Research Article

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Novel Lead-free biocompatible piezoelectric Hydroxyapatite (HA) – BCZT ($\text{Ba}_{0.85}\text{Ca}_{0.15}\text{Zr}_{0.1}\text{Ti}_{0.9}\text{O}_3$) nanocrystal composites for bone regeneration

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Abstract: BCZT ($\text{Ba}_{0.85}\text{Ca}_{0.15}\text{Zr}_{0.1}\text{Ti}_{0.9}\text{O}_3$) is a recent class of lead-free ferroelectric material associated with high piezoelectric coefficient, making it suitable to inspire hydroxyapatite (HA)-BCZT ceramics for bone materials. Nano-crystalline hydroxyapatite (HA) synthesized using the hydrothermal route was characterized via FT-IR, Raman spectroscopy, X-ray powder diffraction (XRD), and Scanning Electron Microscopy (SEM). We also rationalized its formation as a function of operating conditions such as dwell time and temperature in this route. The nano-crystalline BCZT powder was synthesized via a sol-gel technique and its structural and morphological characterization were carried out using Raman Spectroscopy, XRD and Transmission Electron Microscopy (TEM). These investigations facilitated the optimization of HA-BCZT compositions and their electrical poling conditions to achieve enhanced piezoelectric effect. The composites (HA-BCZT) sintered at 1350°C exhibited promising piezoelectric properties. We report the enhanced piezoelectric coefficient (d_{33}) of 7 ± 1 pC/N for 50% HA-BCZT which is significant as compared to that reported in the literature for $\sim 98\%$ BT (barium titanate) - HA composites. We highlight the role of Simulated Body Fluid (SBF) on the intriguing phase-change of Tricalcium Phosphate (TCP) obtained at this sintering temperature, to hydroxyapatite for its essential contribution to promote bone growth. We theoretically support the confirmed *in vitro* biocompatibility of these composites.

Keywords: Hydroxyapatite, BCZT, piezoelectricity, bone regeneration, biocompatibility

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Graphical abstract: Novel lead-free biocompatible piezoelectric HA-BCZT nanocrystal composites for accelerated Bone regeneration

1 Introduction

The intrinsic electrical properties associated with the human bone play a key role in bone healing/remodeling [1, 2]. Hydroxyapatite (HA) that is present roughly about 70% in the human bone [3], is the commonly used bone repair material [4, 5]. There are innumerable reports documenting the use of HA as porous scaffolds for bone substitution [6, 7]. However, the piezoelectricity associated with human bone tissue *in vivo* is ascribed to its composition that produces biological electricity due to the ionic displacement under a deformation force [8–10]. Since the OH

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groups in HA were found to be responsible for the reported piezoelectric activity, the retention of these polar groups is essential for observing spontaneous polarization [11, 12]. The available literature confirms that the reorientation of polar groups in HA leads to the cell growth in HA implants [13].

Given the restrictions for the use of PZT ceramics owing to their toxicity from the Lead, there has been a tremendous research on lead-free alternatives. Recent studies on the use of lead free HA-Barium Titanate (BT) composites have assumed prime importance in bone implants. The studies have confirmed the improved osteoblast growth owing to incorporation of piezoelectricity by the BT in the hydroxyapatite [14, 15]. However, a more promising class of lead-free ceramics has been the BCZT solid solutions that have no toxic elements [16–21]. Further, as recorded in the Table 1, as per the prescribed standards of the EPA, USA, we note the stringent maximum containment levels permissible for the different elements. The chosen composition of $(\text{Ba}_{0.85}\text{Ca}_{0.15}\text{Zr}_{0.1}\text{Ti}_{0.9}\text{O}_3)$ makes it therefore non-toxic. Also unlike Lead that is both carcinogenic and genotoxic [22], there has been no reported carcinogenicity and genotoxicity observed from Ba, Ca, Zr and Ti, except the benign Baritosis obtained via Ba consumption [23]. BCZT $(\text{Ba}_{0.85}\text{Ca}_{0.15}\text{Zr}_{0.1}\text{Ti}_{0.9}\text{O}_3)$ is reported to possess a piezoelectric coefficient as high as 563 pC/N which is roughly three times that of BT reported to possess 190–260 pC/N [24]. Further, the electrical properties of BCZT are strongly linked to its crystal structure and microstructure that have been evaluated in detail [25–27]. It also has a well resolved morphotropic phase boundary, higher domain density and a large strain [28]. Most importantly, they have been demonstrated to be bio-compatible as a polymeric support for the osteogenic differentiation of stem cells within the bone-marrow [29]. All of these properties warrant a lead-free BCZT in the enhancement of piezoelectric

activity of novel HA-BCZT composites for the bone formation in the complex physiological environment.

Therefore, to mimic the bone's inherent electrical properties, various HA-BCZT nano-piezocomposites were fabricated in the ratios of 10, 20, 30, 40 and 50% by weight BCZT. The nano crystallinity plays a key role in controlling the desired properties of the material. For, the morphological effects on the surface and the internal volume of the crystallites are relatively easy to form and stabilize in this phase [14]. Therefore, to achieve the maximum piezoelectricity in these composites with the desired microstructure, the sintering and the poling conditions were optimized. Further, the temperature profile of HA and the role of simulated body fluid in its phase transformation were evaluated. We also verified their potential in bone regeneration by determining the biocompatibility through *in vitro* studies and provided a theoretical rationale for the observations.

The details of the systematic studies carried out on various aspects of the composites under investigation are elucidated in the following sections.

2 Materials and methods

2.1 Preparation of hydroxyapatite at nanoscale

The synthesis of nanostructured hydroxyapatite was carried out by the hydrothermal method similar to that reported by Li *et al.* [30]. The following chemical reagents with analytical purity were dissolved in 45 ml of deionized water (DI): 0.60 g of L-glutamic acid (Sigma), 0.38 g of dibasic sodium phosphate $(\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O})$, 0.07 g of sodium hydroxide (NaOH), 0.05–0.15 g of urea and 0.28 g of calcium nitrate tetrahydrate $[\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}]$. The solution was then sealed and subjected to hydrothermal process at 160°C in a 50 ml Teflon container with a dwell time of 24 h which yielded a colorless precipitate. Thus obtained precipitate was filtered and dried for 24 h in a hot air oven at 80°C and thereafter characterized using XRD, FT-IR, Raman Spectroscopy and SEM. Further, to evaluate the role of temperature and dwell time in the formation of HA, we studied these reaction conditions for the appropriate modification in the aforementioned method prescribed by Li *et al.* The effect of temperature was determined by heating the individual reactant mixtures separately at 100, 120, 140 and 160°C keeping the dwell time constant at 24 h. Subsequently, the effect of dwell time was studied by heating the

Table 1: The elemental comparison of the toxicity factors as provided by the National Primary Drinking Water Regulation Table, EPA, USA 2010

Metal	MCL (ppm)	Carcinogenicity	Genotoxicity
Pb	0.015	Yes (2B)	Yes
Ba	2.00	No (Baritosis - benign)	No
Ca	None	No	No
Zr	25.0	No	No
Ti	0.10	No	No

MCL: Maximum Containment Levels

reaction mixture in the furnace for 4, 8, 16, and 24 h maintaining the temperature constant at 160°C.

2.2 Synthesis of nano-crystalline BCZT powder

The nano-crystalline BCZT was synthesized using the citrate based sol-gel technique [20, 21]. To begin with, titanium isopropoxide $[\text{Ti}[\text{OCH}(\text{CH}_3)_2]_4$, Sigma Aldrich], (6 ml) was stirred in acetyl acetone [Merck Chemicals] (3 ml) for 10 mins to which a solution of citric acid (CA) [Sigma] (2.62 g) dissolved in DI water was added with stirring for 30 minutes to form solution 'a'. Calculated quantities of barium nitrate (3.314 g) $[\text{Ba}(\text{NO}_3)_2]$, Merck], calcium nitrate (0.519 g) $[\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}]$, Merck], zirconium nitrate (0.345 g) $[\text{ZrO}(\text{NO}_3)_2 \cdot \text{H}_2\text{O}]$, Merck], and citric acid (1.5 g) were dissolved in DI water separately and stirred for 10 mins to obtain solution 'b'. The mixture of solutions 'a' and 'b' was stirred for 30 mins at room temperature under a pH of ~7-8 maintained by adding ammonia solution with constant stirring. It was heated to form a yellow viscous gel at 150°C and raised to 200°C till the gel turned into a dark brownish residue which was ground with mortar and pestle to homogenize the fine powder. To remove the organic substituent, the as-prepared powder was calcined at 450°C for 5 h. After grinding, this powder was further heat-treated at 850°C for 5 h to obtain the BCZT powder. This fine powder was structurally and morphologically characterized using XRD and TEM.

2.3 Fabrication of HA-BCZT composites

The mixture of hydroxyapatite and BCZT powders in different % by weight were ground using agate mortar and pestle as tabulated (Table 3). The mixed powder was pressed into discs in a 15 mm die at 25 MPa for 2 mins. Discs were also produced from 100% hydroxyapatite powder as a control for the study. The discs of HA-BCZT composites were sintered at temperatures ranging from 800-1350°C for 2 h whereas the pristine HA and BCZT discs were sintered separately at 800°C and 1400°C for 2 h respectively. These sintered discs were poled at the electric fields between 4-16 kV cm^{-1} at 95°C for a duration of 60 mins [3], to yield the optimum piezoelectric activity.

2.4 Characterization of materials

X-ray powder diffraction was undertaken using the Pananalytical, X-Pert-3 diffractometer at a scan speed 2°/min with $\text{Cu-K}\alpha$ radiation ($\lambda = 1.54 \text{ \AA}$) in the 2θ range of 10° to 80°. The crystallite size (X_c) was determined using the Scherrer equation. FT-IR spectroscopy was performed to identify the functional groups using Thermofischer Nicolet iS10 by KBr pellet technique recorded from 4000-400 cm^{-1} in transmission mode. A Thermofischer DSR Raman microscope equipped with a 780 nm laser was used for Raman spectroscopic studies. The DTA/TG studies were undertaken using the Q600 V20.9 Build 20. The crystallite size and morphology of the synthesized powders and composites were assessed using SEM (JEOL JSM-IT300). The piezoelectric coefficient d_{33} of the poled HA-BCZT pellets was measured using an APC d_{33} meter.

These well characterized composites were then evaluated for their biocompatibility and *in vitro* phase transformations using the following studies.

2.5 Cell adhesion and proliferation studies

We determined the impact of piezoelectricity associated with HA-BCZT composites on the growth of bone cells. In this regard, cells were cultured on unpoled discs of the composite as well as on 100% HA discs as control. MC3T3 cells that are mature osteoblastic phenotypes cultured in MEM- α were used to assess the cellular response to the materials. In preparation for cell culture as carried out by Baxter *et al.* [33], the triplicate of composite discs made of HA-BCZT in the ratios ranging from [100:0 - 50:50] were autoclaved at 120°C under 15 MPa and soaked in complete culture medium overnight. Discs were then allowed to dry in air for 1 h in a 24-well plate in sterile conditions before cells were seeded at 20,000 cells/ cm^2 in 25 μl of complete culture medium. The culture plates were incubated for 2 h at 37°C with 5% CO_2 in order to allow cells to adhere to the ceramic surface before 1 ml of complete culture medium was added to each well. Cells were then incubated for 3 days; the culture medium changed on the second day.

2.6 Cellular metabolic activity

The metabolic activity of the cells was measured using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay kit (CGD-1). The culture medium was removed from the wells and replaced with 500 μl of serum-free medium. After adding 50 μl of MTT solution to each

well, the culture plates were incubated at 37°C with 5% CO₂ for 3 h. The medium was then removed and 200 µl of MTT solvent (DMSO) was added to each well, ensuring all visible crystals formed were dissolved. These solvents were then transferred to a fresh 24-well plate and a colorimetric measurement was carried out using Spectra Max M5 fluorescence spectrometer at 570 nm with a 630 nm reference wavelength.

2.7 Simulated body fluid immersion

To determine the role of body fluids on the phase transformations of HA, we undertook this following study. The HA powder that was calcined at 1300°C for 3h was soaked for different durations in a solution of FBS Fetal Bovine Serum as a natural substitute for SBF [34]. The sample, soaked for 14 days, was taken out of the FBS, centrifuged and washed with acetone and distilled water. Samples were dried overnight in an oven at 120°C and the phase transformation in the sample morphology was analysed by FT-IR.

2.8 Computational Simulations

2.8.1 Quantum Espresso

The hexagonal bravais-lattice of the HA crystal was built and energy optimized to its least energy conformation over 1000 iterations with the PAW pseudopotential in Quantum espresso [35]. The system was optimized for 144 kohnsham states with a kinetic energy cut-off at 30.0 Ry and charge density cut-off at 120.0 Ry through 8 iterations of plain mixing to yield the least energy conformation. The corresponding electron density structure was then visualized in the VESTA software for the different symmetric planes.

2.8.2 Molecular docking Studies

The various proteins that are expressed in the bone formation [36], were extracted from the protein data bank [37], and suitably cleansed of their heteroatoms and solvent. The Heinz team generates the all-atom Interface Force Fields (IFF) for the simulation of nanostructures with high accuracy, inclusive of metals, minerals, and oxides. The 001 HA crystal lattice was obtained from their ready-to-use model of the HA unit cell with the corresponding cleavage plane. These were suitably docked using HEX 8.0.0 software where the algorithm was set to an output search

for the top 100 energy cluster poses, with Shape only correlation type, 3D Fast Fourier Transform mode, Grid dimension of 0.6, a Distance range of 40, and the translation step of 0.8 [38].

3 Results and Discussion

3.1 Characterization of HA and BCZT

3.1.1 FT-IR spectroscopy

The primary peaks signifying the formation of HA are observed in the IR spectra (Supplementary data, Figure S1). As recorded in Figure S1(c), we confirm the phosphate peaks, the asymmetric P-O stretching and the presence of the key OH group. We note the primary peaks of 3423 and 1636 cm⁻¹ for the adsorbed water and 3567 cm⁻¹ corresponding to the stretching vibration of lattice OH⁻ ions. We also note the 668 cm⁻¹ OH deformation mode with a minor deviation from the expected 633 cm⁻¹ that could be attributed to the defects in the crystal formation. The different PO₄³⁻ peaks are observed at 472, 562, 603, 961, 1036, and 1091 cm⁻¹. We note the key 961cm⁻¹ peak corresponding to the asymmetric P-O stretching and the triple degenerate bending vibrations of phosphate in 603, 562 and 472 cm⁻¹. This data are accordingly tabulated (Supplementary data, Table ST1).

Further, we also employed the IR spectroscopy for studying the formation of HA as a function of the temperature and dwell time in the hydrothermal route. Figure S1(a) in the supplementary data captures the IR spectra obtained for the effect of dwell time, while the role of temperature is plotted in supplementary data, Figure S1(b). The observed formation of the crucial peak at 3567 cm⁻¹ corresponding to the stretching vibration of lattice OH leads to the optimization of the reaction conditions namely, the temperature at 160°C and dwell time of 24h in the hydrothermal treatment.

3.1.2 Raman spectroscopy

As observed in Figure 1, we obtain the Raman fingerprint reported for the pristine hydroxyapatite sample [39]. We observe the strong active ν_1 Raman mode of PO₄³⁻ at 960 cm⁻¹ along with the weak ν_2 Raman phosphate modes at 431 and 449 cm⁻¹, and ν_4 Raman active phosphate modes at 591 and 609 cm⁻¹. To confirm the structure of BCZT, we note the Raman active A_{1g} octahedral mode at 823 cm⁻¹

Figure 1: Raman spectroscopy of the HA-BCZT composites of different ratios, the Raman spectra of the pristine BCZT and Hydroxyapatite with their active Raman modes

Table 2: The comparison of the crystallinity and phase purity of hydroxyapatite, the shift in the various Raman Harmonic modes, and the EDX elemental composition for the different synthetic conditions using hydrothermal route

Sample	Scores (H:A)	Alternative Phase	CS (nm)	Harmonic Modes (PO_4^{3-})			Elemental Ratio		
				ν_1	ν_2	ν_4	P	Ca	Ca:P
HT_4	80:59	Monetite	34.57	430	590	961	36	38	1.05
HT_8	67:61	Monetite	55.31	424	598	960	34	31	0.91
HT_16	76:42	OBTP	47.93	430	589	960	29	25	0.86
HT_24	75:23	Apatite	21.27	429	589	960	32	48	1.50
HT_100	27:75	Monetite	45.92	423	594	989	28	18	0.64
HT_120	33:77	Monetite	55.11	423	594	989	28	21	0.75
HT_140	62:61	Monetite	34.57	432	594	965	30	29	0.97
HT_160	71:15	Monetite	21.27	430	590	961	32	48	1.50

*HT: Hydrothermal; H: Hydroxyapatite; A: Alternate Phase, CS: Crystallite Size

alluding to the asymmetric Zr-Ti lattice in the synthesized nano-crystalline BCZT. Further, we note the presence of the sharp band corresponding to the B1/E ($\text{TO}_3 + \text{LO}_2$) modes at 293 cm^{-1} , the strong intense A_1 (TO_3) mode at 509 cm^{-1} , and the intense asymmetric band consisting of the $\text{A}_1(\text{LO}_3)/\text{E}(\text{LO}_4)$ modes at 719 cm^{-1} . The confirmation of the cubic and tetragonal phases are reflected in the presence of the peaks observed at 509 cm^{-1} , and 293 and 719 cm^{-1} respectively. The cluster of peaks in the $180\text{--}300 \text{ cm}^{-1}$ range is attributed to the asymmetric phonon vibrations in the Ti–O bonds, while the observed peaks around 509 and 719 cm^{-1} reflect the Ba–O phonon vibrations [40]. Further, the normalized Raman spectra of the composites

depicted in the Figure 1 demonstrate the increasing intensity of the BCZT peaks in the corresponding HA-BCZT composites with the growing overlap of the peaks corresponding to the individual samples. Thus, Raman spectroscopy affirmatively confirms the formation of the composites.

Further, the effect of the synthesis conditions on the formation of these modes is also monitored using Raman spectroscopy. Table 2 provides the corresponding data pertaining to the Raman spectra (Supplementary data, Figure S2). Here we note that the weak ' ν_2 ' Raman phosphate mode is almost uniform across all the conditions and evolves into the signature peak at the optimal conditions of 24 h in 160°C .

Figure 2: SEM images and the corresponding EDX report of the composition of the Nano-crystalline hydroxyapatite for the various dwell times in hydrothermal synthesis at 10 μm resolution

3.1.3 Morphology

Figure 2 provides the snapshot of the growth of the nano-crystalline hydroxyapatite with the dwell time in the hydrothermal route where the temperature is maintained constant at 160°C , while the growth with respect to temperature keeping dwell time constant at 24 h is captured in the supplementary data (Figure S2). We observe the formation of sheets at the beginning that slowly morph into the rods, flowers and a combination of both. However, as recorded in Table 2, we have the comparison of the EDX data as obtained for the different hydrothermal synthetic routes where we observe that the sample prepared at 160°C for the dwell time of 24 h yielded the closest desirable Ca:P ratio (1.5). We thus note the important role of dwell time and temperature in the formation of the nano-crystalline HA.

To rationalize these Ca:P ratios, the SEM images of the nano-crystalline hydroxyapatite synthesized under different reaction conditions were recorded (Supplementary data, Figure S3-S5). It can be reasoned that the Ca:P ratio plays a crucial role in the resulting morphological features of the composites. This is aptly supported by the recorded EDX for the two kinds of the structural morphologies evident in the structures – namely the flower and the rod shapes. Here we note that the local domains as circled (Supplementary data, Figure S4-S5) have unique ratios de-

pendent on the rod and the flower morphologies. In general, it is evident that the rod morphology has more of Ca packed rather than the elemental composition of P.

3.1.4 X-ray powder diffraction studies

The X-ray powder diffraction studies were used to probe the phase purity of the BCZT nano-crystalline powder. The XRD pattern in Figure 3 confirms it to be a polycrystalline tetragonal perovskite structure with space group $P4mm$. No visible significant peaks of secondary phases were found. Using the Scherrer formula, we determined the crystallite size of BCZT to be close to 44nm. The X-ray powder diffraction peaks obtained for the HA sample by the hydrothermal route are significantly similar to the standard reference in JCPDS for hydroxyapatite (98-028-9993) with the major peak of (211) establishing the formation of HA (Figure 3). Bragg peaks that are encountered in the pattern could be indexed to a space group of $P63$. The evaluated crystallinity and phase purity of the sample demonstrated to be a hexagonal structure with no noticeable secondary phases.

For understanding the formation of the nano-crystalline hydroxyapatite, the XRD studies provide a significant picture. As recorded in Table 2, we have a detailed visualization of the formation in the crystal given the ra-

Figure 3: X-ray powder diffraction pattern of HA and BCZT ($\text{Ba}_{0.85}\text{Ca}_{0.15}\text{Zr}_{0.1}\text{Ti}_{0.9}\text{O}_3$)

tion of the Hydroxyapatite phase to the alternative phase recorded at each condition.

Firstly, we observe the formation of hydroxyapatite phase as early as 4 h itself, albeit in addition to the presence of other phases. We however note the decrease in this contamination of secondary phases to achieve the unequivocal hydroxyapatite phase relatively, with the increasing dwell time up to 24 h. Alternatively, as the function of increasing temperature in the hydrothermal synthesis, we notice the gradual growth in the hydroxyapatite phase and significant loss of the monetite phase only at the optimal temperature of 160°C . Here we also observe that the average crystalline size varies widely with the conditions, forming the smallest nano-crystalline HA only at the optimal conditions of 24 h at 160°C . The corresponding comparison of the various XRD patterns for the formation of the nano-crystalline HA in the different reaction conditions of hydrothermal route are captured in Figure 4.

3.1.5 Piezoelectricity in composites

While the pristine samples of HA exhibited no observable piezoelectricity, the various HA-BCZT composites were optimized for their sintering and poling conditions to maximize the piezoelectric activity as recorded in Table 3. The

Figure 4: The comparison of the X-Ray Diffraction patterns for the formation of the nano-crystalline HA from the different reaction conditions in the hydrothermal route with respect to pure HA

Table 3: The different ratios of the HA-BCZT nanocomposites prepared with their various sintering conditions with the corresponding d_{33} values obtained

Ratio (HA-BCZT)	Sintering ($^\circ\text{C}$)	Actual Amounts		d_{33} (pC/N)
		HA(mg)	BCZT(mg)	
0:100	1400	0	500	304
100:0	800	500	0	-
90:10	800	450	50	-
80:20	800	400	100	-
70:30	800	350	150	-
60:40	800	300	200	-
50:50	1350	250	250	7

TGA curve of the synthesized nano-hydroxyapatite that was dried at 80°C is captured in Figure 5. The net mass-loss during the heat treatment is calculated to be about 8.71%. The observed mass loss is relatively pronounced between 25 – 450°C that is attributed to adsorbed water. The

Figure 5: The normalized TG and DTA curves of the nano-hydroxyapatite powder dried at 80°C recorded at a heating rate of 10°C/min in air

Figure 6: The relative cell proliferation observed for the various HA-BCZT nanocomposites

decomposition of carbonate into CO_2 gas occurred from 600–800°C [41]. From 800 to 1300°C, a slight decrease in TGA curves shows the decomposition of $\text{Ca}_{10}(\text{PO}_4)_6$ to CaO and $\text{Ca}_3(\text{PO}_4)_2$ [42]. Therefore, we undertook the sintering of the HA at 800 to optimize the piezoelectricity. However, the BCZT has been reported to demonstrate the optimal density at lower crystallite size while retaining its piezoelectricity between 1300–1400°C [43]. Therefore, on the formation of the HA-BCZT composites of different ratios, we optimized the sintering conditions in this range of 800°C to 1400°C. While the pristine HA exhibited no observable piezoelectricity, BCZT demonstrated a significant value of 304 pC/N when sintered at 1400°C. We obtained a significant piezoelectric coefficient ' d_{33} ' for the pellet of 50 %w BCZT sintered at 1350°C for 2 h, and poled at 10 kV for 2 h at 95°C to yield $7 \pm 1 \text{ pC/N}$. This translates into an important finding as the prior reports required ~98% BT to achieve 6.8 pC/N possessing minimal HA for bone incorporation [14].

3.1.6 Biocompatibility

For the use of the novel HA-BCZT nanocrystal composites as potential bone implants, we first evaluated their biocompatibility in the growth of bone cells. In this study, we observed the minimal cell growth in the control HA sample that could be attributed to the absence of the intrinsic piezoelectric activity. However, HA-BCZT composites of different ratios demonstrated greater bone cell growth when compared to the control of the pristine HA sample as recorded in Figure 6. The cell proliferation tends to be maximum at 10% BCZT while it tapers down to 50% BCZT; a

trend observed in the HA:BT composites too, that can be rationalized to their structural asymmetry in the composites that plays a key role on the piezoelectricity. Interestingly, while there is no poling to generate the desired piezoelectric phase in the material, we allude the observed increase in the bone cell proliferation to the piezoelectricity. This is attributed to the 'inherent piezoelectric domains' already present in the matrix. Further, as reported in the HA-BT samples, the effect of poling has no influence on the cell growth already achieved [33]. We thus demonstrate the biocompatibility of our composites and their potential use as novel composites for accelerated bone regenerating material.

To understand this phenomena, we examined the cross-section of the 50:50 HA-BCZT composite that yielded maximum piezoelectric coefficient using TEM and SEM as captured in Figure 7. The TEM image of the pristine BCZT is provided in the Figure 7(a), while the SEM image of the fractured surface of the HA-BCZT composite in Figure 7(b) depicts the uniform stacking of the multiple layers as a highly connected network conducive for the co-operative piezoelectric effect. Further, the SEM image in Figure 7(c) shows the indistinguishable grain boundaries of HA and BCZT packed efficiently with non-negligible porosity in the composite conducive for cell growth. The EDX studies for this composite demonstrate the composition of HA-BCZT to be 50:50 as noted in Figure 6(d).

Figure 7: (a) TEM image of $\text{Ba}_{0.85}\text{Ca}_{0.15}\text{Zr}_{0.1}\text{Ti}_{0.9}\text{O}_3$; (b) SEM image of cross-section of 50:50 HA-BCZT at $2\text{ }\mu\text{m}$ (c) SEM image of the 50:50 HA-BCZT surface at $1\text{ }\mu\text{m}$ with inset – EDX spectrum; (d) Elemental ratios observed in the HA-BCZT composite as determined by the EDX studies

3.2 Computational Studies

For corroborating the present findings, we carried out the computational simulations. As evident from the Figure 8, we notice that the inherent symmetric electron density dipole is created across the OH in HA as against the symmetric O-O bond in TCP along the lattice edge that leads to spontaneous polarization in HA unlike TCP. This asymmetry compounded with that present in BCZT plays a key role in the consequent piezoelectricity. Therefore, when the composites were optimized for greater piezoelectricity, we observed greater bone cell proliferation. Further, to justify this observation, we simulated the electron densities of 15 different lattice planes of the least energy conformation of the HA crystal as recorded in the Figure 8 obtained by VESTA. Given that the (001) wulff surface is calculated to have the least surface energy and consequently optimal surface interaction [44], we notice its maximum polarity

for interactions that were evaluated consequently with the proteins in the various stages of the cell growth.

3.3 Molecular docking studies

In this regard, we pursued docking simulations given the available literature on the expression patterns of bone related proteins in the successive stages of proliferation, bone matrix formation/maturation, and mineralization [36]. We pursued the protein ligand docking simulations using HEX 8.0.0 software with the HA and the aforementioned proteins. The key proteins expressed in the bone formation as reported, include Histone H3-H4 and Ribosomal protein S6 in the first stage, while greater expression of TGF- β 1, Fibronectin and Osteonectin in later stages. The molecular docking studies demonstrate the favourable interaction of these different proteins, primar-

Figure 8: (clockwise) The electron density of the TCP versus HA, the electron density map of the different symmetric planes of the optimized HA crystal, and the best docking poses of the interaction of the HA 001 plane (ball model) with the various proteins (ribbon model) noted in the different stages of the bone cell growth

Table 4: The binding scores obtained from the molecular docking studies for the interaction of HA 001 crystal plane with the various proteins in the different stages of the cell growth

Protein	Hex Docking Score (au)
Ribosomal protein S6	-402.16
TGF- β 1	-528.24
Fibronectin	-597.4
SPARC	-593.58

ily through the hydrogen bonding in their helical loops to the polar phosphate and hydroxyl groups present on the HA surface (Figure 8). The docking scores with respect to each of the proteins' best docked pose is tabulated (Table 4) suggesting the impactful contribution of the scaffold in the different phases.

This highlights the need for the presence of the OH groups in the HA (absent in TCP) to drive the bone cell growth and in turn validates the hypothesis of the role of piezoelectricity on bone growth.

3.4 Effect of sintering temperature on the phase transformation of HA

Given the high sintering conditions used in the fabrication of the nanocrystal composites, we studied the chemical stability of HA under these conditions. In this regard, we pursued the role of temperature on hydroxyapatite. On increasing the sintering temperature, we observe the generation of more secondary phases (Supplementary data, Figure S6) which correspond to TCP. As a confirmation of the same, we also pursued the FT-IR studies. The FT-IR spectra (Supplementary data, Figure S7) demonstrate visibly that as the temperature increases, the phase transformation is evident. The hydroxyapatite (HA) transforms to TCP when it is subjected to the sintering temperature of 1300°C as evidenced by the reduction in intensity of the OH peaks at 3571 and 630 cm^{-1} .

Figure 9: The FT-IR spectra of the pristine TCP sample versus the soaked TCP sample in SBF after 14 days

3.5 Role of simulated body fluid

The HA nano-crystalline powder was sintered at 1300°C to obtain dense structure. Unfortunately, HA transforms to TCP under these conditions as elucidated above. Therefore, the purpose of the following investigation is to see whether TCP gets converted back to HA under the influence of body fluid given the importance of the polar OH group in the piezoelectric effect. 100 mg of sintered TCP powder was soaked in 1 ml of Fetal Bovine Serum (FBS) for different durations. The one sample, that was soaked for 14 days, was separated using centrifuge and filtered, and heated up to 80°C for 2 h. This was then evaluated for its functional transformation using the IR spectroscopy for the presence of any hydroxyl group. We observe the generation of the 3571 and 630 cm⁻¹ peaks corresponding to the OH stretching and vibrational mode confirming the transformation of TCP to HA (Figure 9). Thus we have a rational understanding that TCP gets converted to HA with prolonged exposure to the simulated body fluids. This ensures that our high sintering temperatures that led to conversion of the phase of hydroxyapatite to TCP in HA-BCZT composites confirms the reclamation of the HA phase when implanted in the body.

4 Conclusion

The study reiterates the relevance of piezoelectricity in bone regeneration. We evaluated the optimum conditions

to generate a significant piezoelectricity (d_{33}) using lead free BCZT in Hydroxyapatite for assisting in bone regeneration. We report the attainment of the enhanced d_{33} of up to 7±1 pC/N at 50% BCZT as against the optimal values obtained with respect to ~98% BT composite. The morphology of the pristine HA, BCZT and their composites were evaluated to rationalize their observed properties. However, given the phase change at higher temperature with loss of OH⁻ which is noted to be key for the piezoelectricity in HA, we evaluated the role of body fluid that helps in regeneration of this polar group for the formation of the desired HA phase. We further demonstrated their biocompatibility and positive impact on bone cell growth, theoretically and experimentally, opening up a new class of piezocomposites for bone regeneration.

Conflict of Interests: “There are no conflicts to declare”.

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Supplementary information

Figure S1: FT-IR spectra of the synthesized nano-hydroxyapatite prepared by the various conditions in the hydrothermal route: (a) Role of dwell time, (b) Effect of temperature and (c) The optimized ideal conditions as comparable to the reference reported in literature [S1]

Table ST1: Comparison of the assigned FT-IR peaks for the various functional groups of the synthesized hydroxyapatite versus the reference HA sample's values from literature [S1]

IR-HA, Literature (cm ⁻¹)	IR-HA, Experiment (cm ⁻¹)	ATTRIBUTE
3432, 1642	3423, 1636	Adsorbed water
3571	3567	Stretching vibration of lattice OH ⁻ ions
633	668*	O-H deformation mode
470, 568, 602,	472,562,603,	Phosphate group
964, 1041, 1093	961,1036, 1091	
964	961	Asymmetric P-O stretching
633, 602, 568	668,603,562	Triple degenerate bending vibration of Phosphate group

Figure S2: The comparison of the Raman spectra obtained for the formation of nano-hydroxyapatite generated in the different operating conditions of hydrothermal route: (a) Role of dwell time, (b) Effect of temperature and (c) Standard raman reference of HA

Figure S3: S3 SEM images and corresponding EDX report of the composition of the nano-hydroxyapatite for the temperature profile of hydrothermal synthesis at 10µm resolution

Figure S4: SEM images and corresponding EDX report of the composition of the nano-hydroxyapatite in the rod and flower composition at 8h dwell time of hydrothermal synthesis at 10µm resolution

Figure S5: SEM images and corresponding EDX report of the composition of the nano-hydroxyapatite in the rod and flower composition at 16h dwell time of hydrothermal synthesis at 10μm resolution

Figure S6: XRD spectra of the hydroxyapatite with increasing sintering temperature

Figure S7: FT-IR spectrum of the hydroxyapatite with increasing sintering temperature

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