Chapter 6

MECHANICAL BEHAVIOUR AND BIOCOMPATIBILITY OF AL_2O_3-LA_2O_3 SUBSTITUTED BIOACTIVE GLASSES

6.1 Introduction

Rare earth elements were widely applied for biochemical and agronomic fields for having superior physicochemical properties. It is well known fact that Lanthanum has been qualified with antibacterial qualities and cellular immunity [Fei. G. et al. 2007]. Bioactive materials are capable to stimulate biological response and motivate binding at host tissue interface (soft and hard). The 45S5 bioglass® and its derivatives are being used as an implant materials and surgical instruments. The primary 45S5 bioglass® has 45 weight % of SiO_2 as network former, 24.5% Na_2O as network modifiers, 24.5% CaO and 6% P_2O_5 (weight %) were extensively investigated . The 45S5 based bioglass® was widely accepted as the substitute for tissue regeneration and was irrevocably acknowledged when a particular form of 45S5 bioglass® was made by Hench et al at the University of Florida in 1969 [L.L. Hench, et al. 2006]. However, a noteworthy shortcoming of 45S5 bioactive glass was its inability to fast degradation and unsatisfactory for primary load bearing applications [V. Aina, et al. 2009]. That's why several metallic and non-metallic ions such as lithium, titanium, potassium, copper, zinc, manganese, iron, magnesium, cobalt, nickel, strontium, neodymium and gadolinium were incorporated to the glasses to augment such mechanical properties for decades [A.M. Deliormanli, et al. 2015]. Metallic materials were frequently used for various medical applications such as bone and joint

replacement since decades for their high mechanical properties, but there were issues associated with it due to corrosive nature of materials. After 1970, nearly inert ceramic materials such as ZrO_2, Al_2O_3, etc., were used for their better biocompatibility and excellent mechanical properties [El Batal, F.H et al. 2008]. Such bio inert materials failed to make the strong bond with bones and loosened over a period of time after implantation. However, 45S5 bioactive glasses are the most suitable candidate for bone replacement, repairing and regeneration in recent times as they provide better biocompatibility than the any other materials.

The Al_2O_3 used for dental ceramics for the implanting teeth [L. Raffaelli 2012]. Alumina also recognized to hold their bacteriostatic properties with low toxicity. Also, it was observed that an increase in Ce^{4+} ions concentration leads to change in color of transparent glass to yellow-gold color bioglass®. La^{3+} is also an element used for health applications with bioglasses.

However, our current investigation deals with the synthesis and their cytocompatibility assessment of alumina and lanthanum substituted bioglass® for proposed biological implantation. Here we studied the outcomes of Al_2O_3+La_2O_3 (equal wt %) substitution for thermal, biological and mechanical properties in the 45S5 bioactive glass. The bioactivity of these glasses were examined with the formation of HCA layer on the surfaces of the bioglass® using SEM and FTIR after immersing the samples in SBF for several days. The microhardness and flexural strengths were also investigated using digital hardness tester and UTM. Substitution of Al_2O_3& La_2O_3 in 45S5 bioactive glass in place of SiO_2, it was found that these substitution favored ultrafine and regular grained microstructure which provide better mechanical strength [G. Malavasi et al. 2015].

6.2 Material preparation and methods

45S5 bioactive glasses with general formula (45-X) SiO_2, 24.5 Na_2O, 24.5 CaO and 6 P_2O_5 (weight %) was made by incorporation of Al_2O_3 and La_2O_3 (where X= 0.5, 1.0, 1.5, & 2.0 of equal percent of Al_2O_3 and La_2O_3. The Al_2O_3+ La_2O_3 substituted bioglass® composition (AlLa-1 to AlLa-4) were presented in Table 6.1. Analytical reagent grade quartz was used as a source of SiO_2, $CaCO_3$, Na_2CO_3, and $(NH_4)H_2PO_4$ were used as a source of CaO, Na_2O, and P_2O_5, respectively. The batches were prepared and mixed thoroughly for 40 min for homogenization and were melted in an alumina crucible in a globar furnace at 1400±2 ºC. The molten glasses were held at 1400 for 3 hours after that they were poured into the preheated aluminum moulds and transferred directly to muffle furnace at 560 ºC for annealing. These glasses were held for 2hrs then switch off. Annealed glasses were taken out from the furnace at normal temperature. The glasses were crushed and grinded into powder and pelletized for further characterization. The glasses were characterized by XRD, FTIR, and SEM for in vitro bioactivity. In vitro cytocompatibility were investigated with hemolytic, cytotoxic and proliferative assay. It was observed that the substitution of Al_2O_3 and La_2O_3 in the parent glass enhances their cellular cytocompatibility. Micro-hardness and flexural strengths of the glasses were also increased with the incorporation of Al_2O_3 and La_2O_3. Other mechanical properties of these bioactive glasses are also increased, thus the bioactive glasses substituted with Al_2O_3 and La_2O_3 is a suitable candidate for bone implant application.

Table 6.1: ***Weight % composition of the Al_2O_3 –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
AlLa-1	44	24.5	24.5	6	0.5	0.5
AlLa-2	43	24.5	24.5	6	1	1
AlLa-3	42	24.5	24.5	6	1.5	1.5
AlLa-4	41	24.5	24.5	6	2	2

6.3 Results and discussion

6.3.1 Differential Thermal Analysis (DTA) of Bioactive Glasses

The differential thermal analysis curves of the bioactive glasses are shown in Figure. 6.1, and the results dictate that the incorporation of La^{3+} and Al^{3+} ions in the 45S5 bioactive glass decreased both the nucleation temperature from 603 to 599 °C and the crystallization temperature from 765 to 635 °C. Further, it was observed that the greater amount of modifiers in the composition has facilitated in lowering T_g point and the viscosity of the glass melt [C.Y. Kim et al. 1989]. On substitution of equal amount of La_2O_3 and Al_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, 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 which decreased the oxygen bond strength [H. Tripathi et al. 2016]. The authors also found that an increased in the concentration of rare earth ions in the glassy system has allowed early nucleation by the nucleating agents like P_2O_5 in the glass composition. [A. S Kumar et al. 2015].

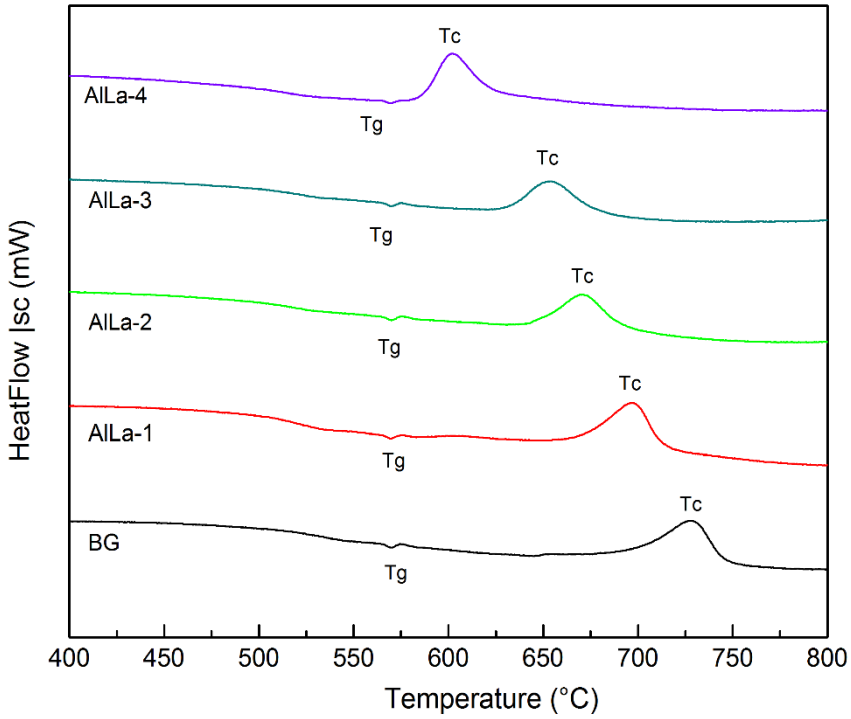

Figure 6.1: DTA of the Al_2O_3-La_2O_3 substituted bioactive glass samples

6.3.2 XRD and FTIR

The XRD spectra of the powdered glass samples show the absence of any peak in Figure 6.2, which indicates the amorphous glassy structure of the melted glasses.

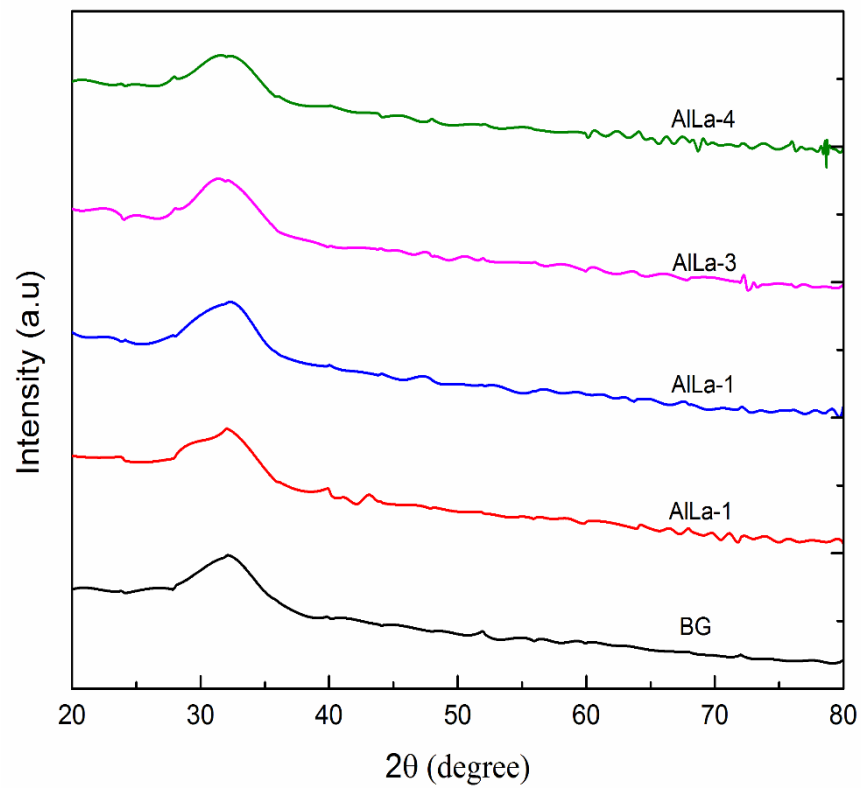

Figure 6.2: XRD of Al_2O_3-La_2O_3 substituted bioactive glass samples.

Figure 6.3 & 6.4 show the FTIR spectra associated with the bioglass® samples before and after immersion in SBF for different time intervals such as 1, 4, 8, 13, and 19 days. It was well known fact that by increasing the intensity of transmittance bands indicates to increase the molecular concentration of group formed at the surface of the bioactive glass samples. The results obtained by FTIR were also suitable with the previous studies made by many researchers [O.P. Filho et al. 1996]. Figure 3 shows the FTIR bands of AlLa-3 sample after immersion in SBF in 13 days. The new bands were found to appear after 13-day after soaking the samples into SBF at 803 cm^{-1} which corresponds to Si-O-Si bending mode of vibration, It was due to the formation of SiO_6octahedra after SBF treated. The bands were subsequent to the frequencies of 1404 and 1539 cm^{-1} are linked with C–O (carbonate) stretching mode and a minor peak at around 3064 cm^{-1} is assigned due to the presence of O–H (hydroxyl) groups present on the surface of the bioglass®.

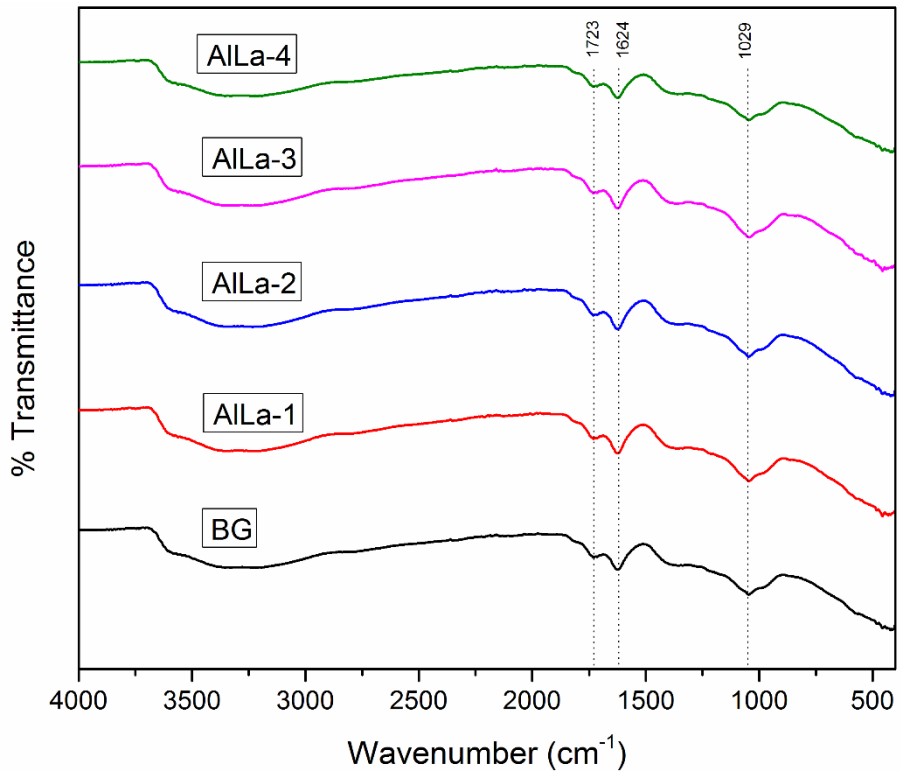

Figure 6.3: FTIR of the Al_2O_3-La_2O_3 substituted bioactive glass samples before SBF treatment**.**

The fluctuation of bands at approximately 900 cm^{-1} are ascribed to the PO_4 (P-O stretching) bands. It was further found that all other samples were observed to exhibit similar characteristics bands. For the long period of the samples in SBF exhibits the same behavior favorably due to the development of HCA layer [M.R.T. Filgueiras et al. 1993].

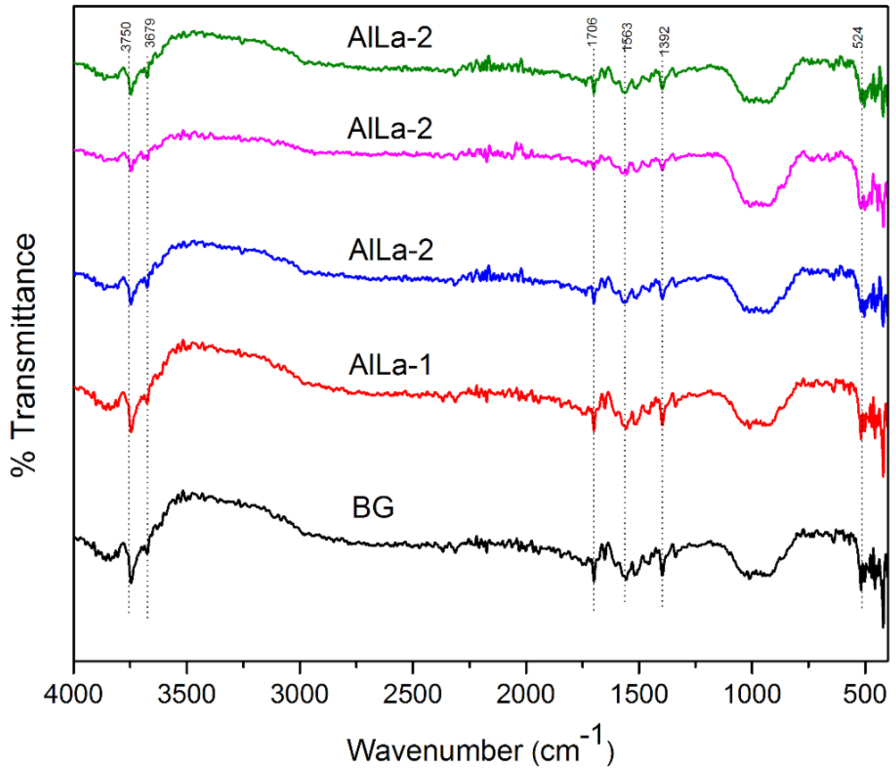

Figure 6.4: FTIR of the Al_2O_3-La_2O_3 substituted bioactive glass after SBF treatment soaked for 13 days**.**

6.3.3 Surface morphology evaluation by SEM

Surface properties of the base and substituted bioactive glass were analyzed in SBF solution (before and after immersion) and evaluated by Scanning Electron Microscopy (SEM). When the bio-glass sample was dipped in SBF solution for 19 days, then carbonated hydroxyapatite (HCA) layer were found on the upper layer of bioactive glass samples. The SEM images of bioactive glass samples prior to SBF soaking (Figure 6.5) indicates the formation of different long cylindrical

structures and unequal grains of bioactive glass samples [H.H. Beherei et al. 2009]. Whereas Figure 6.5 for SBF treated glasses shows that bioactive glass samples were uniformly covered with the irregular shape of cluster and agglomerates.

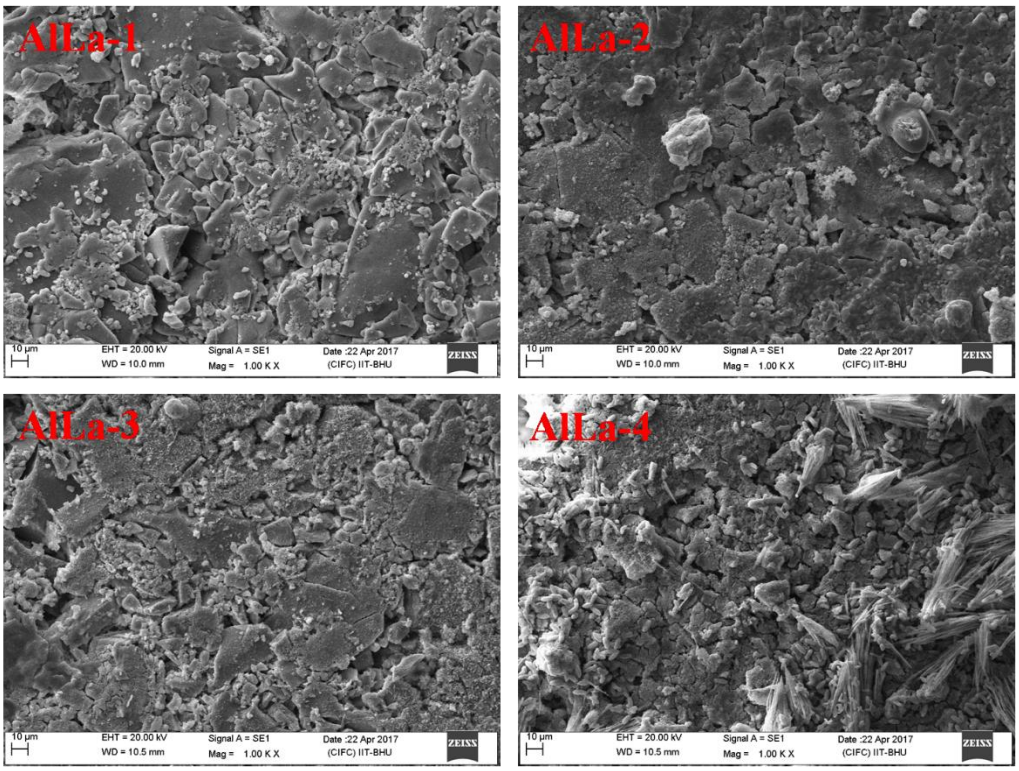

Figure 6.5: Morphological image (SEM) Al_2O_3-La_2O_3 substituted bioactive glass before SBF treatment.

The irregular shaped cluster is signified as the HCA layer which is formed due to the favorable environment of SBF with the glasses. Subsequent to comparing these micrographs it was concluded that all the prepared Al_2O_3& La_2O_3 doped bioglass® promotes the formation of HCA in SBF solution for 19 days shows in Figure 6.6 [M.R.T. Filgueiras et al. 1993]. Formation of the HCA layer was also confirmed by FTIR and XRD analysis after SBF treatment.

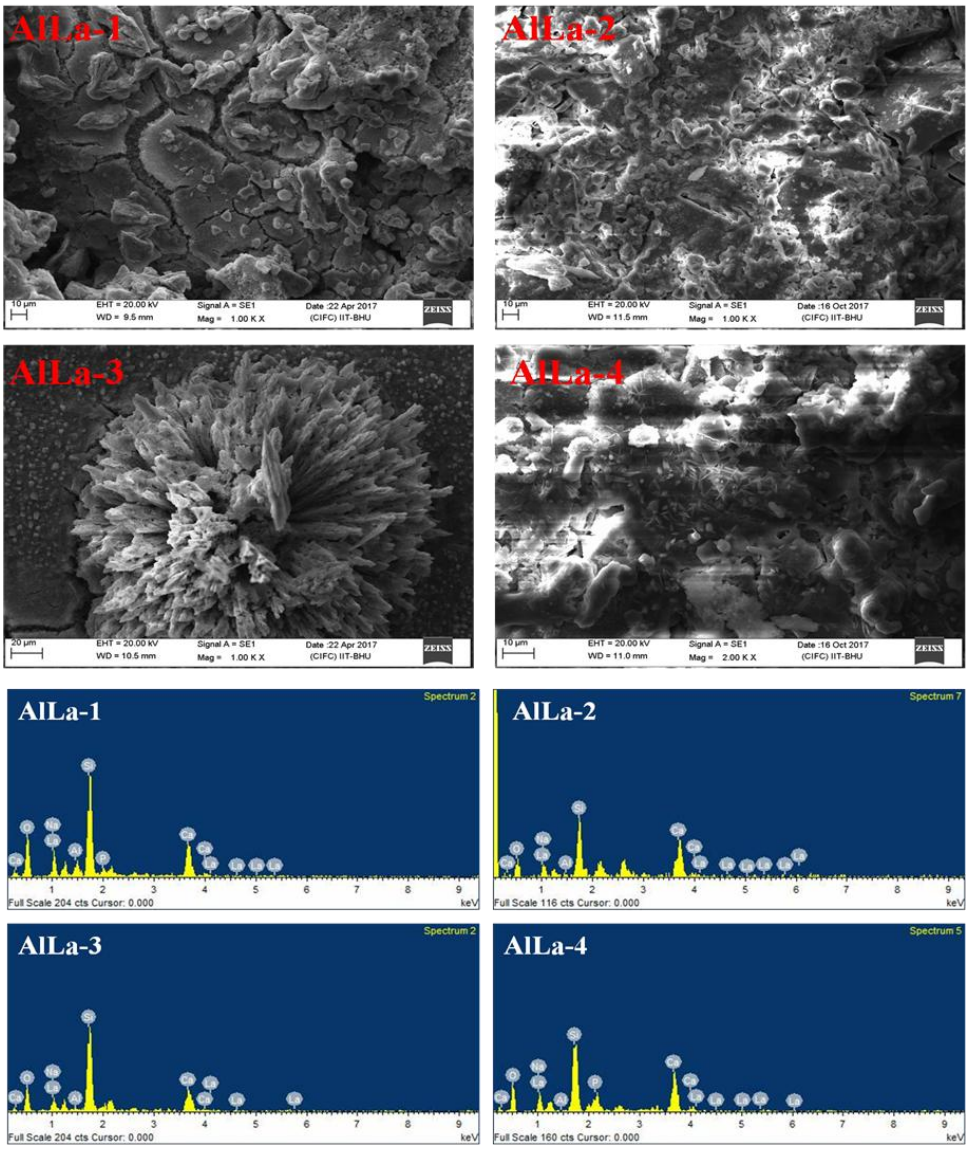

Figure 6.6: Morphological image (SEM) Al_2O_3-La_2O_3 substituted bioactive glass after SBF treatment soaked for 19 days.

6.3.4 Mechanical Properties and Density Measurements

The density of alumina and lanthanum substituted (0.5, 1.0, 1.5 and 2.0 wt %) glasses were measured by Archimedes's principle and found to be 2.702, 2.729, 2.744 and 2.770 gm/cm^3 respectively. Al_2O_3 and La_2O_3 replacement for silica may perhaps found to increase in density due to the result of replacement of lighter elements (Si - 2.32 gm/cm^3) by heavier one (Ce - 6.76 gm/cm^3) and (La - 6.16 gm/cm^3. On the other hand, due to the smaller size of lanthanum ions (ionic radius~1.061 Å) and aluminium ions (ionic radius~1.034 Å) ions it can be

adopted the similar structure and as replacement of the Al^{4+}/La^{3+} ions, it lead to increase the density of the glass [M.E. Santocildes et al. 2015; Y.C. Fredholm et al. 2010].

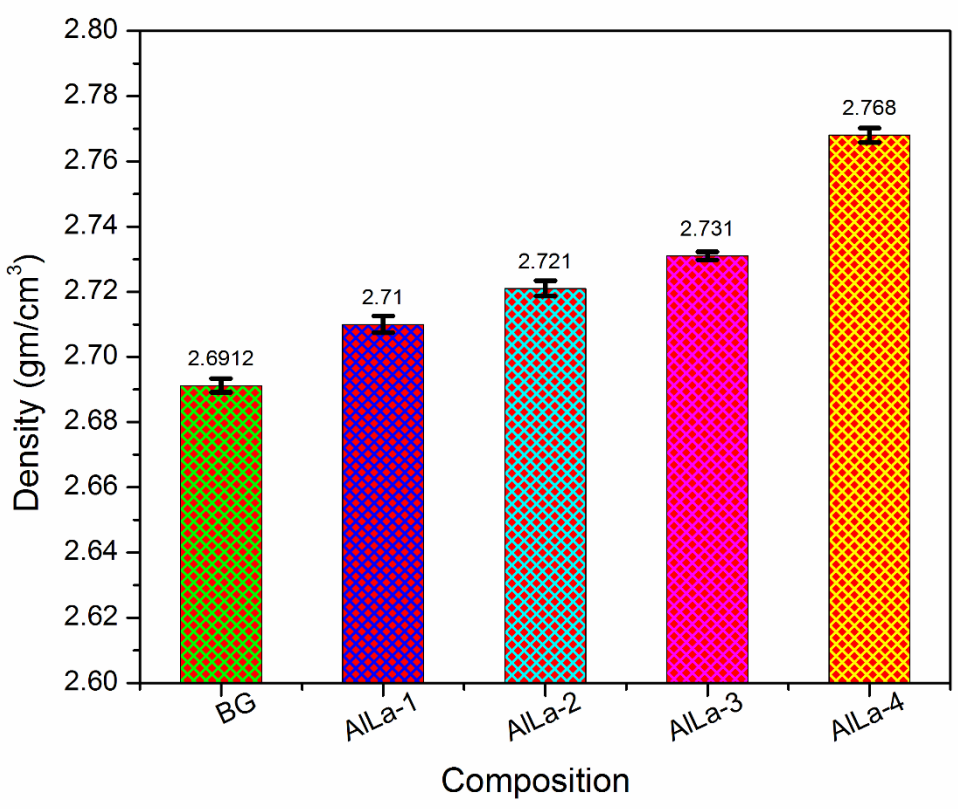

Figure 6.7: Density of Al_2O_3-La_2O_3 substituted bioactive glass samples.

The mechanical analysis result of base glass (Figure 6.7) exhibit micro hardness and flexural strength of 5.92 GPa and 46.87 MPa respectively while the microhardness and flexural strength of substituted bioglass® shows increment in microhardness (AlLa-1, AlLa-2, AlLa-3, and AlLa-4 are 5.97, 6.09, 6.41 and 6.67 GPa) and flexural strength (51.11, 57.33, 64.67 and 69.12 MPa) with increasing concentration of Al_2O_3& La_2O_3. Due to the compactness of molecules of glass powder, the micro hardness and flexural strength were increased by substituting the rear earth ions.

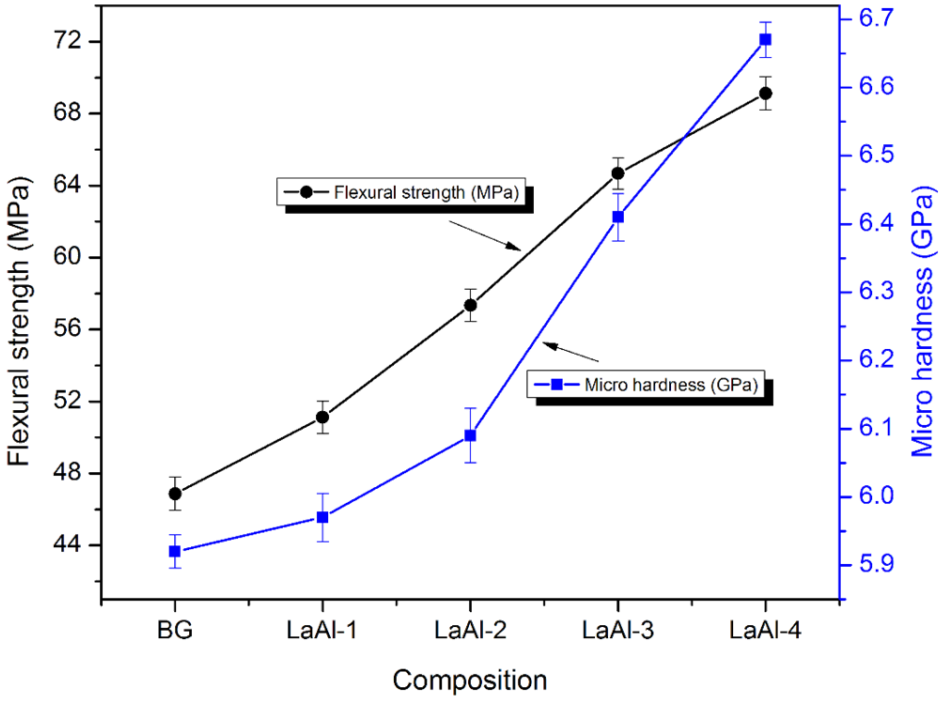

Figure 6.8: Flexural strength and Micro hardness of base bioactive glass and Al_2O_3-La_2O_3 substituted bioactive glass.

6.4 Assessment of biocompatibility

6.4.1 Hemolysis assay

The results of the hemolysis assay revealed the effectiveness and compatibility of glass compositions with blood are shown in Figure 6.9. The rare earth ion substituted bioactive glass powders did not influence the hemolysis of RBCs at lower concentrations, and the results were quite well within the compositional range of 2 wt % [S. K. Mahto et al. 2015]. On the other hand, it was found that the percentage of hemolysis was increased with increasing concentrations of the bioglass® compositions. It was found that increasing concentration of rare earth element decreased the hemolysis level and hence more biocompatible. It was examined that at higher levels (250 and 500mg/ml), AlLa1 caused partial hemolysis, whereas the AlLa-2, AlLa-3, and AlLa-4 samples have relatively better cytocompatibility.

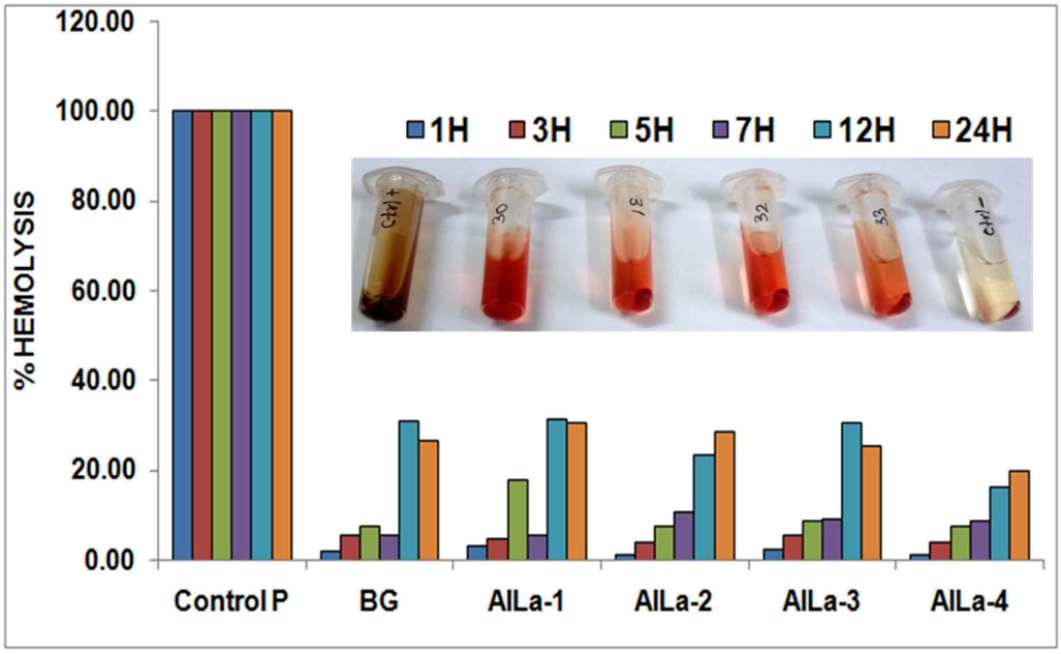

Figure 6.9: Hemolysis assay of Al_2O_3-La_2O_3 substituted bioglass samples.

6.4.2 Cell viability

Mostly, the biomaterials at lower content can show evidence of biocompatibility during in vitro cell culture studies while at higher concentration they showed cytotoxicity. Cell viability of the Al_2O_3 + La_2O_3 substituting samples along with a base glass (BG, AlLa-1, AlLa-2, AlLa-3, and AlLa-4) was assessed against mouse fibroblast, L929 cell lines by MTT assay. Figure 8(c) shows the cell viability (%) with different compositions was 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 h exposure to the sample (800 µg/ml) was recorded as (72.66), (76.94), (81.80), (89.78) and (75.89) % for BG, AlLa-1, AlLa-2, AlLa-3 and AlLa-4, 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, AlLa3 was the most compatible material which was also showed best mechanical strength as compared to all others. This enrichment might be associated with the

controlled release of ions from the bioglass®, which played an important role in cell survival. The substituted glasses with the rare earth elements were further studied for biological assessment. These findings are in good agreement to the prior reports that the rare earth element substituted bioactive glasses did not affect the cell compatibility [H. Tripathi et al. 2015]. We have also carried out comparable studies at higher concentrations (25, 50, 100, 200, 400 and 800 µg/ml) for 48 h for safety evaluation. These results strongly suggest that the replacement of Al_2O_3 and La_2O_3 for SiO_2 has enhanced the cell compatibility. These outcomes could be seen with AlLa-3 at any of the concentrations. Furthermore, the sample AlLa-3 observed significant cell viability 87.78% even at higher concentration (800mg/ml). The AlLa-3 sample contains upto 1.5 wt % of alumina and lanthanumions suggested to be the optimal concentration in these series of bioglasses.

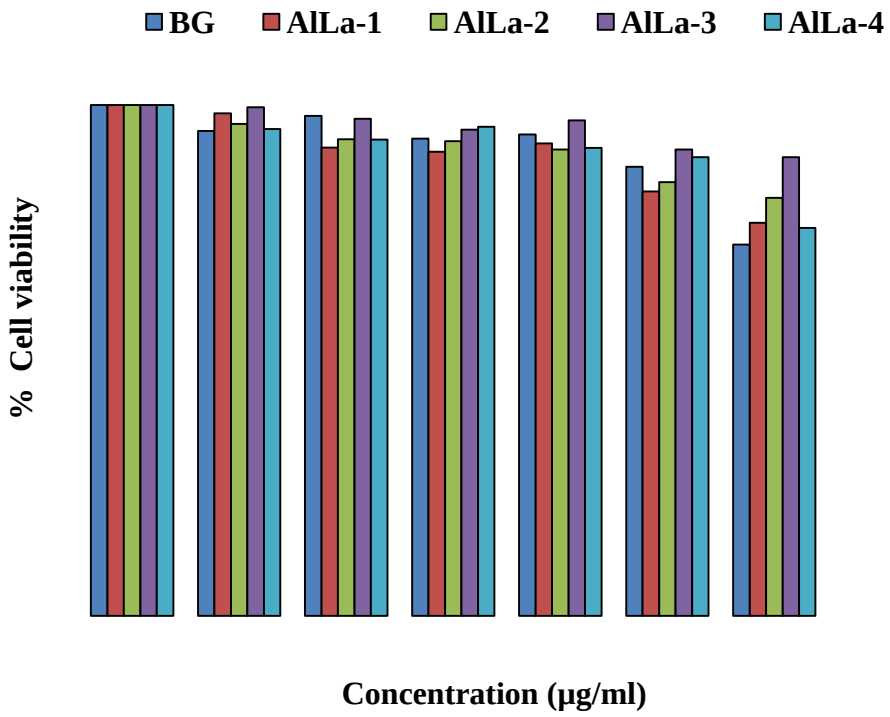

Figure 6.10: Cell viability assay of Al_2O_3-La_2O_3 substituted bioglass samples.

6.4.3 Cell proliferation

It was carried out using the same mouse fibroblast, L929 cell lines cultured with a concentration of 400 µg/ml of the glasses for time intervals (0, 1, 3, 5 days). The proliferations of cells in the presence of the samples were shown in Figure 8.11.

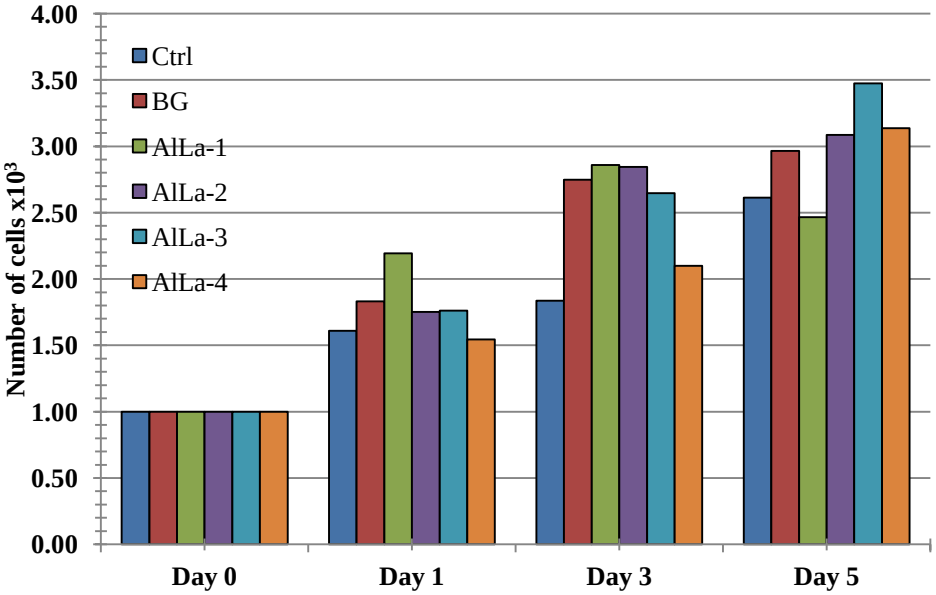

Figure 6.11: Cell proliferation assay of Al_2O_3-La_2O_3 substituted bioglass.

The cell proliferation found to increase slightly with time even treated with the samples (BG, AlLa-1, AlLa-2, AlLa-3, and AlLa-4). Therefore, the replacement of Al_2O_3 + La_2O_3 for SiO_2 is hugely beneficial. Moreover, it is to put emphasis on sample AlLa-3 have significant cell growth even at higher content (800 mg/ml) which is in reasonable with the cell viability data [A.Ali et al. 2018]. It is remarkable to discuss the cell growth which was favored AlLa-3 (Number of cells $3.38x10^3$) the proliferation and similar trend might be seen at all the batches of bioglass® in the present research. Previously reports also examined that decreased the bioactivity of the samples. Hence, the results suggested that the developed glass samples support cell growth and survival which possibility for bone regeneration.

6.4.4 Detection of cell apoptosis

The assessment of cell apoptosis of substituted bioactive glass samples was carried out. The wide spectrum growth inhibition by glass samples has raised the question of whether it can also cause apoptosis of the cells [H. Tripathi et al. 2015]. The fluorescence images of cell morphology of rare earth oxides substituted glasses have shown in Figure 6.12. It could be believe that the inhibitory activity of the samples sometimes cause a cytotoxic effect. But, in this study, we found that all glass samples were not inducing cell death. Although, apoptosis was evaluated by monitoring changes in the cell size and nuclear fragmentation of the mouse fibroblast, L929 cells. The fluorescence images showed that all glass samples were cytocompatible and safe.

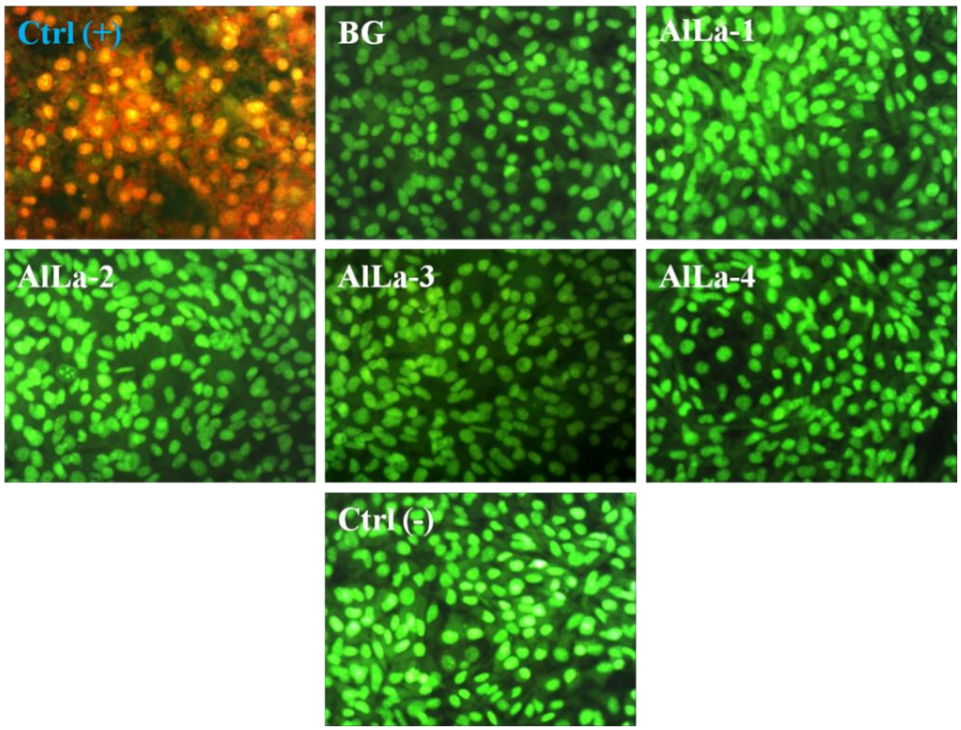

Figure 6.12: Detection of cell apoptosis of Al_2O_3-La_2O_3 substituted bioglass.

Conclusions

The comparative study was made on, physico-mechanical and in vitro test of 45S5bioactive glasses substituted with varying concentration of La_2O_3 and Al_2O_3. It was observed that microhardness and flexural strength increases with increasing concentration of La_2O_3 and Al_2O_3 whereas nucleation and crystallization temperature decreases. SEM and EDX analysed the formation of HCA layer present in the outer surface of bioglass® after immersion into SBF. In vitro cell culture study shows, enhanced the cell proliferation and viability of bioactive glasses. Whereas apoptotic assessment shows that the apoptosis reduced due to the substitution of Aluminium and Lanthanum oxide. Thus, developed REEs (La_2O_3) substituted bioglass® is suitable materials for bone tissue engineering.