ATCC-85), and gastric adenocarcinoma (AGS)(ATCC-1739) cell lines were used to evaluate cytotoxicity of the bioglasses by MTT assay. The biological efficiency of titanium doped fluorophosphate enhancing bone formation was assessed by the capacity of the ionic dissolution products of the bioglasses to enhance the osteocalcin and ALP activity. The phosphate-based titanium nanobioglasses doped with fluoride possesses elastic strength nearer to the normal human bone, higher bone bonding ability and the rate of biodegradation equaling the rate of bone formation than the commercially available bioglass.

KEYWORDS
nanobioglasses, titanium dioxide, fluorophosphates, hydroxyapatite

1 | INTRODUCTION

Metals, ceramics, natural or synthetic polymers, and their composites are used as biomaterials aiding the clinician in the repair. Among these, biomaterials in orthopedics have very many demanding characteristics like tunable density, elasticity, and strength near to that of bone, bioreabsorbability and the ability to bond to live bone and not being toxic to the cells. Hench and Clark paved the way for preparing bioglasses and it is named as 45S5 bioglass. The 45S5 bioglass promotes osteogenesis by
activating osteogenic gene expression and has the potential to bridge the inorganic minerals (HA) with live bone.\textsuperscript{1–3} The drawback of this bioglass implants are low mechanical properties, slow dissolution rate, and gross limitation of load bearing capacity. To meet the orthopedic implant guidelines, metal oxides were coupled with the bioglasses which are particularly appreciated with their high dissolution rates, bioactivity and apatite forming ability.\textsuperscript{4,5} The oxides are classified into three different groups based on their network (ie,) (i) Network forming oxides (eg SiO\textsubscript{2}, P\textsubscript{2}O\textsubscript{5}, B\textsubscript{2}O\textsubscript{3}), (ii) Network modifying oxides (eg PbO, BaO, SrO, ZnO, CaO, K\textsubscript{2}O, MgO, Na\textsubscript{2}O, Li\textsubscript{2}O) and (iii) Network formers and modifiers simply denoted as intermediate oxides (eg oxides of: C, S, Ti, V, Ga, Se, Mo, Te, W, Bi).\textsuperscript{6} From these groups, titanium and titanium based alloys are universally accepted biomaterials because of their bioinert, high strength, fatigue resistance, and simplicity of fabrication. The intermediate oxide TiO\textsubscript{2} is having higher bonding energy for the Ti–O bond when compared to the bonds present in the modifiers (alkali/alkaline earth metal oxides).\textsuperscript{7} The literature survey reported the potential use of adding TiO\textsubscript{2} presence in the bioglasses leads to better network connectivity, glass forming ability and wettability which enhances better bioactivity of the glasses.\textsuperscript{8}

TiO\textsubscript{2} is a bioinert metal and it does not develop any allergic/inflammatory reaction on contact with body tissues. But, TiO\textsubscript{2} is used to induce calcium phosphate surface nucleation in calcium phosphate glass systems. The addition of TiO\textsubscript{2} enhances chemical stability due to the presence of Ti–O–P bonds rather than P–O–P bonds.\textsuperscript{9}

Excessive fluoride on prolonged use is known to produce fluorosis, a soft tissue ossification disease. But an optimized dose helps in new bone formation. To assess the optimal dose of fluoride ion in improving bioconversion, it was added in incremental doses (0-10 mol %) and analyzed. The composition of glass plays a vital role in the bone bonding ability. The present investigation discloses the enhanced longitudinal moduli, bulk moduli, and elastic moduli, etc. by adding TiO\textsubscript{2} and the improved bioactivity by adding fluoride ions. Prior studies have reported the young’s modulus of titanium doped bioglass as around 100 GPa, while that of human cortical bone is 35 GPa.\textsuperscript{10,11} By adding fluorides to the titanium doped bioglass the modulus shifts closer to that of the human cortical bone. The prepared titanium doped fluorophosphate nanobioglasses (TiO\textsubscript{2}P\textsubscript{2}O\textsubscript{5}CaO–CaF\textsubscript{2}Na\textsubscript{2}O) were characterized for their structural, physico-chemical, thermal, morphological, and biological properties. The obtained results are presented and discussed.

2 \hspace{1em} MATERIALS AND METHODS

2.1 \hspace{1em} Bioglasses preparation

The needed inorganic chemicals (Table 1) were purchased from Sigma-Aldrich, India. The sample code (TiFp1, TiFp2, TiFp3, TiFp4, and TiFp5) and the compounds for the preparation of the nanobioglasses were precisely weighed as shown in Table 1. Titanium was taken in the form of titanium oxide at 1 mol % as approved by FDA.\textsuperscript{12} The mixture was ground well and preheated (100-120°C) in an aluminum crucible for a period of 1 hr in a heating furnace. The preheated materials were cooled to room temperature and again grounded in a ball mill into a homogenous compound. The materials were then transferred to a platinum crucible (10% rhodium doped). The crucible was instantly placed in a furnace preheated to 1200°C and the material was kept at this temperature for 1 hour. The molten material was poured into a preheated graphite steel mold and it was allowed cool to room temperature. The product thus obtained was annealed at 573°C for 1 hour and then cooled slowly (1°C minute\textsuperscript{-1}). The bioglass samples were cut into desired size using a diamond cutter.\textsuperscript{13}

2.2 \hspace{1em} Characterization

2.2.1 \hspace{1em} Physical and thermal characterization

The physical, elastic, thermal characters of the nanobioglasses were done in the following manner. The density of the fluoride doped titanium based bioglass pellets were measured by traditional Archimedes’ principle with an accuracy of ±0.5 kg/m\textsuperscript{3}. The young’s, longitudinal, shear and bulk moduli of all the prepared bioglass pellets were evaluated by measuring ultrasonic velocities in an ultrasonic process control system (Model FULL050; Fallon Ultrasonics Inc., Ltd., Ontario, Canada), a 100-MHz digital storage oscilloscope (Model 54600B; Hewlett Packard, Palo Alto, California, USA) using pulse echo method and cross-correlation technique. The Simultaneous Thermal Analysis (STA 449 F3Nevio) was used to obtain the thermal stability of the bioglasses. The 3.5 mg of the substance was heated till 1000°C at 50 K min\textsuperscript{-1} in nitrogen atmosphere.

| Sample Code | P\textsubscript{2}O\textsubscript{5} | CaO | TiO\textsubscript{2} | Na\textsubscript{2}O | CaF\textsubscript{2} |
|-------------|-----------------|-----|------------------|---------------|------------------|
| TiFp1       | 45.00           | 29.00 | 1                | 25.00         | 0.00             |
| TiFp2       | 45.00           | 29.00 | 1                | 23.75         | 1.25             |
| TiFp3       | 45.00           | 29.00 | 1                | 22.50         | 2.50             |
| TiFp4       | 45.00           | 29.00 | 1                | 21.25         | 3.75             |
| TiFp5       | 45.00           | 29.00 | 1                | 20.00         | 5.00             |
2.3 Biological activity

2.3.1 In vitro characterization

The simulated body fluid having ion concentration similar to human blood plasma was prepared as described by Kokubo. The prepared bioglasses (1 g each) were immersed in 100 mL SBF solution for an incubation period of 21 days at 37°C in an incubator with a 5% concentration of CO₂. The pH variation of the SBF solution was monitored throughout the 21 days using Thermo Scientific™ Orion™ 3-Star Benchtop pH meter.

After SBF study, the samples were carefully removed and washed with double distilled water; and after complete drying, it was subjected to further evaluation. The functional group variation of the pre- and post-SBF treated bioglasses were assessed using a Fourier transform infrared spectrophotometer (Model 8700; Shimadzu, Tokyo, Japan) within the spectral range of 4000-400 cm⁻¹. The presence of HAp layer and the crystals on the surface of the bioglasses were obtained from X-ray diffractometer—XRD (Model PW 1700; Philips, Eindhoven, The Netherlands) and the respective diffraction pattern was recorded in the range of 20-80°. The morphological analysis and the semi-quantitative elemental concentration of the pre- and postimmersion samples were examined using scanning electron microscope—SEM (Model Ultra 55; Zeiss, Oberkochen, Germany) coupled with energy dispersive X-ray spectrograph (Model Oxford Xmax50 EDS; Oxford Instrument, England) after gold sputtering.

2.3.2 Cytotoxicity assay

The nontoxic nature of the selected bioglass sample TiFp5 was assessed using cytotoxicity study in cell culture lines. Human gastric adenocarcinoma (AGS) cell line (ATCC-1739) was received from the National Centre for Cell Science, Pune, India. The confluent AGS cell lines were seeded at a density of 10³ cells per mL to evaluate the cytotoxicity at increasing concentration up to 100 µg mL⁻¹. Morphology of the AGS cell lines was observed regularly under a binocular inverted microscope. After 48 hours of incubation, MTT assay was performed to evaluate the viability of AGS cell lines. The percentage of cell viability from triplicates of the bioglasses treated and nontreated cells were calculated by optical density at 590 nm as follows:

\[
\text{% cell viability} = \frac{\text{OD of the bioactive ceramic treated cells}}{\text{OD of the untreated cells}} \times 100
\]

The toxicity was also assessed in osteoblastic cell lines MG-63 cell line (ATCC-1427) and Saos-2 cell line (ATCC-85) as a confirmation, as the target of research was to assess the utility of TiFp in bone tissue engineering.

2.3.3 Alkaline phosphatase activity

A 1* 10⁶ MG63 cells were plated in culture plates and incubated for 48 hours at 37°C in 5% CO₂ incubator. Once the cells were confluent, it was treated with different concentration of TiFp's (from one microgram to hundred micrograms per mL) and incubated. After incubating for 48 hours, cells were washed twice with ice cold PBS and homogenized in 50 µL assay buffer. The insoluble materials were centrifuged at 20 784 g for 3 minutes. The test samples with different concentrations of the ionic dissolution products were added into 96-well plate and then 10 µL of ALP was added to each well. Then, 50 µL of the 5 mmol/L pNPP solution was added to each well containing the test samples. The reaction mixture was incubated for 60 minutes at 25°C in dark condition. A 20 µL stop solution was added to terminate the ALP activity in the sample. The OD values are measured at 405 nm in a micro plate reader.

2.3.4 Osteocalcin secretion

MG-63 osteoblast cells were cultured and seeded on 24 well plate (2*10⁵ cells per well). After attachment of cells to the plates (~18-24 hours), spent media was removed and the cells were washed with Dulbecco’s phosphate buffer saline (DPBS). Then, serum starvation was performed (~18-24 hours) by adding serum-free, antibiotic-free, and phenol red-free media to bring all the cells to the same phase so as to stop further proliferation and enable the cells to respond properly to the sample. The stocks sample was stirred to achieve homogenization prior to the preparation of experimental concentrations (100, 10, and 1 µg) in cell culture media. The prepared concentrations were added to the respective wells in duplicates and incubated for 48 hours.

The extracellular media of all samples with varying concentrations in duplicates were collected in respective Eppendorf tubes and chilled immediately to protect against degradation of osteocalcin by proteases. The same cells were washed with DPBS, and treated with 500 µL of cell lytic solution (specific volume for the density of 10⁶-10⁷ cells). Then, the plate was gently agitated for 15 minutes and observed under microscope to ensure complete lysis of cells. Lysed components of varying concentrations in duplicates were collected and placed in cold condition immediately to protect osteocalcin degradation by proteases. Then, all the samples were centrifuged in 4°C at 12 298 g for 20 minutes. The 25 µL of calibrator, control, and test samples were added into the appropriate wells and 100 µL of working anti-OST-HRP
conjugate was added into all the wells. The plate was incubated for 2 hours at room temperature. The liquids were aspirated from each well. The plate was washed three times by dispensing 400 µL of working wash solution into each well and the contents were aspirated from each well. The 100 µL of chromogenic solution was added into each well and the washing step was followed within 15 minutes. The microtiter plate was incubated for 30 minutes at room temperature under dark condition. The stop solution (100 µL) was added to each well. The absorbance was read at 450 nm (reference filter 630 nm or 650 nm) within 1 hour using Readwell Touch Robonic ELISA Plate Reader.

2.4 | In vivo characterization

2.4.1 | Laboratory animal study

After getting approval from the animal ethical committee, Approval number IAEC-LDC/7/13/1, dated July 4 2013. The animal study was performed as per Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines, young rabbits weighing 1.6 kg were purchased from King Institute, Chennai. Under ketamine anesthesia supplemented with local anesthetic and halothane vaporizer, 1 cm incision was made over the medial epicondyle of femur under image control. The Periosteum was opened and a 2 mm hole was drilled using microscopic magnification. The nonporous titanium fluorophosphate bioceramic rod of diameter 2 mm was pegged into the hole. After saline wash, the wound was closed using single layer 3-0 Ethilon sutures and then 250 mg of ceftriaxone was given intramuscularly.

The rabbit was allowed to move freely immediately after recovery from anesthesia. Fluorescent calcein (Sigma-Aldrich, Japan) (10 mg kg⁻¹) was administrated intramuscularly on the day of surgery and then every week until 2 days before euthanizing to label newly formed bone continuously. After 10 weeks, the animals were euthanized using a lethal dose of ketamine and the lower end of femur harvested, preserved in 10% formalin and used for SEM, EDS, Confocal Laser Scanning Microscope (CLSM) studies. Un-decalcified sectioning was done on the harvested femur perpendicular to the implant rod using a microtome (Model SP1600; Leica, Nussloch, Germany) and getting slices of 1 mm thickness. CLSM (Model Fluoview–IX70; Olympus, Tokyo, Japan) was used and the excitation wavelength was set at 488 nm (Ar laser). Calcein fluorescence was detected through a BP 515–565 nm bandpass filter.

The un-decalcified section of the specimen was dried at room temperature using a desiccator. A thin layer of a gold film was coated on the surface of the bone-bioglass sample using the sputtering technique. SEM (Model Ultra 55; Zeiss, Oberkochen, Germany) coupled with EDS (Model Oxford Xmax50 EDS; Oxford Instrument, England) studies were done on the dried specimen to assess the formation of HAp at the bioceramic-bone interface.

3 | RESULTS AND DISCUSSIONS

3.1 | Physical and thermal characterization

The difference in densities of the various TiFp’s having different proportions of calcium fluoride is shown in Figure 1. The density explains the effect of the chemical composition on the structural reorganization of the atoms and groups present in the bioglass materials. From Figure 1, it is obvious that there is a slight decrease in density (2634-2632 kg m⁻³) when the initial concentration of calcium fluoride is 1.25 mol % and the density increased when the CaF₂ concentration is 2.5 mol %. Until 5 mol % of CaF₂ content, the differences noted in the density values may be attributed to the alteration of the ceramic network. This may be due to the breakage of the P–O–P bonds in the phosphate network resulting in terminal oxygen formation. At high mole percentage of calcium fluoride and one mole percentage of titanium oxide content in the bioglasses it leads to ionic crosslink bridges between the nonbridging oxygen of phosphate network.15-17

Followed by density study, pulse echo method was used to measure various elastic moduli (Young’s, Shear, Bulk, and Longitudinal), which provides an idea regarding the strength and interatomic potential of the biomaterial. The load bearing capacity of the prepared materials used for bone implants is assessed by their elastic properties.18 The variation profile of Young’s and shear moduli (48.02-50.67 Gpa and 18.29-18.82 Gpa) of the materials reflected the variation in the density profile. In the case of longitudinal and bulk moduli, a
slight variation was noted at 3.75 mol% of CaF₂ indicating the weakening of fluoride network (Figure 2 and Table 2). From these studies, TiFp5 showed to maintain the structural rigidity and was taken for further analysis.

The differential scanning calorimetric curve showed thermal transitions like crystallization and melting (Figure 3). The exothermic peak indicates the crystallization process (Tₐ) whereas the endothermic peak denotes the melting phenomenon (Tᵅ) of the glass matrix. The crystallization exotherm was noted at 550°C and the associated enthalpy of crystallization (ΔHₐ) for the sample was found to be 36 Jg⁻¹. From the DSC curve, the melting endotherm was observed at 742°C and the sharpness of the peak reveals the material purity. The enthalpy of fusion (ΔHᵅ) for the material was 171 Jg⁻¹. The recorded TG curve confirmed the thermal stability of the material up to 1000°C, as the material showed no noticeable mass loss from room temperature to 1000°C. A temperature window of around 170°C existing between the exotherm and endotherm noted in the DSC curve for TiFp will be of great use in the stage of scaffolding the material by sintering technique if done.

3.2 In vitro characterization

During the first day of immersion of the nanobioglasses samples, a sudden decrease in pH was observed as a result of phosphoric acid formation in all samples. On the second day, the release of alkaline earth metal (Na⁺ and/or Ca²⁺) increases the pH. The variation of the pH is not linear throughout the 21 days of observation, which signifies that ion leaching that alters the network upon degradation (Figure 4). The dissolution of the ions thus replaces H⁺ ions by cations (Na⁺ and/or Ca²⁺) leading to an increase in hydroxyl ion concentration. After 18 days, the alkalinity of the medium was maintained which is one of the essential criteria for the healing mechanism. For all the bioglass samples, the pH level does not cross a critical level due to the homogenous nucleation of apatite formation. The formation of the new layer and the compounds associated with this new layer were further confirmed by FTIR and X-ray diffraction analysis.

The probable steps involved in the formation of apatite layer in simulated body fluid are: By breaking the backbone of the phosphate layer with either H⁺ ions or F⁻ ions leads to phosphoric acid or the phosphoro fluoridate. Cationic interaction of Na⁺ and/or Ca²⁺ with water results in the formation of hydroxide groups. Calcium hydroxide and the orthophosphoric acid acts as the precursors for hydroxy carbonated apatite.

After 21 days of in vitro degradation, the XRD pattern for the titanium containing fluorophosphate nanobioglasses having different amounts of calcium fluoride are presented in Figure 5. The bioglasses shows high crystalline behavior due to fluorapatite formation. Among the five different bioglass samples investigated, TiFp5 indicates the high concentration of fluorapatite (30.54°) Figure 5.

| Table 2 | Density (ρ), longitudinal modulus (L), bulk modulus (K), Young’s modulus (Y), shear modulus (G), and Poisson’s ratio (σ) for Ti doped nanobioglasses having different amounts of fluoride ion doping using CaF₂ |
|---------|-----------------|-----------------|-----------------|-----------------|-----------------|
| CaF₂ (mol %) | ρ (kgm⁻³) | L (GPa) | K (GPa) | Y (GPa) | G (GPa) |
| 0 | 2634.49 | 67.19 | 42.75 | 48.02 | 18.29 |
| 1.25 | 2632.73 | 67.60 | 43.23 | 47.00 | 18.25 |
| 2.5 | 2646.35 | 68.67 | 43.88 | 48.75 | 18.53 |
| 3.75 | 2643.02 | 67.66 | 43.02 | 48.46 | 18.46 |
| 5 | 2659.81 | 80.01 | 54.96 | 50.67 | 18.82 |

**Figure 2** Longitudinal, Bulk, Young’s and Shear moduli variation in titanium containing fluorophosphate nanobioglasses having different amounts of calcium fluoride
The morphological analysis and the elemental composition of TiFp5 were examined. The preimmersed sample showed a smooth surface and the material is amorphous. Figure 6. The elemental composition profile from EDS indicates the presence of P, Ca, Na, F, and O and their respective concentrations were expressed in atomic and weight %. The postimmersed bioglasses sample SEM results showed rough surface and the EDAX revealed the formation of hydroxyapatite and fluorapatite crystals. When the Ca/P ratio is less than 1.3, the material is found to be highly soluble whereas when this ratio is greater than 1.3, solubility decreases. Before in vitro test, the calcium to phosphate ratio is 0.33 whereas after immersion the ratio reached a value of 1.67 which is an indication of the difference in solubility of the material.

The morphological analysis and the elemental composition of TiFp5 were examined. The preimmersed sample showed a smooth surface and the material is amorphous Figure 6. The elemental composition profile from EDS indicates the presence of P, Ca, Na, F, and O and their respective concentrations were expressed in atomic and weight %. The postimmersed bioglasses sample SEM results showed rough surface and the EDAX revealed the formation of hydroxyapatite and fluorapatite crystals. When the Ca/P ratio is less than 1.3, the material is found to be highly soluble whereas when this ratio is greater than 1.3, solubility decreases. Before in vitro test, the calcium to phosphate ratio is 0.33 whereas after immersion the ratio reached a value of 1.67 which is an indication of the difference in solubility of the material.

The SEM results of the postimmersed sample (B) shown high irregularity of the surface in total contrast to the homogenous amorphous nature of the preimmersion sample (A). The deposited irregular materials are confirmed as HAp and FAp by the EDAX evaluation of the surface (Figure 6 B1). The structural characterization of the prepared bioglasses by FTIR is depicted in Figure 7 and the details observed are presented in Table 3. The broad absorption band observed at
the frequency range 3550-3200 cm\(^{-1}\) is attributed to the adsorbed and/or absorbed water molecules. This band is found to be strong in the postimmersed samples indicating the presence of carbonated hydroxyapatite.\(^{30}\) The titanium phosphate linkage was reflected as P–O–Ti bonds and the frequency shift (2935-2908 cm\(^{-1}\)) in the pre- and postimmersed samples indicates the presence of more hydroxyl group.\(^{31}\) An asymmetric stretching mode of metaphosphate chain (PO\(_2\))
and the phosphate ions in P-O of the samples were present at 1350-1200 cm⁻¹ and 1220-1100 cm⁻¹, respectively. The orthophosphate groups were represented as (PO₄)³⁻ (1060-1000 cm⁻¹). The asymmetric stretching of the phosphate-based glassy network exhibits in 850-1050 cm⁻¹ region. The absorption frequency at 835-720 cm⁻¹ responsible for phosphorofluoridate which is denoted as P-F stretch. Before and after immersion, frequency shift (729-739 cm⁻¹) and the strong absorption explains the sustainability of the phosphorofluoridate compounds. The band at 540-650 cm⁻¹ attributed due to asymmetric stretching of P–O bonds and titanium oxide presence. The titanium doped fluorophosphate nanobioglasses enhances the apatite nucleation in the form of hydroxyapatite and fluorapatite and it is confirmed by the obtained results. The phosphorofluoridate presence in postimmersion strengthens the bioglass in the bone mineralization process.

The bio-compatibility of the bioglasses is evaluated using human adenogastric carcinoma (AGS) cell line. The MTT results show that increasing concentration of bioglass leads to decreased cell viability. The toxic effect is seen at high concentration. This may be due to the agglomeration in the cascade of cellular events such as cell proliferation, attachment, and growth. Overall, the viability is more than 80% even in higher concentration of the dissolved products of TiF₅'s after 48 hours and hence the compound is considered as nontoxic (Figure 8). Since AGS is an epidermal cell line, for bone tissue engineering the cell toxicity of TiF₅'s was also assessed using MG63 cell line and Saos2 cells line which showed no gross toxicity. The morphology of the results was depicted in Figure 9.

ALP enzyme initiates the osteoid mineralization process and act as an important osteoblast biomarker at different stages in the formation of new bone matrix and minerals. In this study, the ALP activity showed higher concentration than in control of MG63 cells in the presence of ionic dissolution

| Wavenumber (cm⁻¹) | Preimmersion | Postimmersion | Assignments       |
|-------------------|--------------|---------------|-------------------|
| 3440              | 3444         | OH in water   |
| 2935              | 2908         | P–O–Ti bond  |
| 1281              | 1276         | P=O Stretch  |
| 1116              | 1116         | P–O Stretch  |
| 1022              | 1002         | PO₃ Stretch  |
| 893               | 888          | P–O–P Stretch|
| 729               | 739          | P–F Stretch  |
| 518               | 518          | PO₃ Bend     |

**TABLE 3** FTIR studies of Ti doped fluorophosphate-based nanobioglasses having different amounts of fluoride ion doping using CaF₂

**FIGURE 8** Cell viability of AGS, MG-63 and Saos2 cells after being treated with different concentration of TiF₅

**FIGURE 9** Morphology of MG 63 cells after being treated with different concentration of TiF₅ at 24 h and 48 h
products of TiFp. This feature confirms the potential utility of fluoride doped titanium phosphate nanobioglasses in osseoinduction (Figure 10).8

Of all the ground substances (more than 20 types found in normal bone tissue), many of them are having varying concentrations throughout the body while some are high in the bone. Osteocalcin is one substance which is secreted only by osteoblasts.36 Hence, its expression was enhanced by dissolution products of TiFp in both intracellular and extracellular level (Figure 11) proves the ability of fluoride doped titanium phosphate nanobioglasses in bone tissue formation.

3.3 | In vivo characterization

The SEM image of the un-decalcified section of femoral condyle of the rabbit at 100× magnification explains the morphological characteristics of the implanted nonporous bioglass size and structure as well as the bone conversion. In Figure 12, the impregnated nonporous rod size got shrunk slowly because of the dissolution of the bioglass material and the apatite formation creates the interface in 10 weeks’ time and is binding the glass to the bone. After ten weeks of in vivo study, a 2.000 mm nonporous rod has eroded to 1.630 mm due to ion dissolution, resorption, and formation of new bone at the interzone. There is a definite distinct layer of transformation all around the bioglass where it was in contact with the bone. In the SEM image shown in Figure 12B (1000× magnification), the distinct interface between the bone and the bioglass is clearly seen. The EDS spectra of the bone-bioglass interface and its mineral composition confirms the new bone formation. The P, Ca, Na, and O spectra of the atoms of mineralization in new bone is present in the interface. The presence of fluoride ions increases the transformation rate considerably.37,38

As a result of osteogenesis, the histological section of the femoral condyle implant with calcein fluorescence explains the new bone formation in dark fluorescent green. The non-fluorescent calcein AM dye is hydrolyzed by cellular esterases to give calcein, which is fluorescent and is retained in the cytoplasm. The intensity of calcein dye measured on a fluorimeter is directly proportional to the activity of cellular esterases, which in turn is proportional to metabolically active cells. The fluorescent green highlighted the transition zone of partially mineralized bone. The presence of fluoride ions in the bioglasses enhances the rate of osteogenic differentiation and proliferation.39 Since the depth of fluorescence

FIGURE 10  Alkaline phosphatase activity of TiFp5

FIGURE 11  Intracellular and extracellular osteocalcin of TiFp5

FIGURE 12  SEM images: In vivo studies of TiFp5, (A) 100× magnification (B) 1000× magnification
was not uniform, sections were taken at various levels. The area of bioconversion was calculated by using the following equation.

\[
\text{Area of bioconversion} = \frac{3.143 (R \cdot R) - 3.143 (r \cdot r)}{3.143 (R \cdot R)} \times 100
\]

where \( R \) = radius of implant; \( X \) = radius of conversion; \( r = R - X \) = radius of residual

The animal studies conducted by various authors are focused toward different effects of the bioglass used. Altering the surface electroconductivity leads to better osteogenesis by Hap.\(^{40}\) In our study, nonporous rod getting bioconversion by penetration of live metabolically active cells to
the extent of 62% in 10 weeks have been proved by CLSM (Figure 9).\(^4,4^2\)

Calculations showed the bone conversion in the case of TiFp5 is around 62% (in a short span of 10 weeks) (Figure 13).

4 | CONCLUSIONS

The present investigation discloses the preparation of titanium containing fluoride ion doped phosphate nanobioglasses (TiO\(_2\)-P\(_2\)O\(_5\)-CaO-CaF\(_2\)-Na\(_2\)O) by melt quenching method. The properties of the prepared materials are altered by network modifier (Ca\(^{2+}\)), network former (P\(^{5+}\)), and intermediate oxide (TiO\(_2\)). The increase in density noted in these bioglasses is attributed to mole percentage of the fluoride from 0 to 10. The measured longitudinal, shear, Young’s, and bulk moduli for bioglasses follow the same trend in the variation in density of the material. The 10 mol % of fluoride shows a similar trend in both the density and the moduli. The correlation of the elastic property with density explains the structural integrity of the bioglasses. The material containing 10 mol % of fluoride shows the density, longitudinal, shear, Young’s, and bulk moduli as 2659 kg m\(^{-3}\), 80.01, 18.82, and 54.96 GPa, respectively. The chemical stability of the biomaterial is expressed by the biomimetic formation of apatite over the surface in SBF. The conversion of amorphous to crystalline phase during in vitro studies were characterized by XRD (Fluorapatite 30.54\(^\circ\)). The titanium oxide added fluorophosphate bioglasses enhances the osteoinduction expressed by increased ALP and osteocalcin levels in cell culture. The inorganic mineral precipitation takes place at the implant bone interface which has the EDAX of HAp and FAp. The femoral condyle un-decalcified section of the implant TiFp5 shows 62% of bone formation after 10 weeks. The in vitro AGS, SAOS-2, and MG63 cell line results reveal 10 \(\mu\)g concentration of bioglasses to be nontoxic. TiFp has shown promising results with the elastic moduli better than the existing metal doped bioglasses and also a better bioactivity by having a bio conversion to metabolically active tissue to the tune of 62% in a short period of 10 weeks.

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