



CHAPTER III
EXPERIMENTAL WORK

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Experimental Work

In the present work, a few compositions in the systems $K_2O:Al_2O_3:2SiO_2$ (leucite) and $K_2O:Al_2O_3:SiO_2$ (kalsilite) were prepared using mechanochemical synthesis route. Furthermore, bioactive glass ceramic composites have been developed by mixing mechanochemically derived leucite/kalsilite, low-temperature frit (LTF) and bioactive glass in different weight ratio. All the samples were characterized by their crystal structure, thermal behavior, microstructure, mechanical and biological properties using

Table. 3.1 List of chemicals used

| S. No. | Materials Used | Chemical Formula | Purity | Manufacturer |
|--------|---------------------|------------------------|--------|-------------------------------|
| 1. | Aluminum oxide | Al_2O_3 | 99.00% | Spectrochem PVT.LTD. India |
| 2. | Potassium carbonate | K_2CO_3 | 99.00% | Loba chemie, India |
| 3. | Silicon dioxide | SiO_2 | 99.90% | Loba chemie, India |
| 4. | Calcium fluoride | CaF_2 | 99.50% | Reidel (India) Chemicals |
| 5. | Magnesium fluoride | MgF_2 | 99.60% | Loba chemie, India |
| 6. | Sodium carbonate | Na_2CO_3 | 99.50% | Loba chemie, India |
| 7. | Potassium nitrate | KnO_3 | 99.00% | Qualigens Chemicals |
| 8. | Borax | $(Na_2H_3BO_4.10H_2O)$ | 99.50% | Loba chemie, India |
| 9. | Feldspar | $K_2OAl_2O_36SiO_2$ | 98.00% | |
| 10. | Magnesium oxide | MgO | 96.00% | Loba chemie, India |
| 11. | Zirconium dioxide | ZrO_2 | 99.50% | Loba chemie, India |
| 12. | Calcium carbonate | $CaCO_3$ | 98.50% | Loba chemie, India |

DTA/TGA, X-ray diffraction, dilatometer, scanning electron microscope, universal testing machine, ion leachability test, FTIR and cell culture study. A list of raw materials used in the synthesis of these systems is given in Table. 3.1.

3.1 Preparation of leucite using mechanochemical synthesis route

Aluminum oxide, potassium carbonate, and silicon dioxide were weighed in stoichiometric ratio 1:1:2 of leucite. Weighed mixes of materials were termed as MCL without CaF_2 and as MCL-C with the incorporation of 2 wt% CaF_2 . The raw materials were first thoroughly mixed in an agate mortar pestle for 30 minutes.

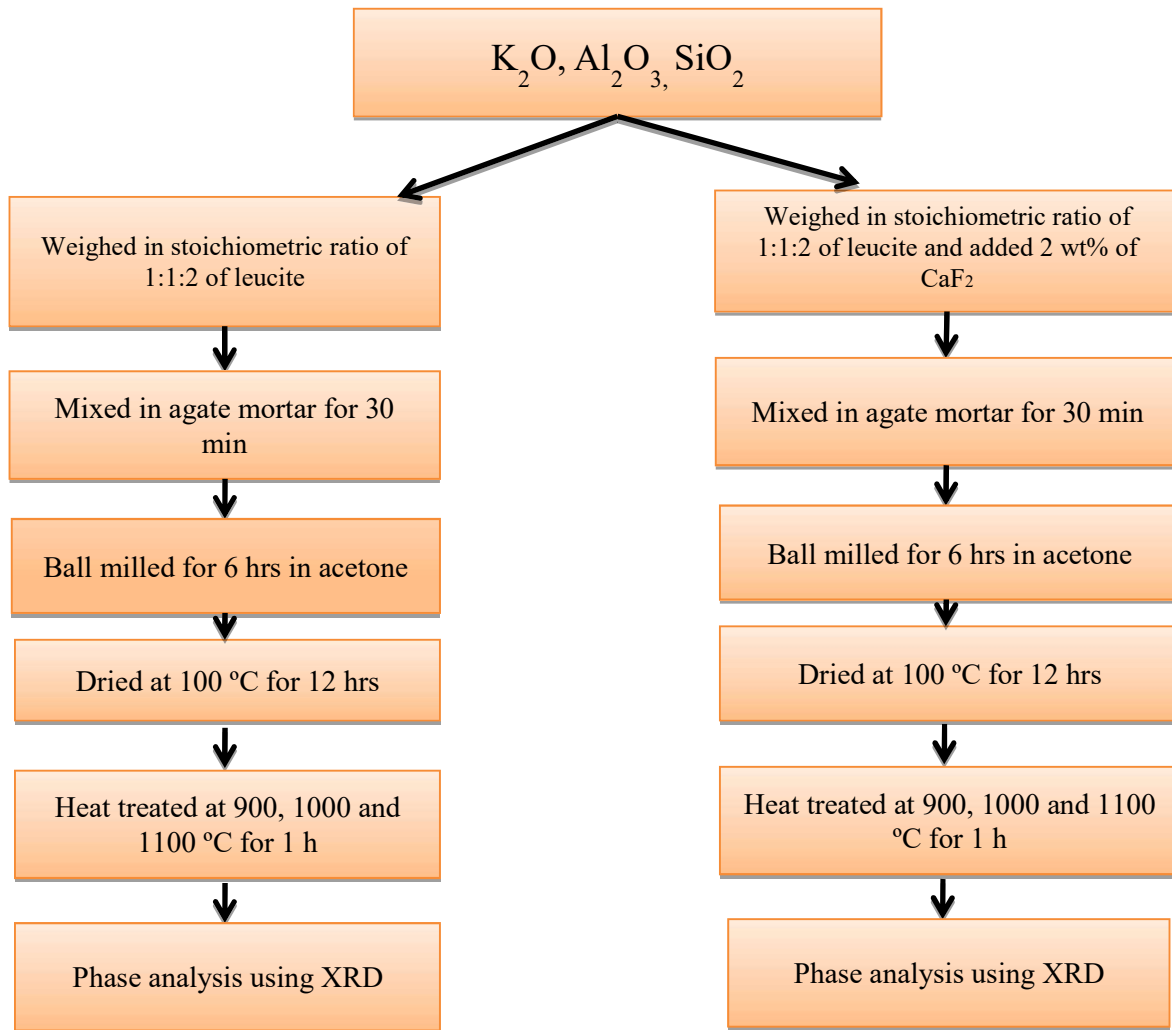


Fig. 3.1 Flow chart of the preparation of leucite

These mixtures were then pulverized in a high-energy planetary ball mill (V.B. Ceramics, Chennai, India) employing zirconia jar with rotating speed of 300 rpm for 6 hrs. The grinding balls made of zirconia having diameter 10 mm were used as a hard grinding medium. Grinding media and the material weight ratio were kept 3:1. The pulverized powders were kept in an alumina crucible and heated in an electric furnace at 900, 1000 and 1100°C respectively at a heating rate of 10 °C/min for a soaking period of 1 h. The furnace was equipped with SiC heating elements and a programmer PID528, manufactured by Selectron Process Controls Pvt Ltd, India. This programmer has the temperature control accuracy of $\pm 1^\circ\text{C}$. The flow chart for preparation of leucite is shown in Fig. 3.1.

3.2 Preparation of LTF

The raw materials for preparation of LTF were taken in a weight proportion to meet 59% SiO₂, 13% Al₂O₃, 9% K₂O, 10% Na₂O, 2% CaO, 2% CeO₂, 1.5% CaF₂, 1% MgO and 1% BaO. This composition was selected because its thermal expansion value was near to dental metal alloys in our experiments. To prepare LTF, starting components were mixed in an agate mortar pestle. The mixture was melt in a platinum crucible at 1350 °C for one h. The melted frit was quenched in deionized water then pulverized to pass a 350 Tyler mesh.

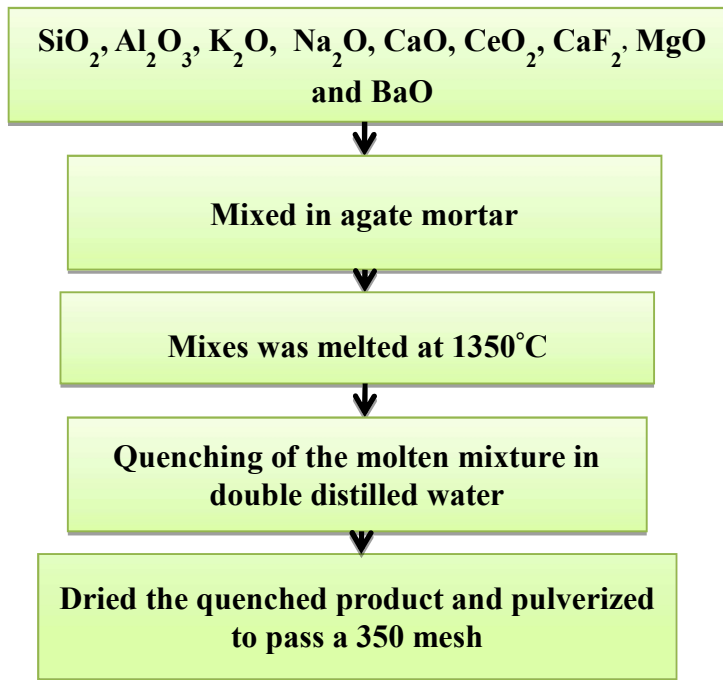


Fig. 3.2 Flow chart for synthesis of LTF

3.3 Preparation and heat treatment of samples containing different wt. ratio of mechanochemically derived leucite and LTF

The rectangular bar-shaped samples were formulated of a composition containing 25 wt. % leucite (leucite samples heat treated at 1000 and 1100 °C showing better phase formation) and 75 wt. % LTF using a uniaxial hydraulic press with a load of 200MPa. These samples were heated in a dental furnace (VITA VACUMAT 40T by Vita International, Germany). This furnace was pre-programmed for the firing of wash opaque, opaque, dentin, incisor, glazer and margin. Our selected program had five steps from room temperature to 800 °C. These five steps of firing cycle include; (i) preheating at 500 °C for 2 min, (ii) heating from 500 to 800 °C for 6 min in a vacuum, (iii) 1 min soaking at 800 °C followed by cooling to 600°C in 1 min. The batch compositions are given in Table. 3.2.

3.4 Preparation of kalsilite using mechanochemical synthesis route

Potassium carbonate, aluminum oxide, and silica were weighed in a stoichiometric ratio of 1:1:1. Weighed mixes of materials with and without the incorporation of 2 wt % of MgF_2 were prepared in the same manner. These mixtures were then pulverized in a high energy planetary ball mill (V.B. Ceramics, Chennai, India) for 3 h and 6 h with the rate of 250 rpm. Milling was done in a 250 ml zirconia cylindrical jar. The grinding balls made of zirconia having diameter 10 mm were used as a hard grinding medium. Ball to powder weight ratio was kept 4:1. The milled powder was heat-treated in an electric furnace at different temperatures 900, 1000 and 1100 °C at a heating rate of 10 °C/min and soaked for 1h. The flow chart for the preparation of kalsilite is shown in Fig. 3.3.

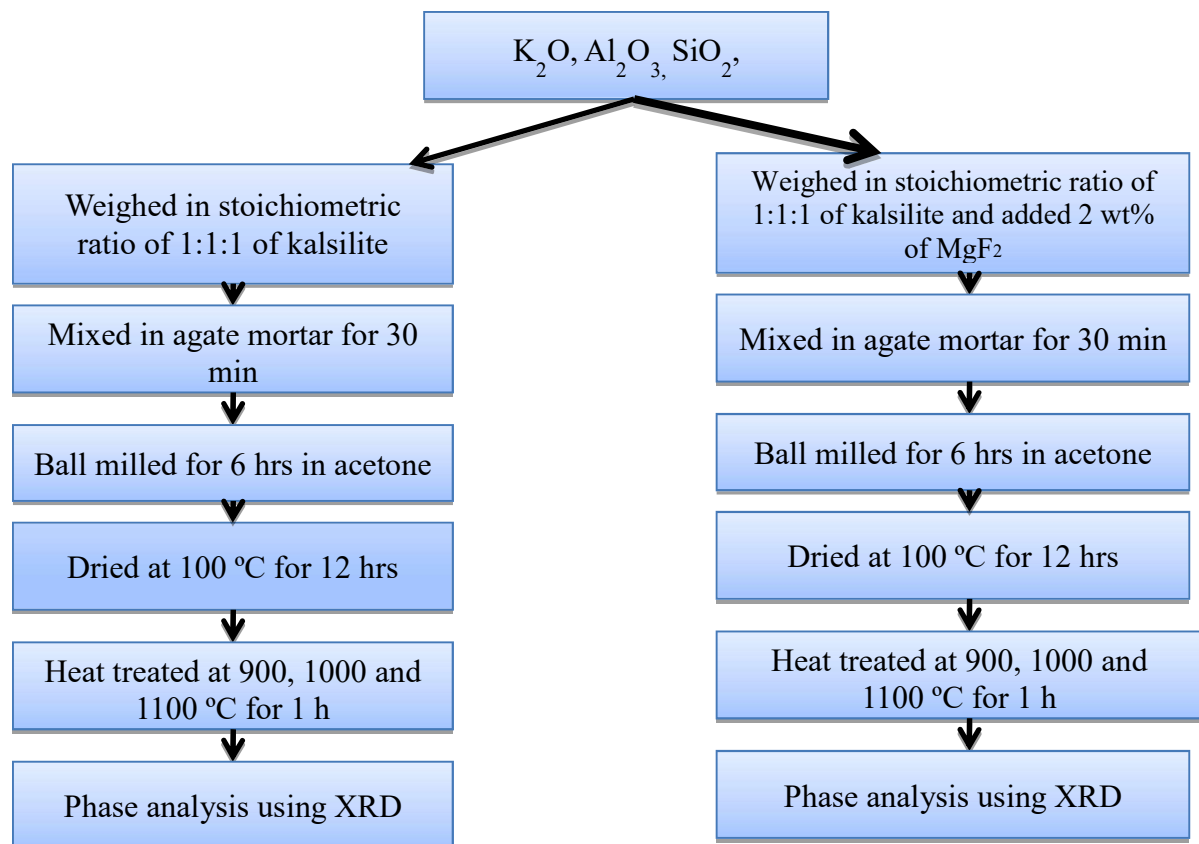


Fig. 3.3 Flow chart for the preparation of kalsilite

3.5 Preparation and heat treatment of samples containing different wt. the ratio of mechanochemically derived kalsilite and LTF

The rectangular bar-shaped samples were formulated of a composition containing a different weight ratio of kalsilite (milled for 6 hrs and heat treated at 1000 °C, showing better phase formation) and LTF (30:70, 25:75 and 20:80) using a uniaxial hydraulic press with a load of 200MPa. The compositions and their abbreviations are given in Table 3.3. These samples were heated in a VITA VACUMAT 40T according to a standard dental veneering firing cycle pre-programmed by VITA. It consists of five steps from room temperature to 800 °C. These five steps of firing cycle are: preheating at 500 °C for two min., heating from 500 to 800 °C for six min., 1 min. soaking at 800 °C followed by cooling to 600 °C in 1 min.

3.6 Preparation of Bioglass

Commercial Bioglass® 45S5 was prepared on a lab scale using traditional melt-quenching in a platinum crucible at 1400 °C. The molten frit was quenched in deionized water followed by drying at 110 °C for 2 h and subsequently pulverized to pass a 350 BSS mesh. A similar process was adopted for the preparation of LTF described elsewhere [2&3]. A flow chart for the preparation of bioglass is shown in Fig. 3.4.

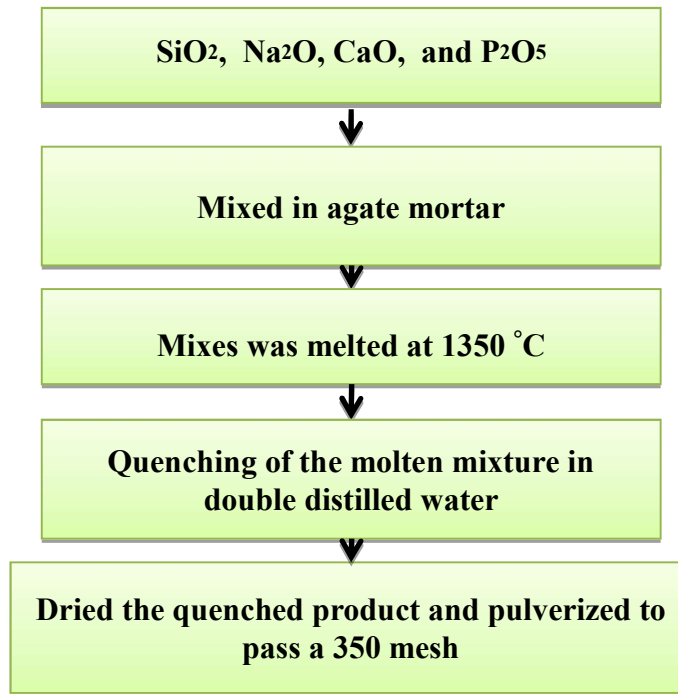


Fig. 3.4 Flow chart for preparation of bioglass

3.7 Preparation of bioactive leucite/kalsilite based glass-ceramic composites

Composites were prepared by mixing 40 wt% of mechanochemically derived leucite (heat treated at 1100 °C) and kalsilite (heat treated at 1000°C), 45 wt% Bioglass and 15 wt% LTF powder. Compositions were chosen by various optimizations such as; thermal expansion, the glossiness of the surface and translucency. The batch was ground in a planetary ball mill (V.B. Ceramics, Chennai, India) for 10 minutes to obtain a homogenous mixture. This ground material was pelletized using a uniaxial hydraulic press by applying a load of 200 MPa. These pellets were heat treated at 960 °C with a heating rate of 80 °C/min using a VITA VACUMAT 40T according to a standard dental veneering firing cycle pre-programmed by VITA under vacuum. The Vacuum was introduced at 500 °C and released at 960 °C.

Table 3.2 Batch composition and their abbreviations

| Sample Coding | Abbreviation | Leucite (wt%) | LTF (wt%) |
|--------------------------|--------------|---------------|-----------|
| L _{1000 25/75} | MCL | 25 | 75 |
| L _{1100 25/75} | ----- | 25 | 75 |
| L _{C1000 25/75} | MCL-C | 25 | 75 |
| L _{C1100 25/75} | ----- | 25 | 75 |

*L₁₀₀₀ and L₁₁₀₀ means leucite heat treated at 1000 and 1100 °C

Table: 3.3 Batch composition of different wt% of mechanochemically synthesized kalsilite and LTF

| Sample Coding | Abbreviation | Kalsilite (wt%) | LTF (wt%) |
|--------------------------|--------------|-----------------|-----------|
| K _{1000 20/80} | MKL | 20 | 80 |
| K _{1000 25/75} | ----- | 25 | 75 |
| K _{1000 30/70} | ----- | 30 | 70 |
| K _{M1000 20/80} | MKL-M | 20 | 80 |
| K _{M1000 25/75} | ----- | 25 | 75 |
| K _{M1000 30/70} | ----- | 30 | 70 |

*K₁₀₀₀ means kalsilite fired at 1000 °C

Table. 3.4 Batch composition of the bioactive glass-ceramic composites

| Composites | Leucite (wt%) | Leucite | | Kalsilite | | Bioglass (wt%) | LTF (wt%) |
|------------|---------------|-----------------------------|-----------------|-----------------------------|---|----------------|-----------|
| | | with CaF ₂ (wt%) | Kalsilite (wt%) | with MgF ₂ (wt%) | | | |
| COMP-1 | 40 | - | - | - | - | 45 | 15 |
| COMP-2 | - | 40 | - | - | - | 45 | 15 |
| COMP-3 | - | - | 40 | - | - | 45 | 15 |
| COMP-4 | - | - | - | 40 | - | 45 | 15 |

To examine the bonding of the composites, pellets made of opaque from VITA VMK 95 opaque 1M2, (Product no. B330212, VITA Zahnfabrik, Bad Sackingen, Germany) were used as the substrates. The substrate powder was pelletized similarly subsequently heating at 960 °C. Coating material was prepared by mixing composites in a modeling liquid using a liquid to powder weight ratio of 0.3 to make a thick paste. It was then spread manually using a spatula on the substrates. These specimens were fired up to 960 °C using VITA VACUMAT 40T. The samples of VITA VMK95 Dentin 1M2 (Product no. B330212, VITA Zahnfabrik, Bad Sackingen, Germany) were also prepared for the comparative study of physical and mechanical properties. The batch compositions and their abbreviations are given in Table. 3.4.

3.8 Formulation of Al₂O₃ added leucite based glass-ceramic composites

Composites (referred as Al-0, Al-2, Al-4 and Al-8) were prepared by mixing the different wt. % of mechanochemically derived leucite, bioglass, LTF and fine alumina. The mixtures were ground in an agate mortar for 20 minutes to get a homogenous mixing. The ground mixtures were pelletized using a uniaxial hydraulic press by applying a load of 200 MPa. The pressed pellets were heat treated at 950°C using a VITA VACUMAT 40T furnace.

3.9 Characterizations

3.9.1 Powder X-ray diffraction (XRD)

X-ray diffraction is a non-destructive analytical technique used to determine the crystal structure and phases of a crystalline material. XRD is based on constructive interference of monochromatic X-rays and a crystalline sample. X-rays are produced by a cathode ray tube, filtered to produce monochromatic radiation, collimated to concentrate, and directed toward the sample. X-ray diffractometer mainly consists of a Debye-Scherrer camera in

which a free counter is used in place of a strip of film, a sample holder, and X-ray detector. Fig.3.6 shows the schematic diagram of X-ray diffractometer. It consists of a source of monochromatic radiation and X-ray detector on the circumference of a circle. This circle is centered on the sample holder and divergent slits located specimen and detector. It limits the scattered radiation, reduces noise. The detector and specimen holder are mechanically coupled with a goniometer so that rotation of the specimen automatically accompanies a rotation of the counter through 2θ degrees through θ degree. This coupling ensures that the angle of incidence on and reflection from, the specimen will always be equal and half of the angle of diffraction.

X-rays are produced by heating a filament in cathode ray tube which produces the electrons, accelerated towards the target sample by applying a voltage. If electrons have the sufficient energy to dislodge the inner shell's electrons of the target, characteristics X-rays are produced. These spectra consist of mostly K_{α} and K_{β} lines. K_{α} consists of $K_{\alpha 1}$ and $K_{\alpha 2}$ lines in which $K_{\alpha 1}$ has a shorter wavelength and twice intensity than that of $K_{\alpha 2}$. The specific wavelength is the characteristic of the target material. A copper target produces monochromatic radiations used in the X-ray and a Ni filter whose characteristic wavelength for K_{α} -line is 1.54059 \AA .

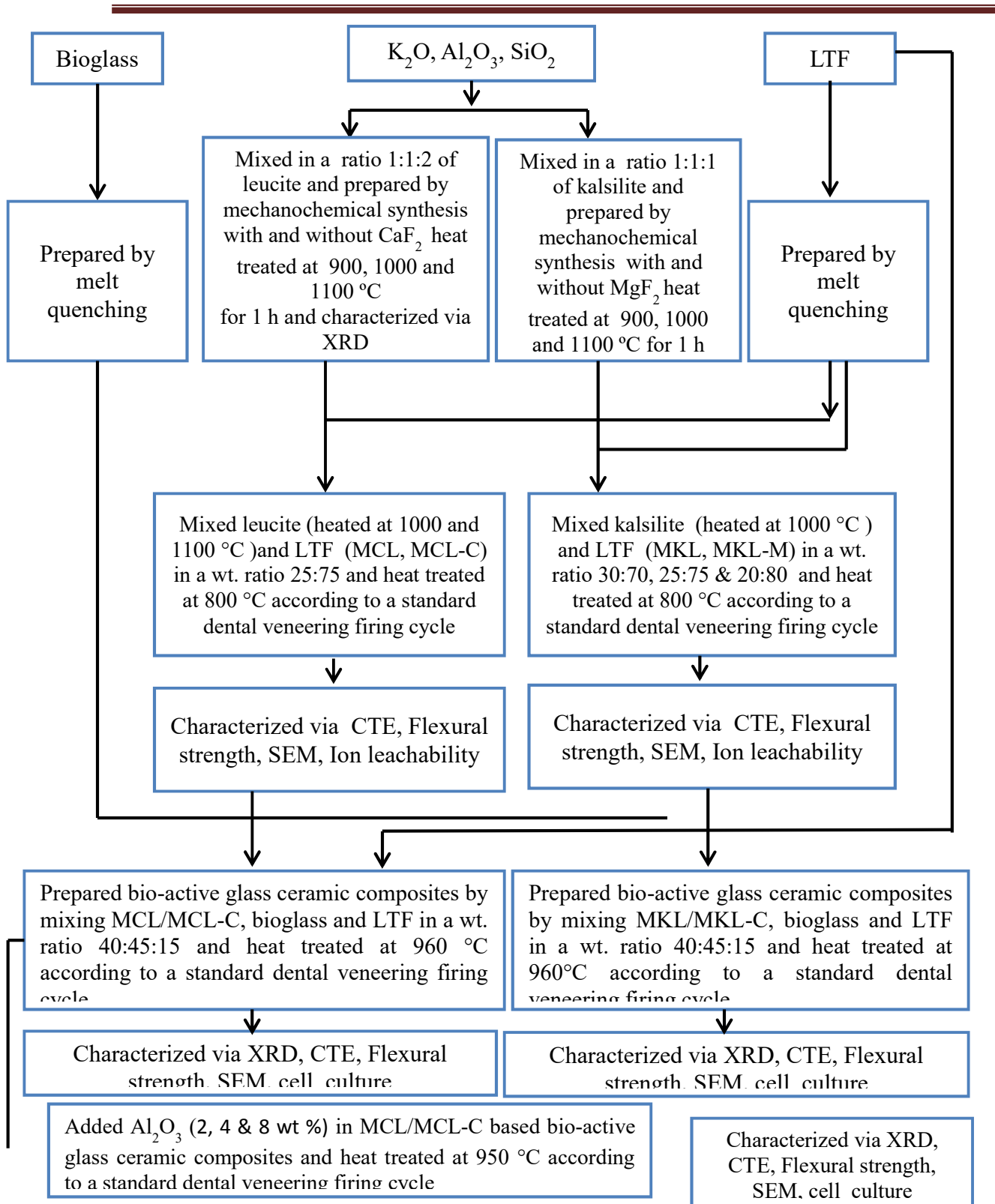


Fig. 3.5 Overall flow chart of the project

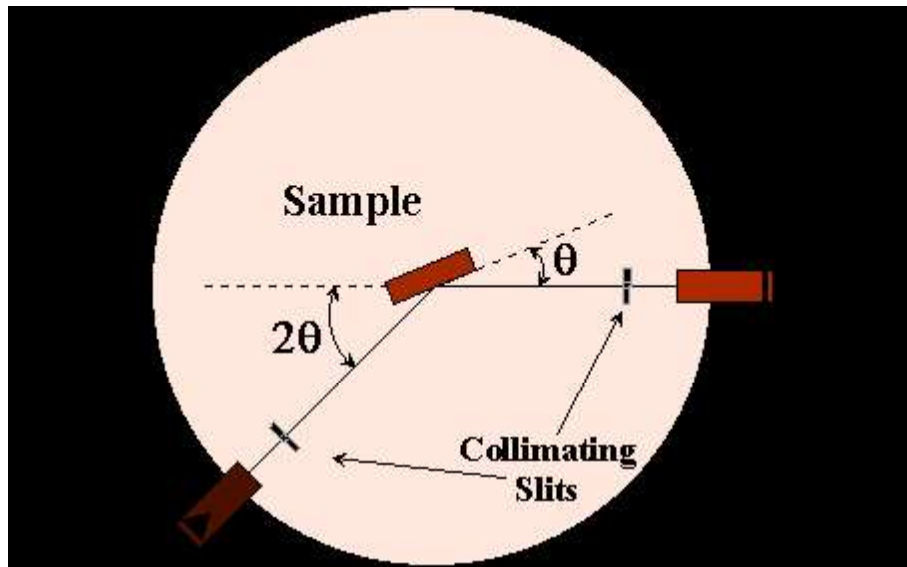


Fig. 3.6 Schematic diagram of X-ray diffractometer [http://www.wikipedia.com]

These X-rays are collimated and directed onto the sample, as the sample and detector are rotated, the intensity of the reflected X-rays is recorded. If the incident X-rays follows the Bragg's equation (Eqⁿ.3.1), a constructive interference occurs and producing X-rays. The detector records these X-rays and converts the signal to a count rate, then output to a device such as a computer monitor.

$$2d\sin\theta = n\lambda \quad (3.1)$$

where d is the interplaner spacing of the planes, θ is the diffraction angle or Bragg angle, n is the order of diffraction and λ is the wavelength of X-rays.

Powder X-ray diffraction patterns of all the milled leucite and kalsilite powder samples (heat treated at different temperatures) were recorded using a Rigaku high-resolution powder X-ray diffractometer employing Cu $K_{\alpha 1}$ radiation and Ni-filter at room temperature. The data were collected in the Bragg angle range of $20^{\circ} \leq 2\theta \leq 80^{\circ}$. Diffraction peaks were indexed using JCPDS standard files, 85-16626, 87-1707, 851421, 74-0387 and

81-1667. Average crystallite size, D of the milled powders was determined using Scherrer's formula:

$$D = \frac{0.9\lambda}{\beta \cos\theta} \quad (3.2)$$

where β is the full width at half maxima (FWHM) excluding instrumental broadening, λ is the wavelength of X-rays and θ is Bragg angle.

3.9.2 Coefficient of thermal expansion (CTE) using dilatometer

A dilatometer measures the change in dimension of a material when it is heated or cooled. A pushrod dilatometer (V. B. Ceramics, Chennai, India) was used to measure the thermal expansion and glass transition temperature of the prepared samples (Fig. 3.7). A pushrod dilatometer consists of an intermediary machine which transmits the dimensional change in the sample due to change in the temperature. A linear thermal expansion is defined as the change in length of the specimen (dL) resulting from a change in the temperature.

It is dimensionless and represented by the ratio of change in length of a sample to its original length (L_0). CTE was calculated using the following formula:

$$\alpha_{T_2-T_1} = \frac{dL}{L_0 (T_2-T_1)} \quad (3.3)$$

Where, dL and L_0 are the change in the length and original length of the sample respectively. T_2 and T_1 are the final and initial temperature respectively.



Fig. 3.7 Image of pushrod dilatometer [http// www.vbceramics.com]

For CTE measurements, rectangular bars of dimension 45 mm ×15 mm ×10 mm were prepared using a rectangular die made of stainless steel under a load of 50 kN. Thermal expansion of all the samples (LTF added samples) was determined using dilatometer in the temperature range 30-650 °C at a heating rate of 6 °C/min. The dilatometer was equipped with SiC heating element with a control accuracy of ±1 °C. It had Nippon PID programmable digital temperature indicator cum controller.

3.9.3 Scanning electron microscope (SEM)

Scanning electron microscope (SEM) uses a focused beam of high-energy electrons instead of light to produce a variety of signals at the surface of a sample by scanning it. These signals give the information about the specimen including surface morphology, chemical composition, crystalline structure and orientation of the materials. The electron beam is thermionically emitted from an electron gun fitted with a tungsten filament cathode. SEM can image the area in the range from 1 cm to 5 μ in width and magnification ranging from 20 X to 30,000 X, the spatial resolution of 50-100 nm.

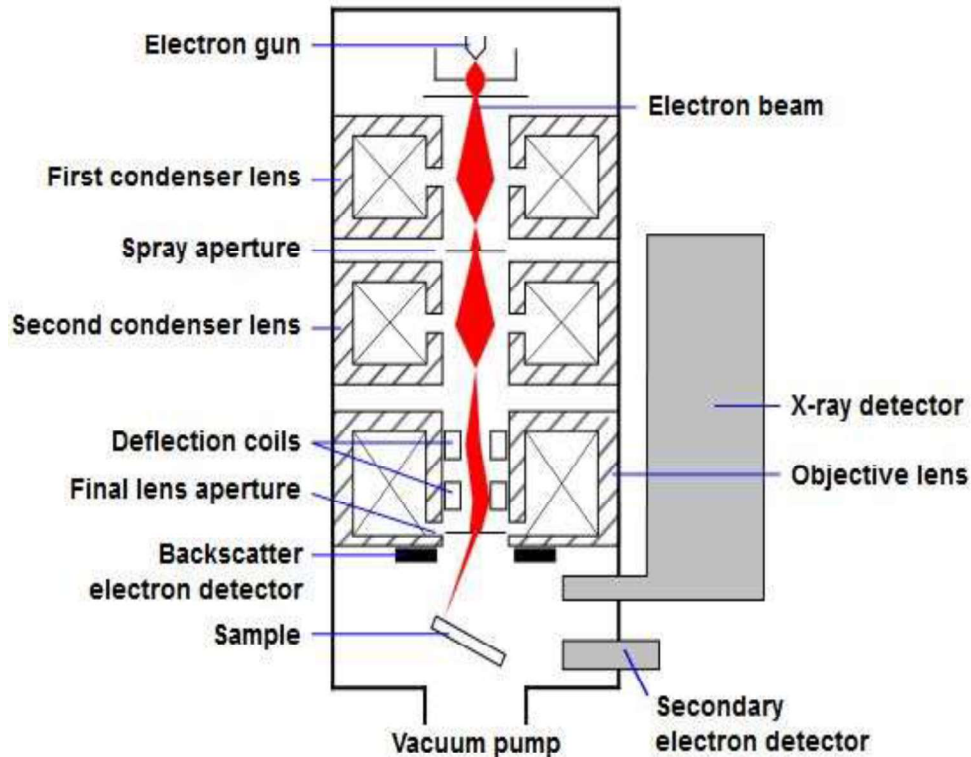


Fig. 3.8 Working principle of SEM [http://www4.nau.edu]

SEM uses a very fine probe of electrons with energies up to 30 or 40 keV passing through scanning coils focused at the surface of a specimen and scanned across it in a pattern of parallel lines. Once the beam hits the surface of the specimen, different types of signals are produced including; backscattered electrons, secondary electrons, characteristic X-rays. Fig.3.8 shows the working principle of SEM.

Secondary electrons are used for showing morphology and topography on samples, and backscattered electrons are used for illustrating contrasts in the composition having multiphase. The intensity of emission of both the secondary and backscattered electrons depends on the angle at which the electron beam strikes the specimen surface. The detector

collects these signals and converts them into a signal that is sent to a monitor screen which produces the final image.

For the microstructural study, all the heat treated samples were polished using emery papers of grade 1/0, 2/0, 3/0, and 4/0 (Sia, Switzerland) followed by polishing with a velvet cloth using a diamond paste of grade 1/4-OS-475 (HIFIN). Then these were etched chemically with diluted hydrofluoric acid (3 % concentration) for 20 sec and washed with double distilled water. Finally, they were dried, and gold sputtered. Micrographs were taken using INSPECT 50 FEI scanning electron microscope. Fig. 3.9 shows an image of INSPECT 50 FEI scanning electron microscope.



Fig. 3.9 Image of Inspect FEI SEM (<http://www.fei.com/products/sem/fei-sem>)

3.9.4 Flexural strength testing

Flexural strength is the force that a material can withstand before it breaks or yields.

Flexural testing is commonly used to determine the flexural strength and modulus of all

types of the materials. It is performed on a universal testing machine (UTM) with a 3 point or 4 points bending fixture. In the present work, flexural strength measurements of all the samples were performed according to ASTM C78/C78M. The specimens were tested in a three-point bending fixture with 20 mm span length between the two supports. The universal testing machine Instron, 3344 (Germany) was used for the measurement. The load and the corresponding deflection were recorded.

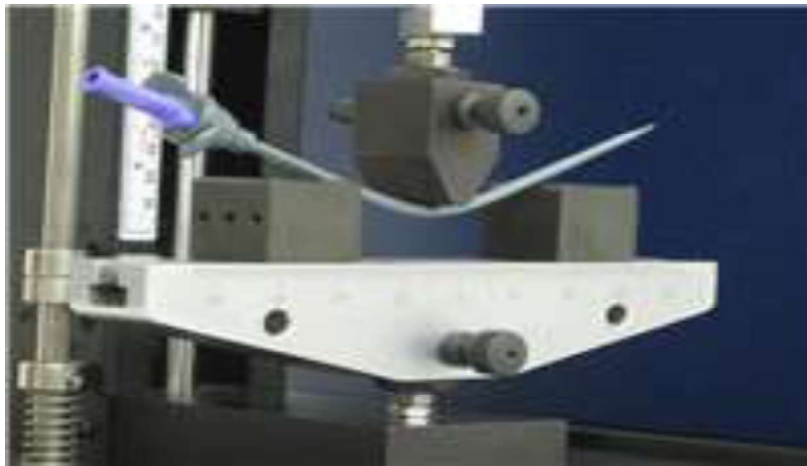


Fig. 3.10 Image of 3-point bend UTM [http://www.google.utm]

The flexural strength of rectangular bar was calculated using the following equation

$$\sigma = \frac{3FL}{2bd^2} \quad (3.4)$$

Where, σ is the fracture strength (kg/cm^2), F is the maximum applied load, L is the span length, b and d are the width and depth of the specimen respectively. Fig. 3.10 shows an image of the UTM.

The standard deviation, S of the flexural strength values is calculated using the following formula:

$$S = \sqrt{\frac{1}{N-1} \sum_{i=1}^N (x_i - \bar{x})^2} \quad (3.5)$$

where N is the number of samples, x_i is the value of one sample and \bar{x} is the mean value.

3.9.5 Weibull statistical analysis

Weibull statistical analysis is a common tool to interpret the strength data by a semi-empirical expression derived from Weibull's statistical theory of fracture as given below [Klein C.A. 2004]:

$$P(\sigma) = 1 - \exp\left[-\left(\frac{\sigma}{\sigma_N}\right)^m\right] \quad (3.6)$$

It describes the cumulative failure probability P as a function of the applied tensile stress σ . σ_N and m are the nominal strength and Weibull modulus respectively. These parameters are determined from a set of experimental data by fitting the estimated failure probability to Eq. (3.4). In the logarithm form Eq. (3.4) is written as:

$$\ln[-\ln(1 - P)] = m \log \sigma - m \log \sigma_N \quad (3.7)$$

Values of m and σ_N were determined from the slope and intercept $\ln[-\ln(1 - P)] = 0$ respectively by fitting the straight line given by Eqⁿ (3.6). Evidently, the strength does not take into account the potential impact of the test method (loading geometry and specimen size) and does not relate to the intrinsic strength in an obvious manner. The failure probability P_i was calculated in the following steps:

- (a) Rank by ascending order ($i=1\dots n$) the observed stresses at fracture and assign cumulative probabilities of failure according to $P_i = (i-0.5)/n$, where i is the rank, and n is the number of broken samples .
- (b) Fit the $\ln[-\ln(1 - P)]$ versus $\ln \sigma$ data points to a straight line using linear fit program.

3.9.6 Flame photometer for ion (alkali and alkaline earth ions) leachability test

Flame photometry is the branch of atomic spectroscopy used for determining the concentration of certain metal ions, e.g., Na^+ , Ca^+ , K^+ , Cs^+ , Li^+ . A flame photometer consists of a source of flame, mixing chamber, optical filter, and a photodetector. Fig.3.11 shows a schematic diagram of a flame photometer.

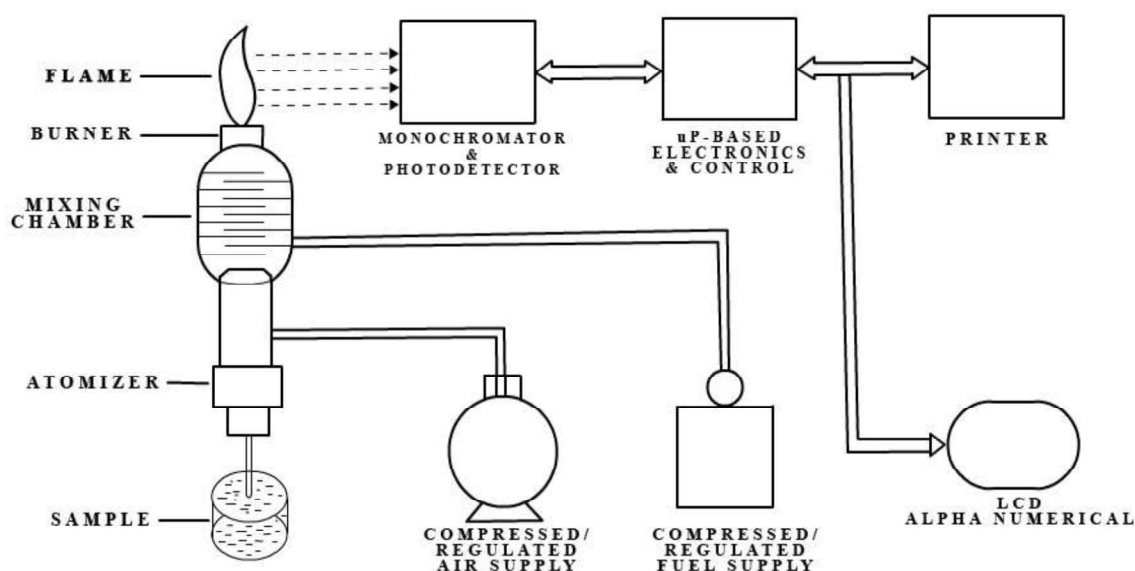


Fig. 3.11 A schematic representation of flame photometer
[<http://www.kaplasscientificsupplier>]

A burner provides the flame at a fixed temperature. Nebulizer transports the similar solution into the flame at a steady rate. The basic principle of flame photometer (Fig.3.12) is that the alkali and alkaline earth metal ions are dissociated due to the thermal energy coming from the flame, resulting in the excitation of atoms to the higher energy levels where they are not stable. By using absorption technique, absorption of light due to electrons excitation can be measured.

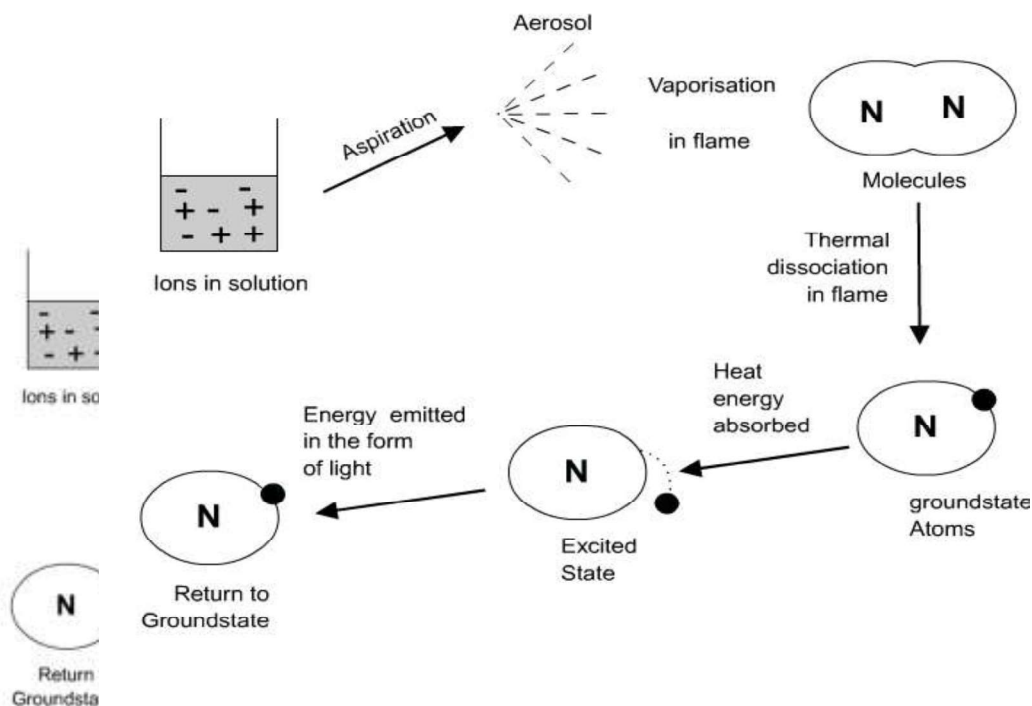


Fig. 3.12 Working principle of flame photometer [http://www.kaplasscientificsupplier]

The loss of energy will result in the atoms come to the ground state followed by emission of some radiations, can be visualized in the visible region. The wavelength of emitted radiation is the characteristics of the atoms.

For ion leachability test, the pellets of mechanochemically derived leucite and kalsilite samples were cleaned with distilled water in the ultrasonic bath and dried in the oven at 100 ± 2 °C for 3 hours. Subsequently, every sample was transferred to a plastic polypropylene (PP) flask with 20 ml of 4% acetic acid. Samples were immersed in the test solution. For each batch, five polyethylene bottles contained one composition of glass-ceramic pellet and 20ml of the acidic agents (pineapple and 4% acetic acid), and the top of the bottles was sealed with a screw cap. The chemical compositions of pineapple and acetic acid are given in Table.3.5. The storage temperatures were different of the various acidic

joints. The bottles were kept in an incubator at 80°C for 4% acetic acid for 168 hours as per ISO 6872.

Table.3.5 Chemical compositions of pineapple juice and acetic acid

| Acidic Agent | Type and form | Composition | Lot number | Manufacturer |
|--------------------------------|-------------------------------|---|------------|---------------------------------------|
| 4% Acetic acid (pH 2.48) | Diluted from 100% acetic acid | Assay (acidimetric), 99.5%; Water-0.5% Non-volatile matter-0.01% Chloride-0.0005% Sulphate (SO ₄)-0.0005% Formic acid (HCOOH)-0.1% Heavy metal (as Pb)-0.0001% Iron (Fe)-0.0001% | 0701 | Loba Chemie Pvt. Ltd (Mumbai, India). |
| 100% Pineapple juice (pH 4.40) | Instant | Pineapple juice 100 vol% | NB401 | Dabur Nepal Pvt. Ltd. |

These temperature and time are equivalent to 22 years of immersion in artificial saliva at 22 °C as reported in the literature [Napankangas R. and Raustia A. 2008, Milleding P. *et al.* 1999]. This immersion time is almost similar to the clinical service life of metal, ceramic restorations [Jaeggi T. and Lussi A. 2006]. For pineapple juice, the bottles were kept in an incubator at 35 °C for 168 hours. A protective clothing of disposable polyvinyl gloves was used to decrease the contamination of the specimens. After removal of pellets from their bottles, the fluids were examined for leaching of ions using microprocessor flame photometer as shown in Fig. 3.13 along with compressor unit. In addition to this, mean and standard deviation (SD) were also determined for the present experiments.

The leaching of ions from the samples was carried out one time per sample. The amounts leached were then associated with the leached area of the specimens, resulting in leaching

values expressed in $\mu\text{g}/\text{cm}^2$. The leached ions were sodium, potassium, and calcium. The leached specimens were coated with gold using the gold sputtering technique to study morphology.



Fig. 3.13 Image of a flame photometer [http://www.kaplasscientificsupplier]

Scanning electron microscope (INSPECT 50 FEI) was used to examine the surface morphology of leached specimens. The values of elemental compositions in storage agents have a significant role in the accuracy and exactness of analytical results.

3.9.7 Fourier transforms infrared spectroscopy (FTIR)

FTIR is a spectroscopic technique used to identify organic molecular groups, functional groups, and compounds present in the sample, all of which have characteristic vibrational frequencies in the infrared range. The basic principle of FTIR is that molecules absorb light in the infrared region of the electromagnetic spectrum. The absorption specifically ascribed to the presence of chemical bonds and functional groups in the sample. The frequency range is measured as wave numbers typically over the range $4000 - 600 \text{ cm}^{-1}$.



Fig. 3.14 Image of FTIR [<http://www.wikiwand.com>]

To confirm the presence of phosphate group (PO_4^{-3}) in the glass ceramic composites, FTIR spectrum were recorded. For this measurement, the samples were prepared in the form of a disc by mixing KBr powder in the ratio 1:100. The mixtures were subjected to a load of 10 tons/cm² in a revocable die using a hydraulic press. The measurements were measured at room temperature in the frequency range 4000–400 cm⁻¹ using a Fourier transform infrared spectrometer (VARIAN scimitar 1000, USA) (Fig.3.14). The test results were compared with functional groups.

3.9.8 Bioactivity

The hydroxyapatite (HAp) forming ability of the coated specimens was observed through immersion in simulated body fluid (SBF) solution for several times. SBF solution was prepared by dissolving AR grade NaCl, KCl, NaHCO₃, MgCl₂, 6H₂O, CaCl₂ and KH₂PO₄ (Loba Chemie Pvt. Ltd., Mumbai, India) in double distilled water and buffered at pH 7.25 with tris hydroxymethyl aminomethane (TRIS) and 1N HCl at 35–37 °C. Its composition was maintained equivalent to the ionic concentration of human blood plasma [Kokubo T. and Takadama H. 2006].

For these tests, specimens of dimension 10 mm diameter and 2 mm thickness were prepared and immersed in SBF (40 ml) up to 21 days. The SBF solution was replaced every three days since there is a decrease in cation concentration due to the changes in the chemistry of the samples. At the end of each selected time, samples were removed from the SBF solution, rinsed with distilled water, dried and stored in airtight containers for further investigation. SBF treated samples were examined by SEM and FTIR to assess the possible formation of HAp layer on the material surface, as a marker of bioactive behavior.

3.9.9 Cell lines and cell culture

Human buccal epithelial cell line SCC-25 was originally obtained from American Type Culture Collection (ATCC), Manassas, USA. The cells were maintained in RPMI 1640 (Invitrogen, Carlsbad, CA), supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen, Carlsbad, CA), henceforth, called as complete medium. The cell line used in the study was free from mycoplasma.

3.9.10 Cell viability assay

Effect of leucite and kalsilite glass–ceramic composites (MCL-0, MCL-C, MCK-0, MCK-M and STD) on the feasibility of SCC-25 tumor cells was evaluated by a colorimetric XTT (sodium 3-[1-(phenylamino carbonyl)-3, 4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate) assay (Roche Molecular Biochemicals, Indianapolis, IN). Tumor cells were plated (5×10^3 cells/well) in a 96-well plate and were exposed to varying concentrations (1, 5, 10, 25 and 50 mg/ml) of different composite materials (MCL 0, MCL C, MCK 0, MCK M and STD) and incubated at 37°C, 5% CO₂, for 18 hours (h). OD was

measured at 450 nm using Synergy HT Multi-Mode Microplate Reader BioTek, USA. The data was presented as the percentage of viable cell calculated from the following formula:

$$\% \text{ Cell Viability} = \frac{\text{Experimental OD}_{450}}{\text{Control OD}_{450}} \times 100 \quad (3.8)$$

3.9.11 Cell proliferation assay

Effect of kalsilite glass ceramic materials ($d < 25 \mu$) on proliferation of SCC-25 cells was studied by MTT assay, 5×10^3 SCC-25 cells/well were added in a 96-well tissue culture plate, and exposed to serial concentrations of (5, 10, 25, 50, 100, 250 and 500 $\mu\text{g/ml}$) kalsilite glass ceramic materials. Plates were incubated at 37°C , 5% CO_2 , for 48 hours (h). The cell proliferation was measured by CellTiter 96® Non-Radioactive Cell Proliferation Assay (MTT) kit from Promega, USA according to the manufacturer's protocol. The plates were incubated for 4h with the MTT reagent and absorbance was measured at 570 nm using Synergy HT Multi-Mode Microplate Reader BioTek®, USA. The data presented as the percentage of inhibition of tumor cells and was calculated from the following formula:

$$\% \text{ Growth Inhibition} = \left[1 - \frac{\text{Experimental OD}_{570}}{\text{Target OD}_{570}}\right] \times 100 \quad (3.9)$$

where Experimental OD is the reading of tumor cells exposed to various concentrations of Kalsilite glass-ceramic materials, and Target OD is the corresponding value of tumor cell only cultured in medium only.

3.9.12 In-vitro cytotoxicity assay

The lytic activity of leucite and kalsilite glass–ceramic composites against tumor cells was measured by non-radioactive cytotoxicity assay using the CytoTox 96 Non-Radioactive Cytotoxicity Assay kit from Promega, USA. Target cells (5×10^3) were added to 96-well tissue culture plates and were exposed to serial concentrations of (10, 20, 30, 40, and 50

mg/ml) leucite and kalsilite glass–ceramic composites and incubated for 18h at 37 °C, 5% CO₂. Percent specific lysis was determined using the following formula:

$$\%Cytotoxicity = \frac{Experimental-Effector\ Spontaneous-Target\ Spontaneous}{Target\ Maximum-Target\ Spontaneous} \times 100 \quad (3.10)$$

3.9.13 Detection of apoptosis

Apoptotic cell death in SCC-25 cells by Kalsilite glass ceramic materials (100 µg/ml) was assessed by binding of FITC-conjugated Annexin V. After 18h of incubation; apoptotic cells were analyzed by staining with FITC-conjugated Annexin V and propidium iodide (PI) for 20 minutes in ice-cold PBS. Cells were washed in Annexin buffer and were mounted on microscope slides with a drop of mounting medium to reduce fluorescence photobleaching. The FITC-conjugated Annexin V positive cells were visualized under a fluorescence microscope (Nikon Eclipse 80i, Nikon, Japan).

3.9.14 Hemolysis assay

For time-dependent kinetics, a concentration of 100 µg/ml Kalsilite glass ceramic materials was incubated with the blood sample. For concentration-dependent kinetics, the blood sample was incubated with varying concentrations (25, 100, and 150 µg/ml) of Kalsilite glass ceramic materials for 4h. Hemolysis assay was performed according to the standard protocol (3). In brief, an aliquot of each blood sample was centrifuged at 600 g for 5 minutes. 25 µl plasma aliquot was diluted with 225 µl Drabkin's reagent (Sigma) in a 96-well plate and mixed for 2 minutes under lateral agitation (300 rpm). After 10 minutes equilibration at room temperature, optical density was recorded at 540 nm in Synergy HT Multi-Mode Microplate Reader BioTek, USA. Blood hemoglobin was determined by measuring the absorbance of a 100-fold dilution of the whole blood in Drabkin's reagent at 540 nm. Saponin (2mg/ml final blood concentration) and PBS were used as positive and

negative control respectively. A sample of plasma without additives was considered as basal conditions. A standard calibration curve was obtained with the solutions containing 0.07 to 3.8 mg/ml bovine hemoglobin (Sigma) treated with Drabkin's reagent. The results are presented as percent hemolysis indicating the free plasma hemoglobin (mg/ml) and were measured as released hemoglobin divided by the total blood hemoglobin (mg/ml) multiplied by 100. All measurements were performed in triplicate.

3.9.15 SEM evaluation of cell morphology

Three sintered pellets of each leucite and kalsilite glass–ceramic composites were used to assess cells morphology on their surface at different culture periods. SCC-25 cells were cultured up to 10 days to evaluate asset the cell's response on the surface of the material following extended interaction time. The composite blocks with cells were fixed in 2.5% (v/v) glutaraldehyde in 0.14M sodium cacodylate buffer (pH 7.3) (both Sigma–Aldrich) at 4 °C, dehydrated in a graded series of alcohols (50%, 70%, 90%, and two changes of 100% ethanol). The blocks were treated with hexamethyldisilazane (TAAB Laboratories, UK) for 1–2 minutes and placed in a desiccator to dry overnight. After 24h, the samples were mounted on aluminum stubs, sputter-coated with gold/palladium using a Polaron E5100 coating device (Polaron CVT, Milton Keynes, UK) and observed using a Cambridge Stereoscan S90B SEM (Cambridge Instruments, Crawley, UK) at 15 kV. Representative samples (n = 3) of each of the surfaces were used for observation and recording of images.

3.9.16 Statistical analysis

In this study, n reflects the number of times experiments were performed independently in triplicate. The mean \pm SD were calculated for each experimental group (n=3-4). Differences between groups were analyzed by unpaired Student's t-test and one- or two-

way ANOVA analysis of variance depending on the requirement. One or two-way ANOVA followed by Holm-Sidak post-hoc multiple comparison tests was used to conduct pairwise comparisons using PRISM statistical analysis software (Graph Pad Software, Inc., San Diego, CA, USA). Significant differences among groups were calculated at $P < 0.05$ or less (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ in control versus experimental group).

3.9.17 Bulk density (BD) and Apparent porosity (AP) The bulk density and apparent porosity of the heat treated specimens were determined according to standard ASTM C20-00 test method. The AP and BD were calculated using the following formulas:

$$BD = \frac{D}{W-S} \quad (3.11)$$

where, BD is the bulk density (gm/cm^2), D is the dry weight of the specimen, W is the soaked weight after boiling the samples for 2hrs in de-ionized water, and S is the suspended weight.

$$AP = \frac{W-D}{W-S} \times 100 \quad (3.12)$$

where AP is the Apparent Porosity (%), D is the dry weight of the specimen, W is the soaked weight after boiling the samples for 2 hrs in de-ionized water, and S is the suspended weight.