Chapter 3

Experimental

In this chapter, the synthesis method, and characterizations of $Mg_{1-x}Ca_xSi_{1-x}Zr_xO_3$, (x = 0, 0.1, 0.2, 0.3 and 0.4) MCSZO-X and hydroxyapatite ceramics are described. To analyse the phases, microstructure, surface chemistry and physical characterization of the developed bioceramics, various phase evolution techniques including X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR), microstructural characterization (SEM and EDX), contact angle measurement and X-ray photelectric spectroscopy (XPS) were used. The effect of electrostatically and electrodynamically treatment on in vitro cellular response was also demonstrated. In addition, the electrostatically treated antibacterial response of prepared bioceramics have also presented. Further, in - vivo experiment is also performed to investigate the toxicity of MCSZO-X nanoparticles in the rat model.

3.1. Synthesis

3.1.1. Synthesis of $Mg_{1-x}Ca_xSi_{1-x}Zr_xO_3$ bioceramics

$Mg_{1-x}Ca_xSi_{1-x}Zr_xO_3$, (x = 0, 0.1, 0.2, 0.3 and 0.4) [MCSZO-X, where X = (0 – 4), corresponding to x = 0 – 0.4] bioceramics were fabricated via solid-state synthesis route, using MgO, $CaCO_3$, SiO_2 and ZrO_2 as precursors [Figure. 3.1]. After stoichiometric calculations, corresponding amounts of these powders were milled in a nylon jar using a roller ball mill at 600 rpm for 24 h. Here, the ethanol and zirconia balls were used as wetting and grinding media (powder to ball ratio, 4:1), respectively. After the overnight drying of slurry in oven at 100 ºC and crushing, the obtained powder was calcined at temperature of 1300 ºC for 10 h. Subsequently, the calcined powder was crushed and again ball milled for 12 h and dried in an oven.

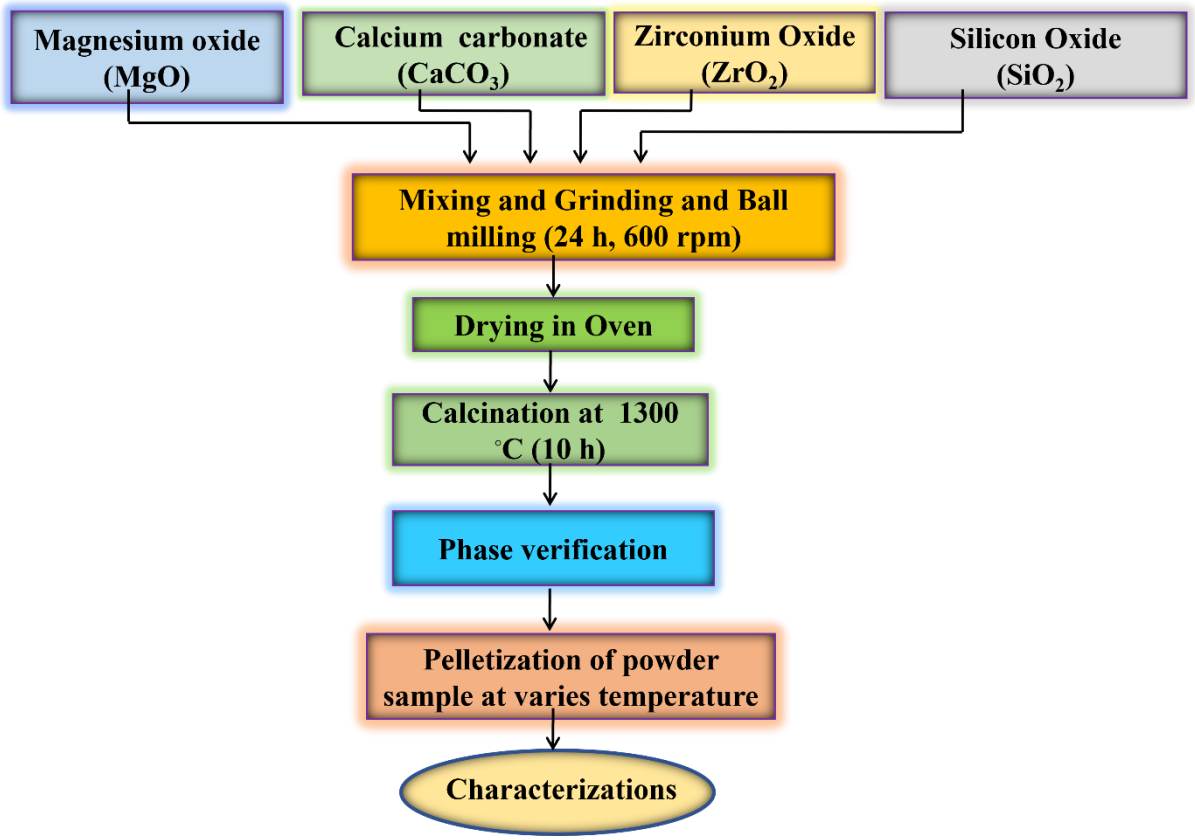

Figure 3.1. Flow chart showing the solid - state synthesis for MCSZO-X bioceramics.

3.1.2. Synthesis of hydroxyapatite bioceramics (HA)

Hydroxyapatite (HA) was synthesized using the suspension precipitation route [1]. A mixture of CaO (Merck) and H_3PO_4 (Himedia) was stirred at 80-90°C with a magnetic stirrer at 200 rpm, at a pH of 8-10, and the obtained solution was left overnight for precipitation [Figure. 3.2]. The precipitate was dried and calcined for 2 h at 800°C.

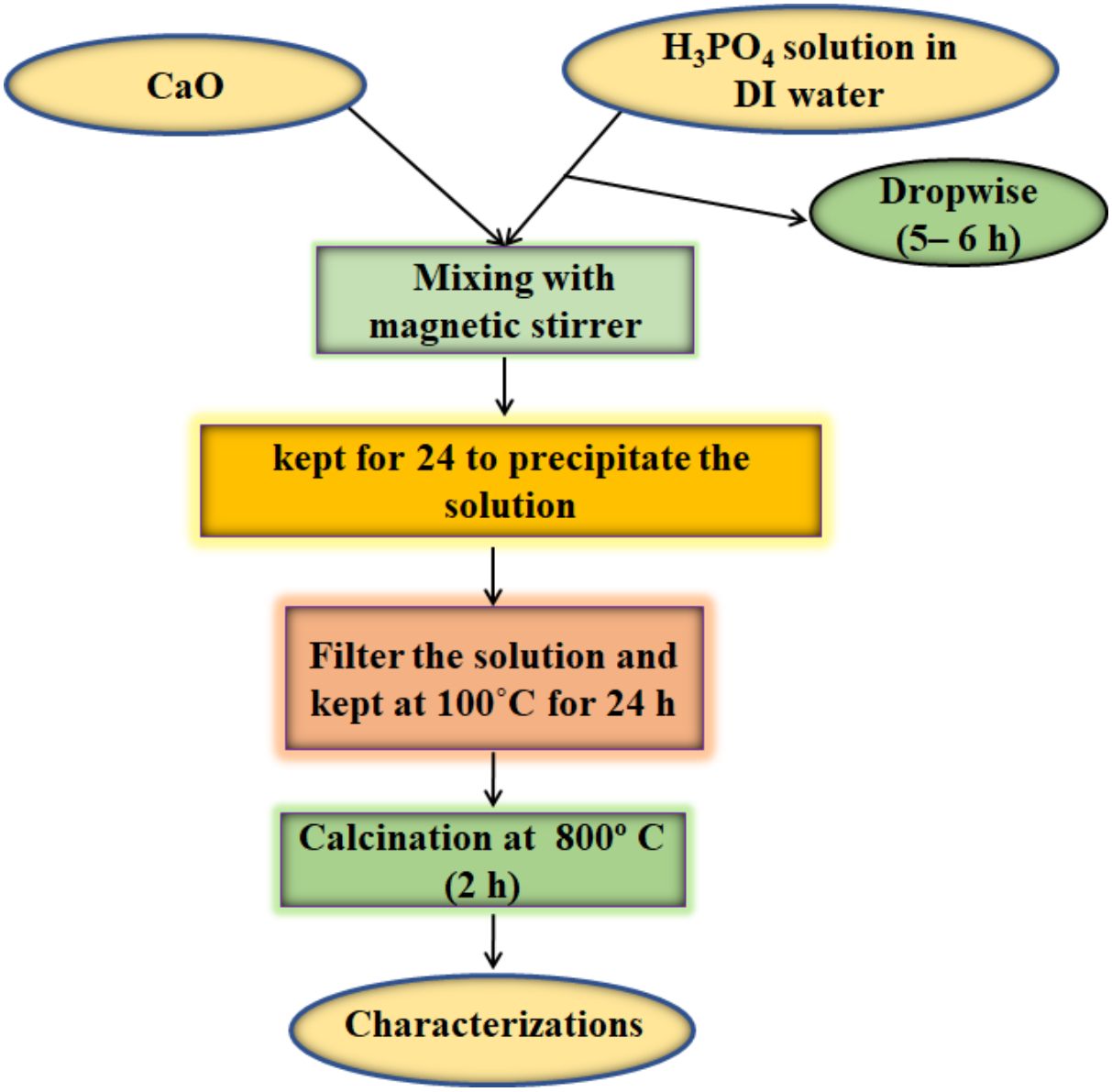

Figure 3.2. Flow chart showing the synthesis protocol for HA bioceramic.

3.2. Processing and densification of prepared MCSZO and HA bioceramics

The calcined powder of MCSZO-X and HA was hydraulically pressed into 12 mm diameter and 1 mm thickness pellets, followed by cold isostatic pressing at 330 MPa to achieve the higher densification. The MCSZO-X and HA discs were fired at the optimal sintering temperatures, ranging from 1320°C to 1380°C for 3- 8 h and 1200 °C for 2 h [Table. 3.1]. The density of sintered MCSZO-X (X = 0 – 4) and HA pellets samples was measured by Archimedes's principle.

$$Density\ of\ samples\ (\rho) = \frac{Dry\ weight}{weight\ loss} = \frac{w_d}{w_d - w_l} \quad (3.1)$$

$$Densificaton\ (\%) = \frac{Density\ of\ samples}{theoritical\ density} \times 100 \quad (3.2)$$

Where w_d represent the dry weight of samples (in air) and w_l represent the suspended weight (in water).

Table 3.1. Optimized sintering temperature at duration of 2 h in heating rate 5°C/min parameters for the MCSZO-X (X = 0 - 4) and HA compositions

S. No.	Samples	Sintering temperature (ºC)
1.	MCSZO-X (X=0)	1380
2.	MCSZO-X (X=1)	1350
3.	MCSZO-X (X=2)	1350
4.	MCSZO-X (X=3)	1350
5.	MCSZO-X (X=4)	1350
4.	Hydroxyapatite (HA)	1200

3.3. Phase and microstructural analyses of MCSZO and HA bioceramics

3.3.1. Phase evaluation

X-ray diffractometer with Cu-Kα radiation were used to identify the phases of sintered MCSZO-X (X = 0 - 4) samples. X-ray peak profile analyses were performed to analyse the XRD data. The crystallite size and lattice strain were determined using the Scherrer, modified Scherrer, size-strain methods and Williamson-Hall plot.

The crystallite size (D) for MCSZO-X ceramic samples were calculated by the Scherrer formula as [2]

$$D = \frac{k\lambda}{\beta Cos\theta} \qquad (3.3)$$

Where, β, λ and K are the Full width half maxima (FWHM), wavelength (1.54046 Å) and shape factor (0.94) of the X-rays, respectively [Table 1]. The modified Scherrer formula can be written as,

$$\ln\beta = \ln\left(\frac{k\lambda}{D}\right) + \ln\left(\frac{1}{\cos\theta}\right) \qquad (3.4)$$

Small crystallite size and lattice distortion, caused by lattice dislocations, broaden the XRD peaks [2]. The lattice strain influences the XRD pattern in addition to the size of the crystallites. As a result, peak broadening equals the summation of peak size and strain-induced broadening [3]

$$\beta = \beta_D + \beta_\varepsilon \qquad (3.5)$$

$$\beta\cos\theta = \left(\frac{k\lambda}{D}\right) + 4\varepsilon\sin\theta \qquad (3.6)$$

Where, ε represents lattice strain.

The WH plot provided information about the line broadening of the diffracted peaks, which was found to be isotropic. This suggest that the diffracting domains were also isotropic, and microstrain was a contributing factor. When line broadening is isotropic, it is possible to determine the size and strain parameters accurately by using an average SSP plot [4, 5]. This method employs a Lorentzian function to determine the crystallite size and a Gaussian function to describe the strain profile [4-6].

As a result, we have,

$$(d\beta \cos\theta)^2 = \left(\frac{k\lambda}{D}\right)(d^2\beta \cos\theta) + \left(\frac{\varepsilon}{2}\right)^2 \qquad (3.7)$$

In this equation, d and K represent the interplanar spacing and shape parameter, respectively.

3.3.2. FTIR spectroscopy

The Fourier transform infrared spectra (FTIR) analyses of sintered MCSZO- X (X = 0 - 4) and HA samples were obtained (Bruker Model Tensor 27, Germany) within the wave number range of 4000 - 500 cm^{-1} to identify the functional groups present in the prepared samples.

3.3.3. Microstructural analyses

The surface morphology of fractured MCSZO-X (X = 0 - 4) samples were obtained using scanning electron microscopy (SEM, EVO 18 Research, Zeiss) and energy dispersive X-ray spectroscopy (EDS) was used for elemental analyses.

3.4. Development of bioelectrets

The electrets of well-polished MCSZO-X (X = 0 - 4) as well as HA pellets were developed by corona poling unit at the voltage and temperature of 20 kV and 500 ºC, respectively, for a duration of 30 min. Afterwards, the samples were cooled down under the exposure of polarization voltage. The surface of the sample, exposed to the corona, exhibited a negative pole (negative end of electrets), while the opposite surface exhibited a positive pole (positive end of electrets). Thereafter, the influence of the electrets on cellular response, leaching behaviour, surface hydrophilicity, surface chemistry and antibacterial response was examined.

3.5. Ion leaching behavior of MCSZO-X bioceramics in stimulated body fluid (SBF)

The influence of formation of electrets on leaching behaviour of MCSZO-X (X = 0 - 4) was analyzed by the immersion of these samples in the simulated body fluid (SBF, pH; 7.4), at 37 °C, for different time periods (7, 14, and 21 days) [7]. The pH of the SBF was kept to be 7.4 during entire protocol. After that, the pellets were removed from SBF.

The acquired solution from all samples was diluted 10 times with DI water and filtered through a 0.22-micron filter. The solution was kept for inductively coupled plasma-atomic emission spectrometer (Thermofisher Scientific, ICP-AES, iCAP6000 series) to measure ionic concentration of Mg^{2+}, Ca^{2+}, Si^{4+} and Zr^{4+} in the SBF solution.

3.6. Contact angle measurement

The influence of formation of electrets on hydrophilicity of MCSZO- X (X = 0 - 4) samples were analysed by measuring the contact angle with 1 ml of culture media (DMEM)/deionized (DI) water, by contact angle measurement unit (KRUSS GmbH Germany, model DSA10).

3.7. X-ray photoelectron spectroscopy (XPS)

X-ray photoelectron spectrometer (Thermofisher Scientific, Kα X-ray photoelectron spectrometer) was used to investigate the effect of formation of electrets on the surface chemistry of MCSZO-X (X = 0- 4) samples. Casa XPS software was used for the analyses of XPS data. The pass energy during survey scan was kept to be 200 eV. However, during elemental analyses, pass energy was kept to be 50 eV.

3.8. *In vitro* cellular assessment

Electrodynamically stimulated *in vitro* cellular response of the prepared MCSZO-X (X = 0 - 4) electrets and HA bioceramics were evaluated, quantitatively and qualitatively using human osteoblast-like MG-63 cells (National Centre for Cell Science, Pune, India). Dulbecco's Modified Eagle's medium (DMEM) media, supplemented with Fetal bovine serum (15 % FBS) and antibiotic (1 %, Antibiotic-Antimycotic) was used as growth media. The samples were submerged into 70 % ethanol solution and sterilized under UV exposure for 1 h in biosafety cabinet. Thereupon, phosphate buffer saline (1 X PBS) was used twice for washing the samples. The dried samples were seeded with 10^4 cells/well on all the positively, negatively and uncharged MCSZO-X (X = 0 - 4) electrets and HA samples and incubated for different periods (3, 5 and 7 days) in CO_2 incubator (95% humidity, 5% CO_2). After seeding

the cells on the electrets and controls, the samples were incubated for 12 h [Figure. 3.3], which was then followed by stimulation with electrodynamic field (1 V/cm) using digital oscilloscope (ScientiFic SMO702) with pulse duration of 400 µs for 5 min. The similar procedure was repeated one more time after the further incubation of 12 h. The electrets and uncharged samples were used as reference to analyse the results. For the statistical analyses, the SPSS-16.0 software was used to analyse the significant difference in various examined assays by one way ANOVA technique and Tukey's tests at significant value of $p \leq 0.05$.

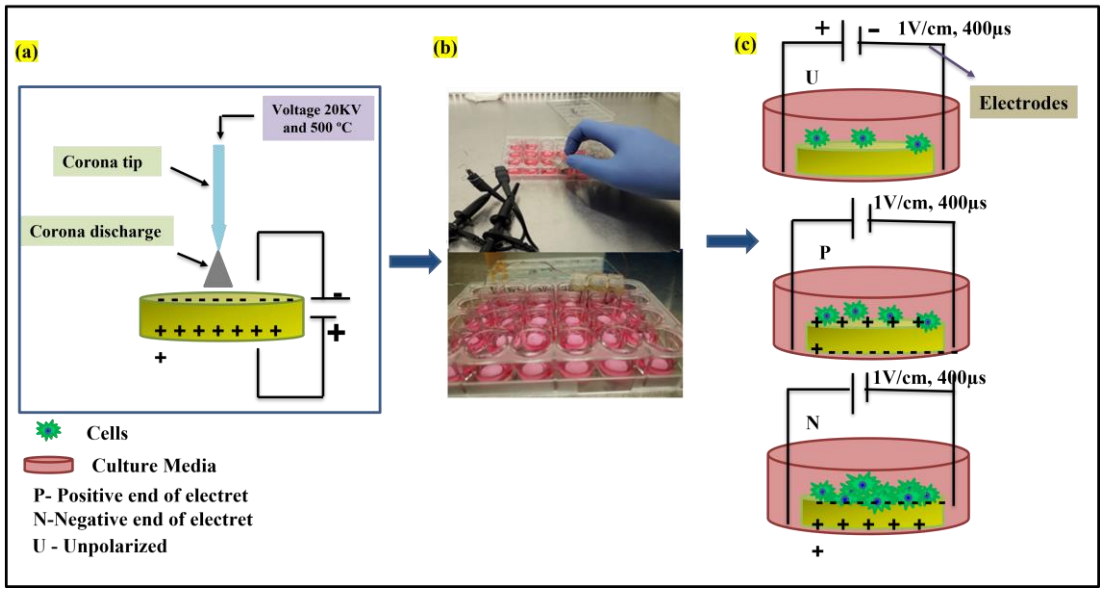

Figure 3.3. A schematic illustrating the combined effect of electrostatic and electrodynamical stimulation in a cell culture experiment. a) bioelectret development, b) , and c) application of electrical stimulation while the cells are being adhered on uncharged, negative and positive end of electrets.

3.8.1. Cell attachment/ proliferation assay

The MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay was performed to examine the viability of MG-63 cells on uncharged and electrodynamically stimulated MCSZO-X (X = 0 - 4) electrets and HA samples, as per ISO 10993-5 standard. After the completion of incubation, the samples were rinsed twice with 1X PBS.

Subsequently, MTT solution (MTT: DMEM media in 1:10 ratio) was added and kept in incubator for 6 h to form formazan crystals which were dissolved in DMSO. The obtained solution (150 µl, triplicate) was moved into the 96 well plate and the optical density was measured @ 595 nm using ELISA micro plate (Biorad iMarkTM) reader. Thereafter, the percentage viability of MG-63 cells on the samples was calculated as [8],

$$\%\,\boldsymbol{Viabilty} = \frac{\boldsymbol{mean\ absorbance\ of\ sample}}{\boldsymbol{mean\ absorbance\ of\ control}} \times \mathbf{100} \qquad (\mathbf{3.8})$$

3.8.2. Morphological assay

The morphology of the adhered MG-63 cells, seeded on uncharged and electrodynamically treated MCSZO-X (X = 0 - 4) electrets and HA control samples were evaluated using fluorescence microscope (Nikon Eclipse LV-100 ND). The adhered cells were fixed in 3.7% formaldehyde solution. Titron X-100 was used to permeabilize the cells and later, the bovine serum albumin (BSA) was added as a blocker. Alexa Fluora 488 Phalloidin and DAPI dyes were used to stain the cytoskeletons and nuclei of adhered cells, respectively.

3.8.3. Alkaline phosphatase (ALP) activity

Alkaline phosphatase enzyme plays a crucial role in regulating the osteogenic activities as well as excellent marker for early stage biomineralization [9]. Therefore, ALP activity test was performed to evaluate the combined action of electret and electrodynamic field treatments on cell differentiation on the developed scaffold [9, 10]. In this experiment, the growth media for osteogenic cells was prepared by the addition of 10 mM β-Glycerol phosphate and 0.2 mM L-ascorbic acid in the DMEM media which was supplemented with 1% antibiotics and 15 % FBS. The MG-63 cells were cultured on uncharged and MCSZO-X (X = 0 - 4) electrets and control samples, for 7 and 14 days. After 12 and 24 h of incubation, electrodynamic stimulation (pulse duration 400 s, 1 V/cm) was applied to the wells to examine the synergistic effect of electrodynamic stimulation and electret formation on ALP

activity. The cells, seeded on each sample were lysed with Triton X-100 for 5 min, which was followed by addition of p-nitrophenyl phosphate (200 µl) and then incubated for 1h at 37 °C. Towards the end, the enzyme reaction was terminated using 1.5 ml of 0.6 N NaOH. The optical density of the resulting solutions was finally measured in triplicate at 405 nm using an ELISA microplate reader (Thermo Fisher, skanlt software). The ALP activity of all MCSZO-X (X = 0 – 4) samples was standardised using the BSA standard curve.

3.8.4. Intracellular Ca^{2+} ions measurement

The voltage-gated calcium channels are suggested to be activated and opened by local electrical stimulation, allowing extracellular Ca^{2+} to enter the cells, which ultimately led to increase in the concentration of intracellular Ca^{2+} ions [11, 12]. This elevated concentration of intracellular Ca^{2+} results in execution of gene transcription and promotes the synthesis of transforming growth factor as well as bone morphogenetic protein [11, 12]. Therefore, external stimuli increase the intracellular Ca^{2+} and thereby, promote the cellular functionality [12]. For this purpose, an intracellular Ca^{2+} indicator Fura-2 acetoxymethylester (Fura-2 AM, Thermofisher Scientific) dye was used to study the effect of electrodynamic stimulation on the calcium channel passageways. In this experiment, 10^4 cells/ml MG-63 cells were seeded on the uncharged and electrets of MCSZO-X (X = 0 - 4) and HA samples. Following this, electrodynamic field was applied on each sample, after 12 and 24 h of incubation. After further incubation of 48 h, the culture media was discarded, followed by washing of the samples using HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfolic acid) buffered saline (HBS, pH; 7.4) solution, containing 10 mM glucose, 1 mM MgCl2, 10 mM HEPES, 145 mM NaCl, 1 mM CaCl2, 5 mM KCl, After this, dye solution (prepared with 5 µM Fura-2 AM, HBS, 0.1% BSA) was added to the samples. The samples, with dye loaded cells, was kept in incubator for 1 h. After this, the dye containing solution was discarded, followed by washing with HBS solution.

Thereafter, HBS (added with 2.5 mM probenecid) solution was added to the samples and further incubated for 20-25 min in absence of light. The dye-leakage from the cells were prevented by probenecid [13]. The obtained solution from each sample was taken in 96 well (black colored) plates. The absorbance of final solution from each sample was measured at the 510 nm of emission intensity. Fura-2 dye solution shows maximum emission, corresponding to intracellular calcium free and calcium bound solutions, at the excitation of 380 and 340 nm, respectively. For this reason, the wavelengths of excitation intensity were kept at 340 and 380 nm. In this aspect, the concentration of intracellular Ca^{2+} can be examined by calculating the ratio of maximum fluorescence intensity at the calcium bound and calcium free excitation wavelengths. The reading was obtained using fluorescence plate reader (Synergy H1) at every 10 s upto 10 cycles.

3.9. *In vitro* antibacterial assessment

The *in vitro* antibacterial response of MCSZO-X (X= 0 - 4) electret samples were evaluated with E. coli (GN, Microbial Type Culture Collection, MTCC 443) and *S. aureus* (GP, Microbial Type Culture Collection, MTCC 435S) bacteria. For this purpose, both, quantitative and qualitative analyses such as, MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] as well as live/dead assays were performed. The mechanism of electret induced antibacterial response has been revealed via various assays, such as, catalase activity, superoxide production, protein estimation and lipid peroxidation assays. The freeze-dried bacteria, S. aureus and E. coli, were procured from MTCC Chandigarh, India. Both, the bacteria were grown in nutrient broth media and kept at 37 ºC for a duration of 12 h. The bacteria culture was diluted with media to the optical density (OD) of 0.1 before seeding. For the statistical analyses, the SPSS-16.0 software was used to analyse the significant difference in various examined assays by one way ANOVA (Analysis of the Variance) technique and Tukey's tests at significant value of $p \leq 0.05$.

3.9.1. Quantitative assessment

3.9.1.1. MTT assay

To evaluate the viability of *S. aureus* and *E. coli* bacteria on the uncharged, positive, and negative ends of MCSZO-X electrets, as well as on HA (control), the MTT assay was performed. The uncharged and MCSZO-X electrets and HA pellets were seeded in 24 well plates with 150 µl of bacterial culture and incubated at 37 ºC for 10 h. After incubation, the culture media was removed, and the samples were washed twice with 1 X PBS. Subsequently, 500 µl MTT solution (MTT: PBS: 1: 10) was added in each well to form the formazan crystals and further incubated for 2h. The MTT solution was replaced with 500 µl of dimethyl sulfoxide (DMSO) and kept in incubator for 5 -10 min to dissolve purple coloured formazan crystals. Thereafter, the solution was transferred to 96 well plate in triplicate to measure the optical density (OD) at wavelength of 595 nm using ELISA microplate reader (Bio-red)[14, 15]. Also, the antibacterial ratio for uncharged and MCSZO-X electrets and HA samples was determined as follows [16],

$$\textit{Antibacterial ratio}\ (\%) = \frac{OD_{blank} - OD_{sample}}{OD_{blank}} \times 100 \qquad (3.9)$$

3.9.2. Qualitative assessment

3.9.2.1. Live/ dead assay

The bacteria seeded samples were incubated for 10 h at 37 ºC, then washed twice with 1 X PBS. The SYTO 9 and propidium iodide (PI) dyes were used for staining of live and dead bacterial cell. The combination of both the dyes (SYTO 9 and PI, 1 : 1) was added (1-2 µl) to the each sample and left in the dark, for 30 min. Further, Nikon Eclipse, LV 100 ND fluorescence microscope was used to observed both, the living and dead bacteria cells.

3.10. Enzymatic response

E. coli and *S. aureus* bacteria were seeded on the uncharged and MCSZO-X electrets and HA samples and then incubated for 8 h. In order to understand the mechanism of antibacterial

response, induced via electrets, various enzymatic assays, such as, lipid peroxidation (LPO), super oxide dismutase (SOD), protein estimation and catalase activity assays, have been performed.

3.10.1. Super oxide radical (SOD) assay

The quantity of antioxidant superoxide (O_2^-) enzymes was calculated using SOD assay. In this assay, uncharged and MCSZO-X electrets and HA samples were cultured with *S. aureus* and *E. coli* bacteria, for 8 h at 37ºC. Further, lysozyme (1 mg / ml) was added, and the solution was incubated for additional 1 h. Thereafter, EDTA (Ethylenediamine tetracetic acid, 0.5 mM) and NBT (Nitro blue tetrazolium, 0.75 mM) were added to form SOD buffer. Further, 60 µM riboflavin (in DI water) were added into the prepared solution. The colour of the final solution changes after 6 min of direct contact with fluorescent light. The absorbance of final solution was measured at wavelength of 560 nm [17, 18].

3.10.2. Catalase assay

The catalase activity was assessed by measuring the dissociation of hydrogen peroxide (H_2O_2) radicals on the uncharged and MCSZO-X electrets and HA samples. These MCSZO-X and HA samples were seeded with *E. coli* and *S. aureus* bacteria. 100 µl of bacterial culture solution was mixed with freshly prepared 30 mM H_2O_2 and 50 mM phosphate buffer (pH = 7.2 - 7.4) to create the reaction solution. The absorbance of the final solution was measured at a wavelength of 240 nm after every 30 seconds for 3 min. The catalase activity/sec of the H_2O_2, decomposed on electret and uncharged surfaces of MCSZO-X and HA, was measured as [19],

$$\textbf{\textit{Catalase activity}}\ (\boldsymbol{K}) = \frac{\mathbf{2.3}}{\boldsymbol{\Delta t}} \times \boldsymbol{log}\frac{\boldsymbol{E_1}}{\boldsymbol{E_2}} \qquad (\mathbf{3.10})$$

Where, A_1 and A_2 are the absorbance values at time, t = 0 and t = 30 sec, respectively.

3.10.3. Protein estimation assay

The purpose of this assay was to quantify the amount of oxidized protein in the form of carbonyls, obtained from both, uncharged and MCSZO- X electrets and HA samples. The three reagents A, B and C have been used in this assay. The reagent A is prepared with 2% Na_2CO_3 and 0.1 N NaOH, and reagent B was prepared using 1.35% potassium sodium tartrate and 0.5% $CuSO_4$. The Reagents C was made with the mixture of A and B (2% of Reagent B and 48% of Reagent A). In this test, 980 µl of DI water was mixed with 20 µl of centrifuged supernatant. After that, 500 µl of follins and 5 ml of reagent C were added to the solution and allowed to left at room temperature until the solution turned blue. Hereafter, the absorbance was taken at 750 nm. Lowery method was used to calculate the level of oxidized protein in uncharged and MCSZO-X electret samples, using BSA as a reference [20].

3.10.4. Lipid peroxide (LPO) assay

When both *S. bacteria* and *E. coli* was cultured on the surfaces of uncharged and MCSZO-X electrets and HA pellets, the degree of oxidative stress was measured using LPO assay. Free radicals such as, hydroxyl radicals, lipid oxyls, singlet oxygen, etc. start a chain reaction called lipoperoxidation, which produces reactive aldehydes like malondialdehyde (MDA) and 4-hydroxynonenal as its by product (4HNE) [21]. In the LPO experiment, a test tube containing 500 µL of centrifuge supernatant was supplemented with 500 µL of Tris-HCl solution (0.1 M). The mixture was then incubated for 2 h at 37°C. Afterwards, 1 ml of trichloroacetic acid was added into the solution and the prepared solution was centrifuged at 4 °C and 3000 rpm, for a duration of 10 min. The supernatant was combined with an equal amount of thiobarbituric acid and then heated at 100 ^{o}C for a duration of 10-12 min until the color of the solution was changed to light red. Before measuring absorbance at 532 nm, 1 ml DI water was added into the final solution.

Reactive aldehydes, such as malondialdehyde (MDA), which are the products of the lipoperoxide reaction were used to evaluate the LPO activity as [22],

$$\text{MDA/mg protein} = \frac{\text{OD}(532)\times\text{reaction Volume}\times10^{9}}{\text{Sample volume}\times1000\times\text{Extinsion coefficientof MDA}} \quad (3.11)$$

Extension coefficient of MDA is taken to be $1.56 \times 10^{5}\ \text{M}^{-1}\text{cm.}^{-1}$

3.11. *In-vivo* toxicity assessment of MCSZO-X (X = 0 -4) nanoparticles

3.11.1. Sample preparation and material characterization

The synthesis procedure for MCSZO-X (X = 0 -4) nanoparticle with particle sizes at the micron level is provided in section 3.1.1. The solid-state method was employed to prepare these powders within a compositional range of X = 0 to X = 4. The particle size of prepared bioceramics reduces via ball milling (Fritsch Pulverisette 5) for 12-14 h at 300 rpm, using ethanol media (The weight ratio of ball to powder kept at 10:1). The ball milled powder was dried for overnight at 100 ºC.

3.11.2. Phase and morphological characterization

The phase of the MCSZO-X nanoparticles was determined by mean of X-ray diffraction with Cu Kα radiation (XRD, X-ray Diffractometer, Rigaku Miniflex II Desktop) with a wavelength of 1.5418 Å. High-resolution scanning electron microscopy (Nova Nano SEM, FEI) was used to determine the particle size and morphology of the ball-milled MCSZO-X nanoparticles. The elemental distribution in MCSZO-X nanoparticles was assessed using energy-dispersive X-ray spectroscopy (EDS).

3.11.3. Eluate solution preparation

The MCSZO-X ball-milled particles were autoclaved at 121ºC for 25 - 30 min. Following this, the sterilized powders were dispersed in saline (0.9 % w/v NaCl) at three concentrations as 0.25 (C1), 2.5 (C2), and 25 (C3) mg/ml, respectively. Saline served as the medium for injecting MCSZO-X nanoparticles into rats.

To achieve a uniformly dispersed nanoparticles, the eluates were undergone ultrasonication for 15 min after every 6 h, over a period of 3 days. Prior to injection, the eluates were sterilizated once again.

3.11.4. Leaching behavior

The leaching behavior of ready eluates (C1, C2 and C3) of MCSZO-X nanoparticles were examined in saline after 1, 3, and 7 days of incubation. ICP-AES was used to evaluate the amounts of leached ions (Mg, Ca, Si and Zr) from different elute of MCSZO-X samples. Before measurements, the resulting solutions were diluted 10 times in distilled water, followed by filtration using a pore size of 0.22 micron (syringe filter).

3.11.5. Cellular response of MCSZO-X nanoparticles

The cellular behavior of MCSZO-X (X= 0 -4) was studied using MG-63 cells. The procured cells were grown in a CO_2 incubator under specific conditions of 37 ºC temperature, 5 % CO_2, and 95 % humidity. The DMEM with a growth factor of 15 % FBS and 1 % antibiotic solution were used as a growth media. The culture media was replaced with fresh media after every 3 days. After, 90 – 95 % confluency, the cells were trypsinized using a 0.25% trypsin / EDTA solution and 10^4 cells/ml seeded onto glass cover slips (gelatin-coated) in a 24-well. The well plate was then incubated for a period of 12 h for the adhesion of cells to the substrate. After the 12 h incubation period, the 100 µl of three eluates (C1, C2, and C3) of M0, M1 M2, M3, and M4 samples was added into the adhered cells. The cells with nanoparticles were further incubated for 1 and 3 days, respectively, to observe quantitative cellular responses. The viability of cells grown on MCSZO-X samples were assessed using MTT test. After cell seeding of 1 and 3 days, 500 µL of MTT solution was added of sample. After 6 hours of incubation at the prescribed environment, the well plate was taken out, and the solution was replaced with 500 µL of DMSO to dissolve the formazan crystals.

Then, triplicates of 150 µL of the dissolved culture solution for each samples were moved to a 96-well plate. The absorbance of the obtained solution was measured using an ELISA reader at the wavelength of 570 nm.

3.11.6. *In vivo* study

3.11.6.1. Animals

In the present study, adult male Wister rats weighing 200±50 g were used. Rats were housed under controlled conditions of humidity and temperature (21±2ºC) with a 12-hour light-dark cycle. Food and water were provided ad libitum, and rats were randomly allocated to different experimental groups (n=5/group). All animal procedures were carried out in accordance with the guidelines established by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), under the authority of the Government of India, based in New Delhi. The animal experimentation protocols were properly authorized by the Institute Animal Ethics Committee at the Indian Institute of Technology, Banaras Hindu University, situated in Varanasi, Uttar Pradesh, India (IAEC) approval number: IIT(BHU)/IAEC/2023/II/083).

3.11.6.2. Experimental procedure

Rats were administered with 100 µl of MCSZO-X nanoparticles (Figure. 3.4) suspended in sterile saline (25 mg/ml). Animals were randomized into seven groups with n=5 rats in each group. Group 1 served as a control and group 2 injected with saline (intraarticularly). Remaining five designated as M1, M2, M3, M4 and M5 treated with MCSZO-X nanoparticles. The animals were closely observed for any signs of physical changes during the initial 30 minutes following the experimental procedures. Subsequently, they were monitored at regular intervals over the next 24 hours, with particular attention given to the first four hours. Animals were daily observed for a duration of 7 days. Body weight was recorded at day 1, 3, 5 and 7 post injection and compared to see if any changes were

observed. Deaths and other physical changes were recorded. After seven days, blood samples were collected through retro-orbital plexus puncture followed by animals were sacrificed with high dose of anesthesia and different organs (kidney, heart and liver) and knee joint were harvested for histopathological studies.

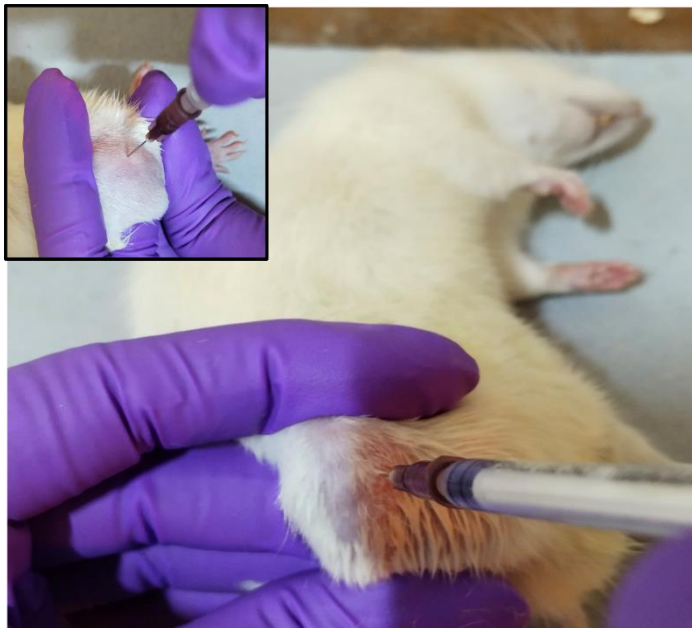

Figure 3.4. Representation of intra-articular injection of MCSZO-X (X = 0 – 4) nanoparticulate in the Wistar rat knee (synovial) joint.

3.11.6.3. Hematology and Biochemical assay

EDTA-coated vials were used to collect the blood samples for hematological analysis, and hematologic toxicity was assessed using an automated hematological analyzer (Cell-Dyn Ruby Hematology Analyzer). Hematological parameters like mean corpuscular volume (MCV) and white blood cells (WBC) were estimated. Biochemical testing was performed on serum obtained after the centrifugation (4000 RPM for 10 min) of blood samples to evaluate the activities of creatinine and alkaline phosphatase (ALP) in the rats injected with nanoparticles and compared with control and saline treated group. A diagnostic kit called AUTOSPAN Liquid and MKB Alkaline Phosphatase kit (manufactured by ARKRAY

healthcare Pvt. Ltd., India) was used for the determination of ALP activity. The investigation was conducted as per guidelines, provided by the manufacturer.

Additionally, creatinine level was also measured by the kit (Crystal Chem, IL, USA), and the examination was carried out in compliance with the guidelines provided by the manufacturer.

3.11.6.4. Histopathology analyses

Using an ethanol solution, the fixed tissues, including the kidney, liver, and heart, are dehydrated. After that, the paraffin blocks were made after fixing with paraffin wax. Hematoxylin and eosin (H&E) stains were used for staining the tissue blocks after they were divided (10 μm) for histopathological evaluations. The tissues in the fixed knee joint were first dehydrated with ethanol solution, and then decalcification was done with nitric acid solution (10%). Like other organs, the embedded paraffin blocks of knee joint tissues were ready for histopathology. Nikon Eclipse LV 100 ND fluorescence microscope photographs of stained tissues have been examined.

3.12. Statistical analyses

For the statistical analyses, the SPSS-16.0 software was used to analyse the significant difference in various examined assays by one way ANOVA technique and Tukey's tests at significant value of $p \leq 0.05$.

3.13. Closure

MCSZO-X (X = 0- 4) bioceramics were synthesized by solid-state route, using MgO, $CaCO_3$, SiO_2 and ZrO_2 as precursors. The calcined powder was pelletized using a uniaxial hydraulic press, followed by cold isostatic pressing. The processing parameter to be optimized. The Scherrer, modified Scherrer, size-strain and Williamson-Hall plot methods were used to determine the crystallite size and lattice strain of prepared bioceramics. The surface chemistry and wettability were investigated by conducting XPS and contact angle measurements to assess the impact of bioelectrets. Further, electrodynamically stimulated *in*

vitro cellular response of the prepared MCSZO-X electret bioceramics were evaluated, both, quantitatively and qualitatively. In addition, *in vitro* antibacterial response of electrostatically treated MCSZO-X (X= 0 - 4) samples were evaluated. Both, *in vitro* cellular and antibacterial test results, were used to predict the potentiality of these prepared bioceramics for orthopaedic implant applications. Also, toxicity assessment investigations were carried out *in vivo* on the MCSZO-X nanoparticles, such as hematology, biochemical analyses and histopathology.

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