Chapter 3

MECHANICAL PROPERTIES AND BIOCOMPATIBILITY OF CERIUM AND LANTHANUM SUBSTITUTED BIOACTIVE GLASSES

3.1 Introduction

Rare earth elements possess superior physicochemical properties and they are widely applied for biochemical and agronomic fields. It has been reported that lanthanum possesses antibacterial effects and cellular immunity [G. Fei et al.2007]. Bioactive materials are capable of stimulate a biological response and mediate binding to host tissue (soft and hard). The bioglass® and its derivatives have used as implant materials for medical applications. The primary bioglass® has 45 % SiO_2 as network former, 24.5% Na_2O as network modifiers, 24.5% CaO as network intermediate and 6% P_2O_5 as network former has been investigated [L.L. Hench. 2006]. The developed first-generation 45S5 based bioglass® is widely accepted as the substitute for tissue regeneration and was irrevocably distorted when a particular form of 45S5 bioglass® were made by Hench et al at the University of Florida [C.A. Beckham, 1971; Jr. T. K Greenlee et al. 1972]. A significant shortcoming of 45S5 bioactive glass was its slow degradation rate and not found satisfactory for load-bearing applications [V. Aina, et al. 2009]. That's why 45S5 bioactive glass was modified through doping of suitable metallic and non-metallic oxides over the last four decades such as lithium, titanium, potassium, copper, zinc, manganese, iron, magnesium, cobalt, nickel, strontium, neodymium and gadolinium to modify their physical and biological response have

been studied by many researchers [A. Ali, et al. 2018; A.M. Deliormanli, et al. 2015].

In the past, metallic materials were widely used for various medical applications such as bone and joint replacement, but there were some issues associated with it due to corrosive nature of materials. After 1970, small size (50 microns) ceramics materials such as ZrO_2, Al_2O_3, etc., were used because of their better biocompatibility and excellent mechanical properties [M. Sherief, et al. 2016; F.H. ElBatal, et al. 2008]. Although, materials do not form strong bond with bones and it looses over a period of time after implantation [A. Vazquez, et al. 1997; J.L. Rygel, et al. 2009]. In recent biological application, bioglass® is suitable for bone as well as teeth implant for the human body and provided better biocompatible than the other materials. Previously, metallic oxides are tightly integrated to the bone by physicochemical bonding between elements to bones.

The present work deals with the synthesis of cerium and lanthanum substituted bioglass® for biological implantation. Here we studied the outcomes of CeO_2+La_2O_3 (equal wt %) substitution for thermal, biological and mechanical properties in the 45S5 bioactive glass. The CeO_2 is used for dental ceramics for the implanting teeth [C. Leonelli, et al. 2003]. Cerium also recognized to hold their bacteriostatic properties with low toxicity [S. Rajeshkumar, et al. 2018; N. Badri, et al. 2017]. It was further found that an increase in Ce^{4+} ions concentration leads to change in color of transparent glass to yellow-gold color bioglass®. The La^{3+} is also an element used for health applications with bioglasses [A. Benedetto, et al. 2018; G. Partridge, et al 1974]. We should not forget that the substitution of lanthanum or cerium associated with phosphorus (P) to form an insoluble phosphate salt in lysosomes., but the direct demonstration of that turned into not

viable using conventional Transmission Electron Microscopy, as long as this method had the difficulty to provide no greater than an ultra-structural analysis [N. Badri et al. 2017]. After SBF treatment, it was observed that formation of HCA layer on the surfaces of the bioglass found using FTIR. The morphology of the samples was found by using SEM, and microhardness and flexural strength were investigated by digital hardness tester and UTM respectively. It was a significant achievement to the better mechanical strength of bioglass® by replacement of these rare earth oxides, and this is favored by formation ultrafine and regular grained microstructure [E. Willbold, et al. 2015].

3.2 Materials and methods

In the present study, bioactive glasses of general composition formula (45-X) SiO_2, 24.5 Na_2O, 24.5 CaO and 6 P_2O_5 (wt %) where X= (0.0, 0.5, 1.0, 1.5, & 2.0) (wt %) equal amount of CeO_2 and La_2O_3. The wt% concentration and the equal ratio of CeO_2 / La_2O_3 substituted bioglass® (CeLa1-CeLa4) compositions are presented in Table 3.1. Fine quartz with high purity (>99.5%) AR grade has been used as a source of SiO_2. The AR grades of $CaCO_3$, Na_2CO_3, and $(NH_4)H_2PO_4$, were used as a source of CaO, Na_2O, and P_2O_5, respectively. In the present study SiO_2 is replaced by the equivalent amount of both rare earth oxides in the batch as given in Table 3.1. The all five batches were weighted and mixed for 40 min in the ball milling machine at 200 rpm and the obtained homogeneous powder containing fine particles (< 60μm) were melted in a 100 ml alumina crucible (purity >99.9%) in globar furnace at 1400±2 ºC with air as furnace environment. The temperature of furnace was controlled by automatic PID controller within ± 2 ºC for the all-glass batches heating from 25 ºC to 1000 ºC is carried out at the rate of 10 ºC min^{-1}. Further, the samples were held at 1000 ºC for 1 hour and heated

from 1000 ºC to 1400 ºC at the rate of 10 ºC min^{-1} after two hours of melting glass at 1400±2 ºC, the molten glass was poured into the preheated aluminum mould and transferred directly to muffle furnace at 560 ºC for annealing. After, 4 hours inside muffle furnace, the furnace was switched off and transparent glass was taken out from the furnace at normal temperature. Prepared bioglass® samples were further characterized for in vitro bioactivity and physico-mechanical properties. Microstructures of developed glass were evaluated by SEM. Bioactivity of glass samples were assessed in simulated body fluid (SBF) for different time intervals and it was evaluated using XRD, FTIR, and SEM. Furthermore, with the substitution of CeO_2 and La_2O_3 in the base glass enhances its cytocompatibility and cell supportive properties. Also, micro-hardness and flexural strength of base bioglass® was enhanced with the incorporation of CeO_2 and La_2O_3, Density of glass also increases with increasing concentration of CeO_2 and La_2O_3. Thus, the developed CeO_2 and La_2O_3 incorporated bioactive glasses have superior mechanical properties which make these bioactive glasses a suitable candidate for bone implant application. Some cutting parts of the glass was used for making the powder with the help of planetary ball milling machine in order to use for XRD, FTIR, DTA and biocompatibility assessment. Some fine powder has been used to make the pellet for SEM, microhardness and flexural strength measurement. Some of the glass has cut into a square shape and further heat-treated for control crystallization to make glass-ceramic.

Table 3.1: ***Weight % composition of the CeO_2 –La_2O_3 substituted bioactive glasses.***

Sample Id	Wt%					
	SiO_2	Na_2O	CaO	P_2O_5	CeO_2	La_2O_3
BG	45	24.5	24.5	6	0.0	0.0
CeLa1	44	24.5	24.5	6	0.5	0.5
CeLa2	43	24.5	24.5	6	1.0	1.0
CeLa3	42	24.5	24.5	6	1.5	1.5
CeLa4	41	24.5	24.5	6	2.0	2.0

3.3 Results and discussion

3.3.1 Differential thermal analysis

The thermal analysis curves of the bioactive glasses are shown in Figure 3.1 and the results dictated that the incorporation of Ce^{4+} and La^{3+} ions in the 45S5 bioactive glass reduced both the nucleation temperature from 602 to 598 °C and the crystallization temperature from 766 to 635 °C. Further, it was observed that the higher amount of modifiers in the composition has facilitated in lowering T_g point and the viscosity of the glass melt. On substitution of CeO_2-La_2O_3 for SiO_2 content in the composition caused the shift of exothermic peaks to lower temperatures as compared to that of the base glass.

Therefore, a lower energy is required to promote crystallization in the glass. This may be attributed due to the presence of larger cations in the system which increased interference in glass network. The modifiers occupied the interstitial position in the glass structure and thereby reduced oxygen bond strength. The process of heat treatment, the concentration of rare earth ions in the glassy system has allowed an early nucleation by the nucleating agents like P_2O_5 in the glass

composition. Thus the glass network becomes weaker and, T_g and T_c decreased [Azevedo et al. 2012].The differential scanning calorimetry (DSC) performed by the authors for their non-charge balance (NonCB) bioactive glass samples had also shown that the glass transition temperature (Tg) decreased with substitution of CeO_2 and La_2O_3upto 2.0 wt% in a concentration dependent manner .

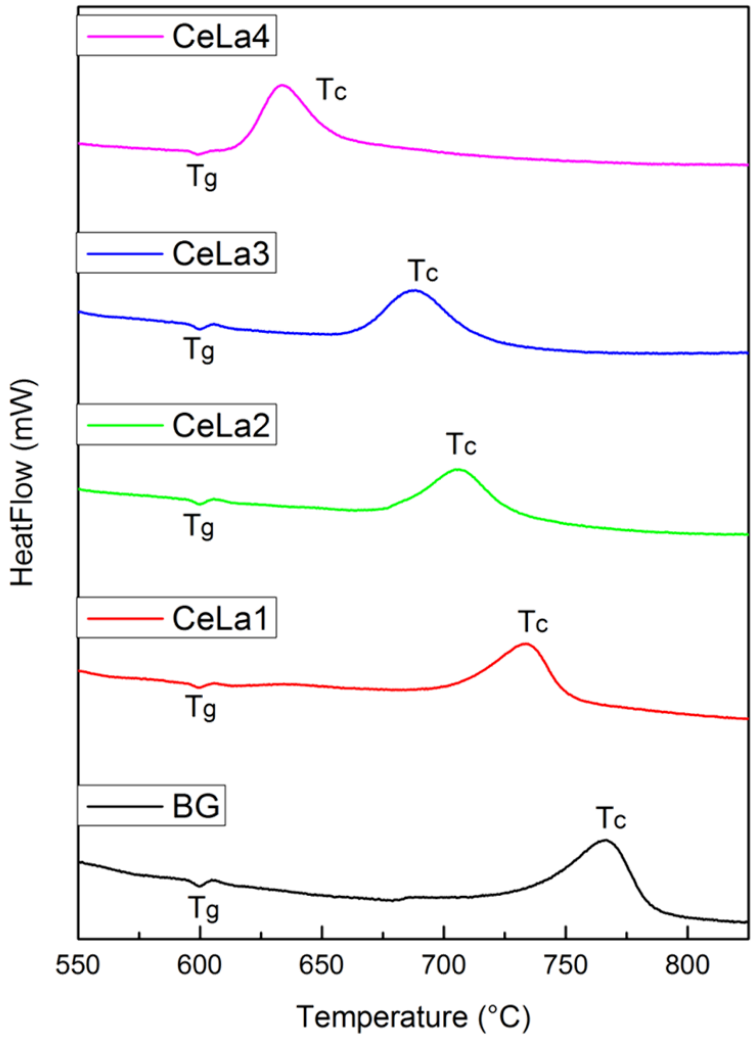

Figure 3.1: DTA Curve 45S5 bioactive glass CeO_2-La_2O_3 substituted bioactive glass

3.3.2 XRD and FTIR

The XRD spectra of the powdered glass samples (SiO_2-Na_2O-CaO-P_2O_5-CeO_2-La_2O_3) show the absence of any peak in Figure 3.2(a), which indicate the amorphous glassy structure of the melted samples. After the control crystallization

two major peaks have been found $Na_2Ca_2Si_3O_9$ and $Ca(PO_3)_2$ which is dipicted in Figure 3.2(b).

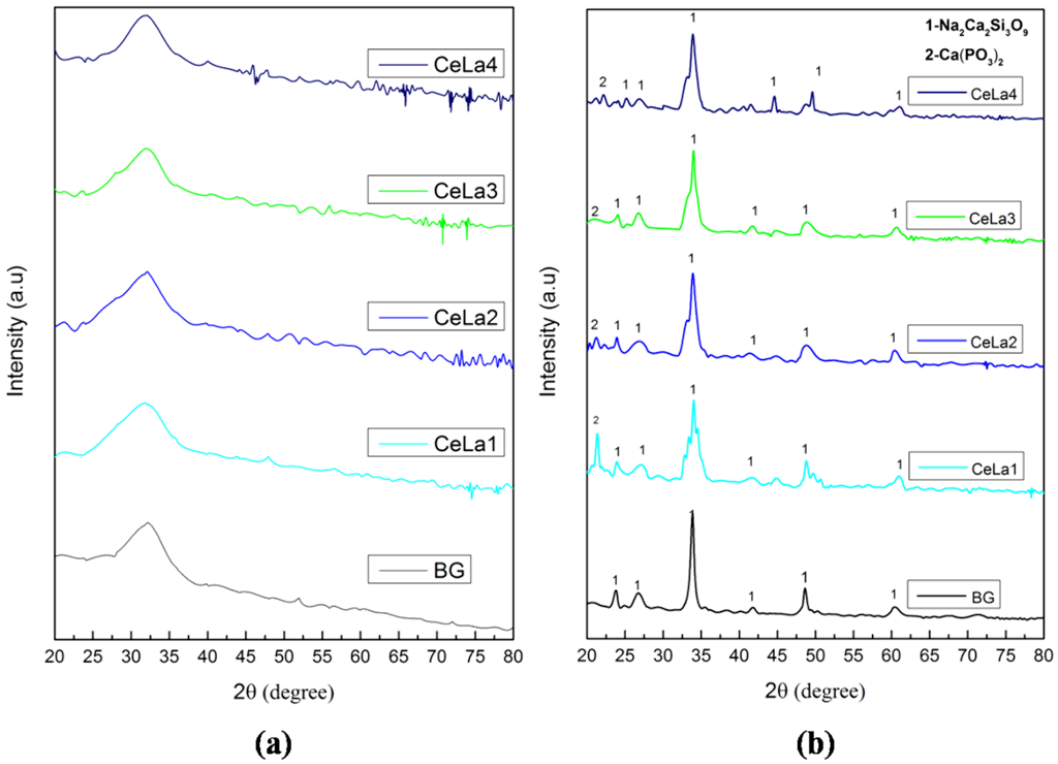

Figure 3.2: XRD image of CeO_2-La_2O_3 substituted, (a) Bioactive glass & (b) Glass-ceramics.

FTIR spectra of CeO_2-La_2O_3 substituted bioactive glasses before and after immersion in SBF solution for different time periods such as 1, 4, 8, 13, and 19 days are shown in Figure 3.3 (a) & 3.3 (b). In general, it is well known that an increase in the intensity of absorption bands indicates an increase in the molecular concentration of species formed at the surface of the bioactive glass treated with SBF solution progressively with rising soaking time. The present results obtained by FTIR spectrometry were a good agreement with the earlier studies made by previous workers [S.M. Salman, et al.2012; O.P. Filho, et al. 1996]. Figure 3.3 (b), shows the infrared spectral bands of CeLa4 sample after immersion in SBF in 13 days. The new bands were found to appear after 13-day immersion in SBF at around 803 cm^{-1} which corresponds to Si-O-Si bending mode of vibration due to

the formation of SiO_6octahedra in the SBF treated sample. The bands corresponding to the frequencies of 1404 and 1539 cm^{-1} are associated with C–O (carbonate) stretching mode and a minor peak at around 3064 cm^{-1} is due to the presence of O–H (hydroxyl) groups on the surface of the sample. The fluctuation bands at approximately 900 cm^{-1} are attributed to the PO_4 (P-O stretching) groups. It was further observed that all the samples were observed to exhibit similar characteristics bands. The prolonged period of soaking of the sample in SBF exhibits the same behavior favorably due to the development of hydroxyl carbonate apatite (HCA) layer [M.R.T. Filgueiras, et al. 1993; I. Rehman et al. 2000].

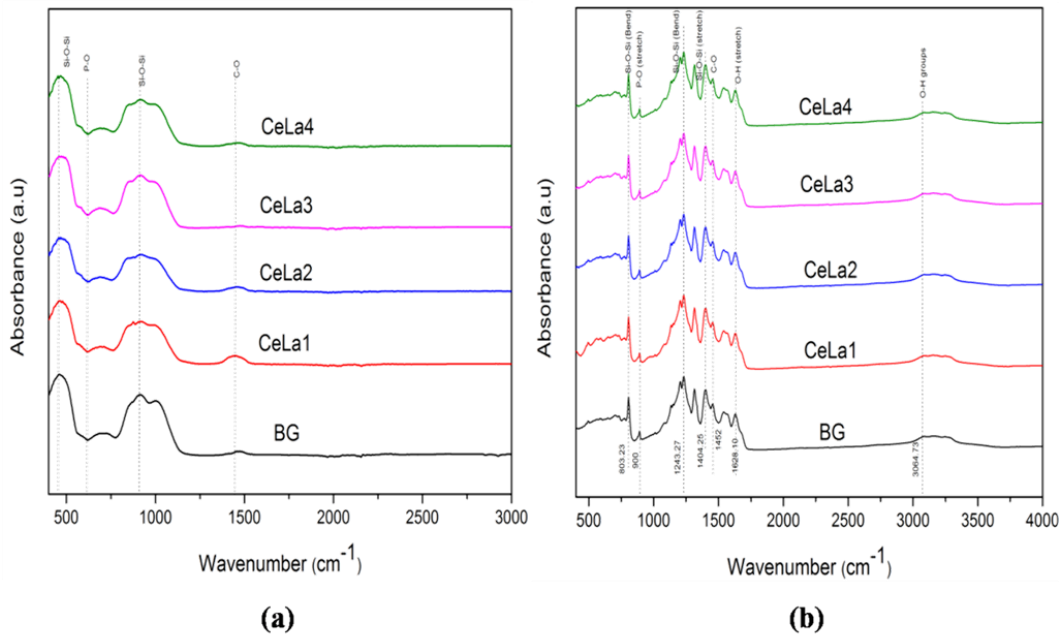

Figure 3.3: FTIR image of CeO_2-La_2O_3 substituted bioactive glass, (a) Before SBF & (b) After 13 days in SBF.

3.3.3 Surface morphology evaluation by SEM

Surface properties of the base and substituted bioactive glass were analyzed before and after immersion in SBF solution by Scanning Electron Microscopy (SEM). The glass pellet was gold coated by sputtering machine before SEM (Inspect 50 FEI) analysis. When the bio-glass sample was immersed in SBF

solution for a particular time interval, then hydroxycarbonate apatite (HCA) layer was found on the surface of bioactive glass samples. The SEM images of bioactive glass samples prior of SBF soaking (Figure. 3.4) indicates the formation of different cylindrical rod type structures and irregular grains of bioactive glass samples similar to the results observed by previous researches [H.H. Beherei et al. 2009].

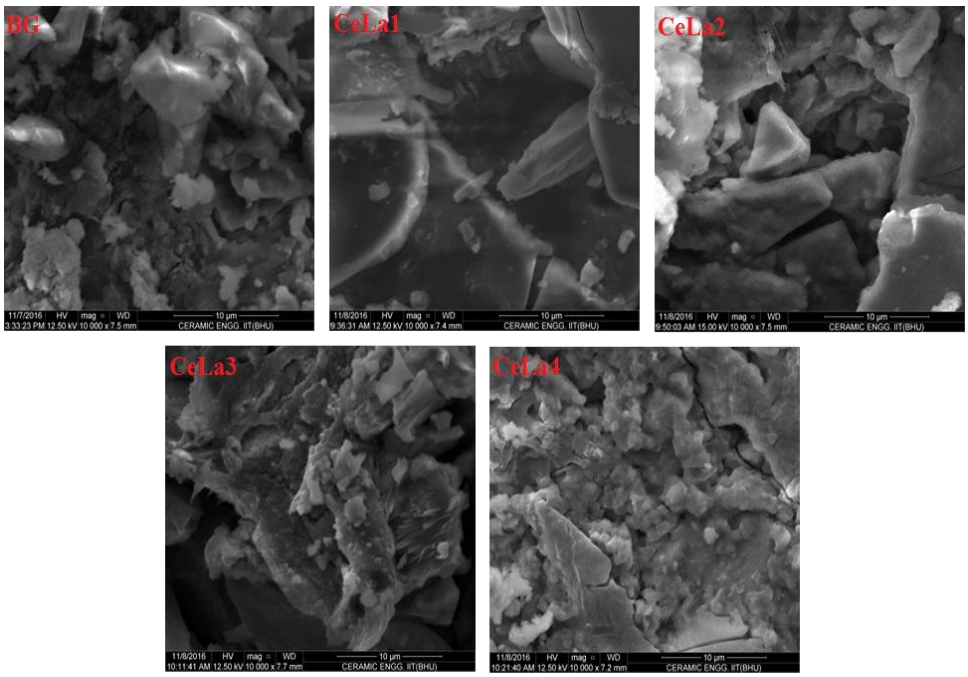

Figure 3.4: SEM image of CeO_2-La_2O_3 substituted bioactive glass before SBF treatment.

Figure 3.5 shows the SEM micrographs of bioactive glass samples after soaking in SBF solution for 19 days. It was observed from the image that bioactive glass samples were uniformly covered with the irregular shape of HCA particles and their agglomerates. On SBF treatment HCA clusters change in a finer structure after 19 days of soaking due to partial dissolution and re-precipitation phenomena in solution. Such activity was due to the refreshing of SBF solution and signifying the formation of a continuous layer of HCA. After comparing these micrographs it was concluded that all the prepared CeO_2& La_2O_3 doped bioglass® promotes the formation of HCA on the surface after immersion in SBF solution for 19 days

[S.K. Arepalli et al. 2016]. Formation of the HCA layer was also confirmed by EDX analysis as shown in Figure 3.5.

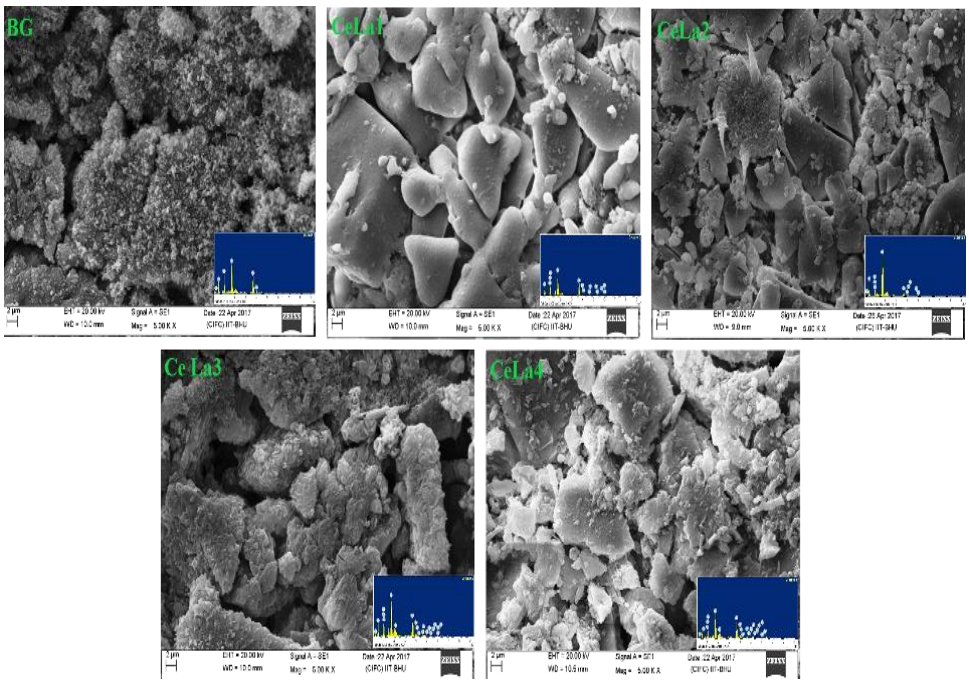

Figure 3.5: SEM image of CeO_2-La_2O_3 substituted bioactive glass after 19 days in SBF.

3.3.4 Mechanical properties evaluation

Figure 3.6 shows that the density of the glass prepared from melting root. The density of cerium and lanthanum substituted glass (0.5, 1.0, 1.5 and 2.0 wt %) were measured by Archimedes's principle and found to be 2.702, 2.729, 2.744 and 2.770 gm/cm^3 respectively. Substitution of cerium and lanthanum for silica increases in density due to the replacement of lighter atoms (Si - 2.65 gm/cm^3) by heavier one (Ce - 6.76 gm/cm^3) and (La - 6.15 gm/cm^3. On the other hand, due to the smaller size of lanthanum ions (ionic radius~1.061 Å) and cerium ions (ionic radius~1.034 Å), it adopted the similar structure and as replacement of the Ce^{4+}/La^{3+} ions, it leads to increase the density of the glass [V.K Vyas et al. 2016; A. Ali et al. 2018].

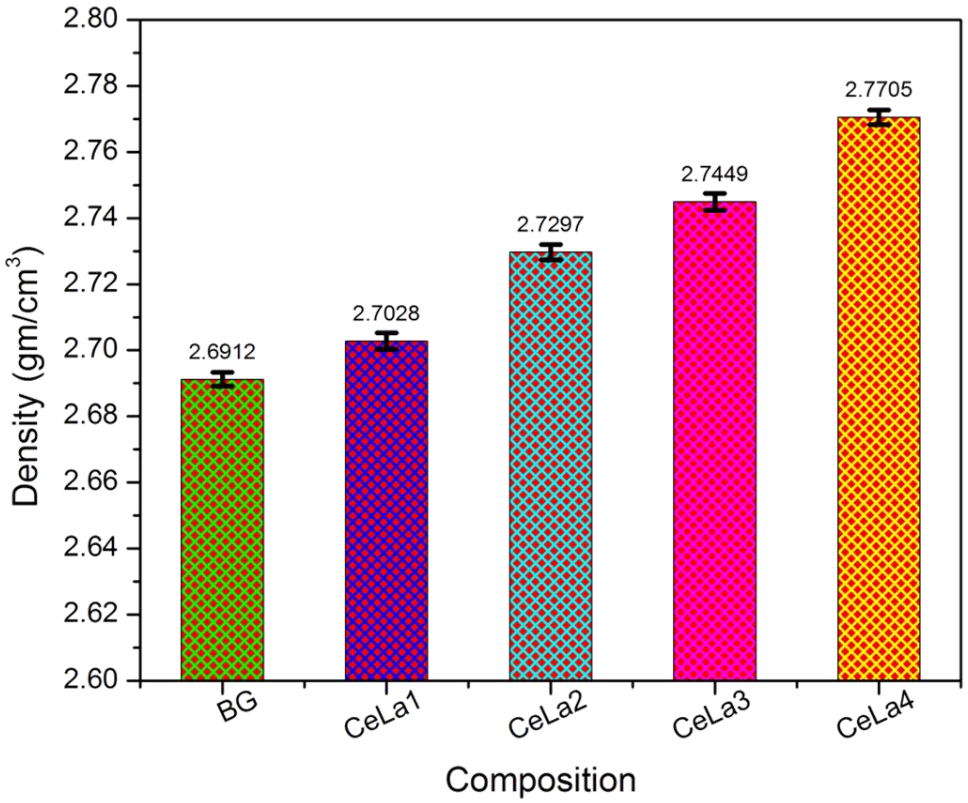

Figure 3.6: Density of CeO_2-La_2O_3 substituted bioactive glass samples.

Table 3.2: ***Density (gm/cm^3) of CeO_2-La_2O_3 substituted bioglass.***

Sample	BG	Cela1	Cela2	Cela3	Cela4
Density(gm/cm^3)	2.691	2.702	2.729	2.744	2.770

Microhardness and flexural strength of base glass sample is 5.92 GPa and 46.87 MPa respectively while the microhardness of substituted bioglass® are (CeLa1, CeLa2, CeLa3, and CeLa4 are 5.97, 6.09, 6.41 and 6.67 GPa) and flexural strength of substituted bioglass® are (51.11, 57.33, 64.67 and 69.12 MPa) as presented in Figure 3.7. It was observed that with increasing concentration of CeO_2& La_2O_3, microhardness and flexural strength of substituted bioglass® increased due to the compactness of glass structure.

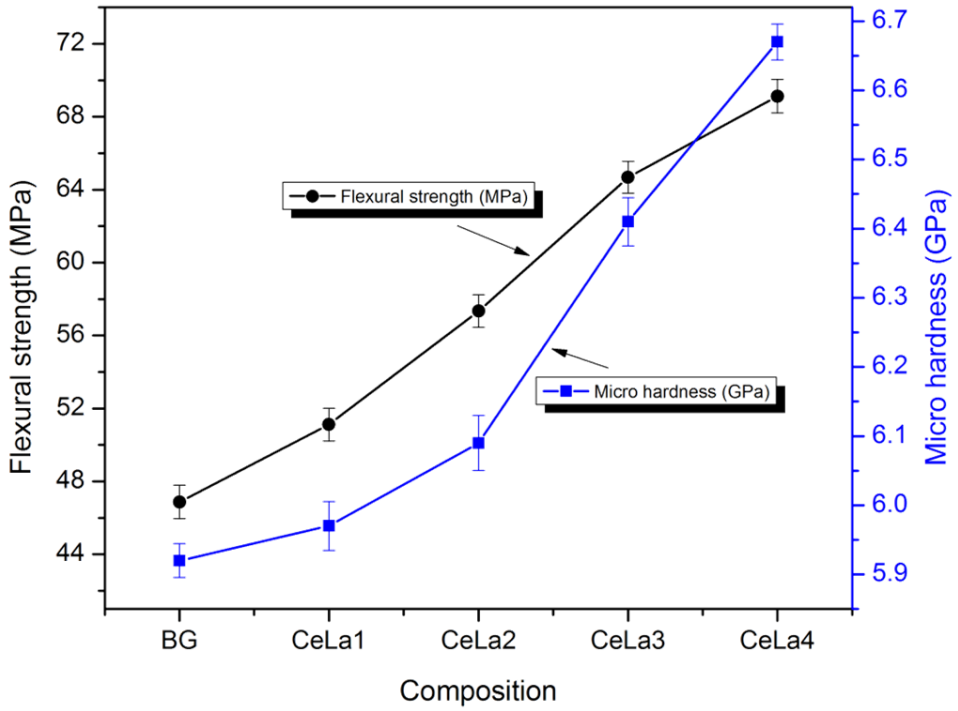

Figure 3.7: Flexural strength and Micro hardness of base bioactive glass andCeO_2-La_2O_3 substituted bioactive glass.

Table 3.3: ***Corresponding values of Micro hardness (GPa) and Flexural strength (MPa) of CeO_2-La_2O_3substituted bioactive glass.***

Samples	45S5 (BG)	CeLa1	CeLa2	CeLa3	CeLa4
Micro hardness (GPa)	5.99	6.17	6.23	6.39	6.61
Flexural strength (MPa)	56.87	61.11	67.34	74.68	89.13

3.4 Assessment of biocompatibility

3.4.1 Hemolysis assay, RBC integrity, and size distribution

The results of the hemolysis assay exhibited the effectiveness and compatibility of glass compositions with blood are shown in Figure. 3.8(a). The rare earth ion substituted bioactive glass samples did not affect the hemolysis of RBCs at lower concentrations, and these results are quite well within the limit of 2 wt % [S.K. Mahto et al. 2015]. However, it was found that the percentage of hemolysis was

increased considerably with increasing concentrations of the samples. Interestingly it was further found that with increasing concentration of rare earth oxides, and the level of hemolysis decreased and hence here it was more biocompatible. For higher levels (250 and 500mg/ml), CeLa1 caused partial hemolysis, whereas the CeLa2, CeLa3, and CeLa4 samples have revealed better blood compatibility.

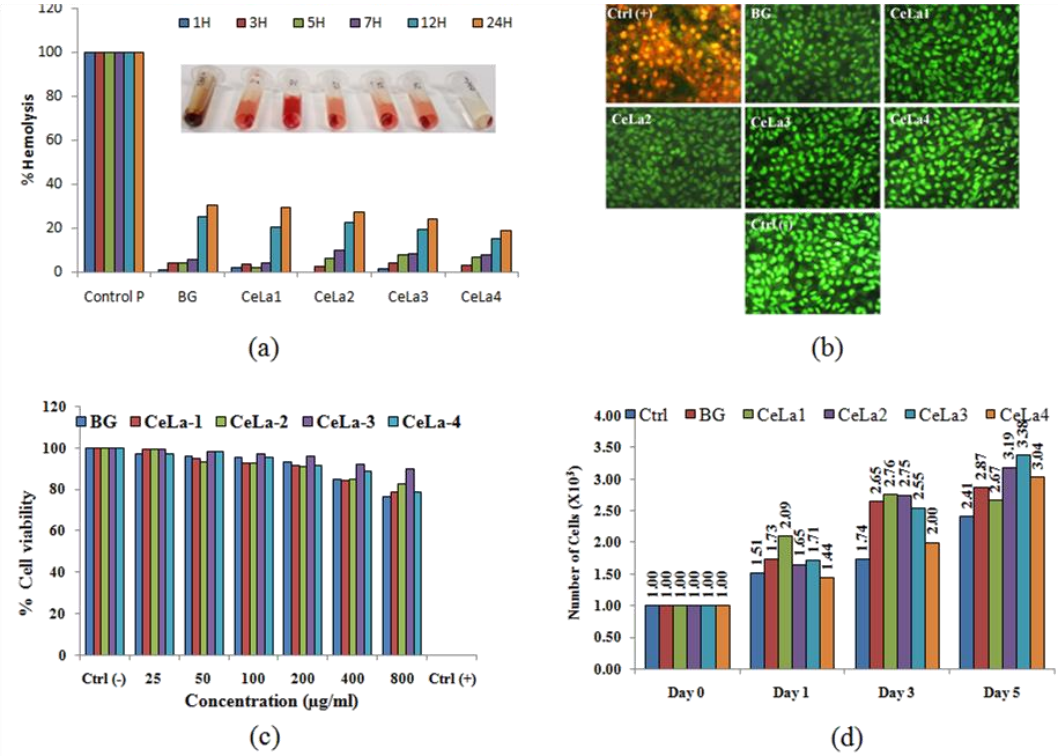

Figure 3.8 (a) Hemolysis assay **(b)** Fluorescent microscopic images for cell apoptosis **(c)** Cell Viability **(d)** Cell proliferation of CeO_2 - La_2O_3 substituted bioactive glass samples respectively

3.4.2 Cell viability

Generally, most of the biomaterials at lower concentrations can exhibit biocompatibility during in vitro cell culture studies while at higher concentration they showed cytotoxicity. Cell viability of the CeO_2 + La_2O_3 containing samples along with a base glass (BG, CeLa1, CeLa2, CeLa3, and CeLa4) were assessed against mouse fibroblast, L929 cell lines by MTT assay. Figure 3.8(c) shows the

percentage cell viability with different concentrations of the samples incubated for 48 h at 37 °C in 5% CO_2. The cell viability was calculated by considering the viability of the cells without treatment of any materials is 100 %. The cell viability after 48 hr exposure to the sample (800 µg/ml) was recorded as 76.66%, 78.94%, 82.80%, 89.78% and 78.89% for BG, CeLa1, CeLa2, CeLa3 and CeLa4, respectively. This study showed all materials were cytocompatible as even at high concentration, the bioglass® did not affect cell viability significantly. Among all five samples including base glass, CeLa3 was the most compatible material which was also showed best mechanical strength as compared to all others. This enhancement might be associated with the controlled release of ions from the glass surface, which played an important role in cell survival [S.M. Salman, et al. 2012].

The glasses substituted with the rare earth elements were further studied for biological assessment. These findings are in good agreement to the earlier reports that the rare earth element substituted bioactive glasses do not affect the cell compatibility [H. Tripathi et al. 2015]. However, we have also carried out similar studies at higher concentrations (25, 50, 100, 200, 400 and 800 µg/ml) of the samples for 48 h for safety evaluation. These results also suggest that the substitution of CeO_2 and La_2O_3 for SiO_2 has improved the cell compatibility. This effect could be seen with CeLa3 at any of the concentrations studied presently. Moreover, the sample CeLa3 possessed significant cell viability 89.78 % even at higher concentration (800mg/ml). The CeLa3 sample contains upto 1.5 wt % of cerium and lanthanumions suggested the optimal concentration in these series of glasses.

3.4.3 Cell proliferation

The cell proliferation assay was carried out using the same mouse fibroblast, L929 cell lines cultured with a concentration of 400 µg/ml of the glasses for different

time intervals (0, 1, 3, 5 days). The proliferations of cells in the presence of the samples were shown in Figure 3.8(d). The cell proliferation was found to increase significantly with time in samples (BG, CeLa1, CeLa2, CeLa3, and CeLa4). A controlled rate of release of ions from the sample into biological fluid was found to play a vital role in cell survival and growth which had also been observed by previous workers [H. Tripathi et al. 2015; A. Ali et al. 2018]. Therefore, the substitution of CeO_2 + La_2O_3 for SiO_2 is immensely beneficial. Furthermore, it is emphasize that sample CeLa3 possessed a significant cell growth even at higher concentration (800 mg/ml) which is in reasonable conformity with the cell viability data. It is interesting to discuss here that the cell growth was favoured CeLa3 (Number of cells $3.38x10^3$) seems to be hold up the proliferation and similar trend could be seen at all the concentrations in the present investigation. Earlier reports also show that decreased the bioactivity of the samples. Thus, the results suggested that the developed glass samples support cell survival and growth which signify a potential scope for bone regeneration.

3.4.4 Detection of cell apoptosis

Qualitative assessment of cell apoptosis was carried out in the presence of substituted bioactive glass samples. The broad spectrum growth inhibition by samples has raised the question of whether it can also cause apoptosis of the cells [S.K. Mahto et al. 2015]. The fluorescence images of cell morphology of all the rare earth oxides substituted glasses have shown in Figure 3.8(b). It may be reasonable to consider that the inhibitory activity of the samples sometimes may cause a cytotoxic effect. But, in this study, we found that all glass samples were not inducing cell death. Apoptosis was determined by monitoring changes in the

cell size and nuclear fragmentation of the mouse fibroblast, L929cells. The fluorescence images showed that all glass samples were cytocompatible and safe.

3.5 Conclusions

The comparative study was made on, physico-mechanical and in vitro bioactivity of 45S5bioactive glasses substituted with varying concentration of La_2O_3 and CeO_2. It was observed that microhardness and flexural strength increases with increasing concentration of La_2O_3 and CeO_2 whereas nucleation and crystallization temperature decreases. SEM and EDX image showed that HCA layer present in the surface of samples after immersion into SBF solutions. In vitro cell culture study shows, enhanced cell proliferation and viability of bioactive glasses. Whereas apoptotic assessment shows that the apoptosis reduced due to the substitution of cerium and lanthanum oxide. Thus, developed REEs substituted bioglass® be a suitable candidate for bone tissue engineering.