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architectures can be observed from the optical images of the scaffolds after 4 days of incubation. The scaffolds with the diamond architecture were completely covered with metabolically active cells after 6 days of incubation. To quantify the results, the measured absorbance of the amount of formazan product of mitochondrial metabolism that was recovered from the cell-seeded scaffolds labeled with MTT is plotted in Figure 3.

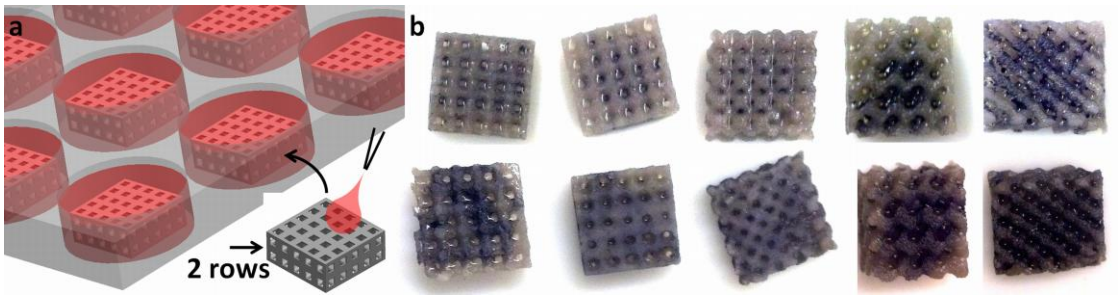

Figure 2. (a) Schematic of the static cell seeding on the scaffold with at least two rows of repeating units. The cell seeded scaffolds were later transferred to a 12-well cell culture plate, (b) Optical images showing the MTT labeling of MLO-A5 cells on the on the scaffolds (L-R: cubic, spherical, X, gyroid and diamond) after cell culture intervals of 4 days (top row) and 6 days (bottom row).

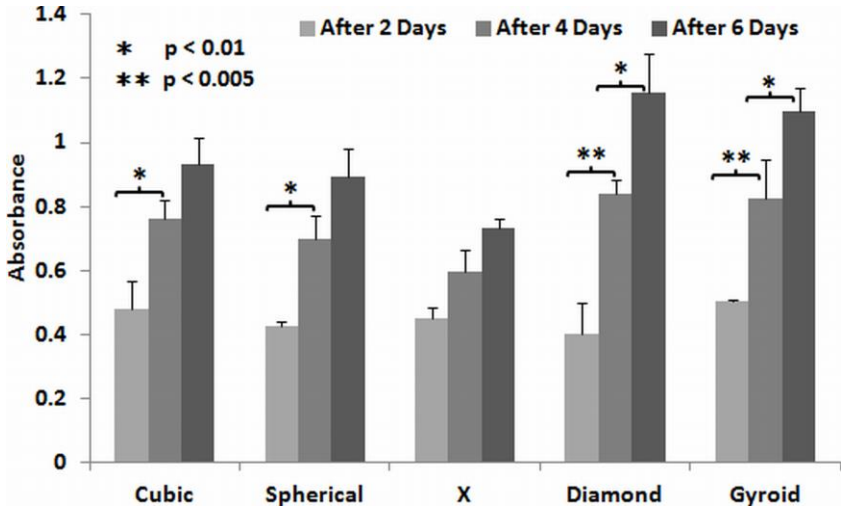

Figure 3. The absorbance values representing the measurement of cell growth plotted for all the scaffolds after 2, 4, and 6 days of incubation. Diamond and gyroid architectures show better overall cell proliferation compared to the other architectures.

The absorbance values plotted in Figure 3 for different scaffolds after 2, 4, and 6 days of incubation indicate the number of metabolically active cells on the scaffold, thereby directly corresponding to the amount of cell proliferation. To understand the cell proliferation trends from 2 to 4 days, it can be observed from Figure 3 that all the scaffolds provide significant cell proliferation except the scaffold with the X architecture. Further, the diamond and gyroid scaffolds show higher significance ($p < 0.005$) in comparison to the cubic and spherical scaffolds. Whereas the cell proliferation from 4 to 6 days was significant for the gyroid and diamond architecture scaffolds ($p < 0.01$), the increase in cell proliferation for the other scaffolds was not significant. These results indicate that although the cubic and spherical scaffolds provide

significant growth up to 4 days, the gyroid and diamond scaffolds provide significant growth up to 6 days of incubation, and the scaffold designed with the X architecture provides the least amount of cell growth. To interpret the results among the scaffolds with different architectures based on their incubation period, no significant difference was noticed after 2 days of incubation as the scaffolds have similar values of absorbance. However, the differences were observed after 4 and 6 days of incubation. After 4 days of incubation, the scaffolds having the diamond and gyroid architectures offered significantly higher cell proliferation ($p < 0.05$) in comparison to the X scaffolds. However, this difference was not significant in comparison to the cubic and spherical scaffolds. After 6 days of incubation, the cell proliferation on the diamond and gyroid scaffolds further improved in significance in comparison to the X scaffolds (from $p < 0.05$ after 4 days to $p < 0.005$ after 6 days). Also, the cell proliferation on the diamond scaffolds was significantly higher than the cubic and spherical scaffolds (with no significant difference among them after 4 days, changing to significant with $p < 0.05$ after 6 days).

3.2. Cell proliferation on 13–93B3 scaffolds

The average absorbance value of the formazan extracted after 2 days of incubation from the 13-93B3 scaffolds was ~0.3 without any significant difference among the scaffolds with different architectures. In comparison, the absorbance values representing the cell growth on 13-93 scaffolds after 2 days was ~0.4 (Figure 3). The cell proliferation results on the 13-93B3 scaffolds indicate that the amount of formazan recovered from the scaffolds after 4 and 6 days of incubation is decreasing, which is in sharp contrast to the result observed with the 13-93 scaffolds. Figure 4 shows the comparison of the absorbance values of the formazan product recovered from 13-93 and 13-93B3 scaffolds. The absorbance values in almost all the cases after 4 and 6 days were measured to be ~0.2 or less, which is too low for the result to be significant.

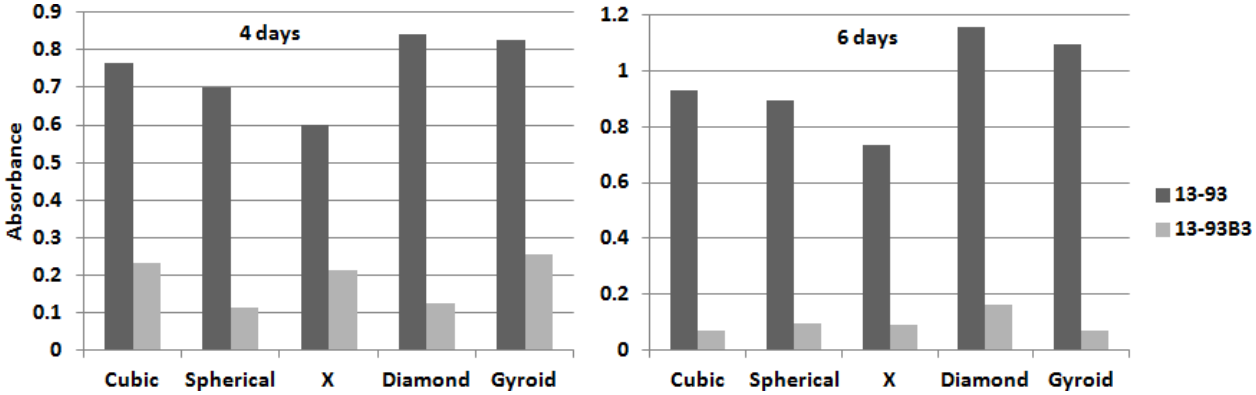

Figure 4. Comparison of the absorbance values for 13-93 and 13-93B3 scaffolds after 4 and 6 days of incubation. The graphs indicate the reduced number of metabolically active cells on the 13-93B3 scaffolds compared to the 13-93 scaffolds.

The results of *in vitro* assessment of borate based glasses reported in the literature are mixed. There have been some reports on the cytocompatibilty of the borate glass and the osteogenic differentiation of the human mesenchymal stem cells [21]. There have been other studies which reported a reduction in the absorbance value with an increase in the boron ion concentration in the cell culture media [22]. The reduction of metabolically active cells after 4 and 6 days in our study (Figure 4) may be due to the fact that an increase in the boron ion

concentration leads to an increase in the pH value of the culture media (pH – 9). Such an environment could drastically affect the growth of cells. It should be noted that this result was observed even after replacing the media every day for the 13-93B3 scaffolds in order to reduce the likelihood of a change in pH. In comparison, the media for 13-93 scaffolds was replaced every other day. Though the *in vitro* assessment of the 13–93B3 scaffolds showed poor cell survival rates, because of the toxicity attributed to boron ion release and its concentration, the results could be regarded as a false negative based on the *in vivo* assessment of the borate glasses which has been shown to demonstrate markedly faster bone growth and healing, as reported in the literature [7]. In fact, borate glasses have also been shown to even promote angiogenesis and healing in difficult-to-treat soft tissue wounds in diabetic patients [7]. The investigation of the effect of pore geometries in bone growth using the 13–93B3 scaffolds *in vivo* will be a subject of our future work.

3.3. Effect of pore geometry on cell proliferation

Apart from the differences in the cell proliferation based on the material used to fabricate the scaffolds, the surface roughness of the scaffold, which is a characteristic of the SLS process and is irrespective of the geometry, could also affect the way cells proliferate on the scaffolds. Our *in vitro* assessment results indicated that during the initial 2 days of incubation, the difference in the amount of cell proliferation among the scaffolds with different pore geometries did not exist and became significant only after 4 and 6 days. This could be due to the rough surface of all of the scaffolds fabricated by the SLS process. Figure 5a shows a scanning electron microscopy (SEM) image of a strut with rough edges due to the layer-by-layer fabrication process. Another SEM image of the surface area surrounding a pore on the scaffold, irrespective of the material and scaffold pore geometry, is shown in Figure 5b. This amount of surface roughness could provide a favorable attachment surface for seeded cells on SLS-based scaffolds. In fact, at the time of cell seeding, all of the cell suspension volume applied to the scaffolds adhered to the samples and no suspension was observed on the teflon sheet after the scaffolds were aseptically transferred to the 12-well plate (100% seeding efficiency). Therefore, during the initial days of incubation, the cells would proliferate on the rough surface of the scaffold as shown in the schematic in Figure 5c. This could be the reason for not having any significant differences among the different architectures after seeding all the scaffolds with equal number of cells and 100% seeding efficiency. The effects of pore geometry would start to prevail only after the rough surface is covered by the cells, i.e., after 4 and 6 days.

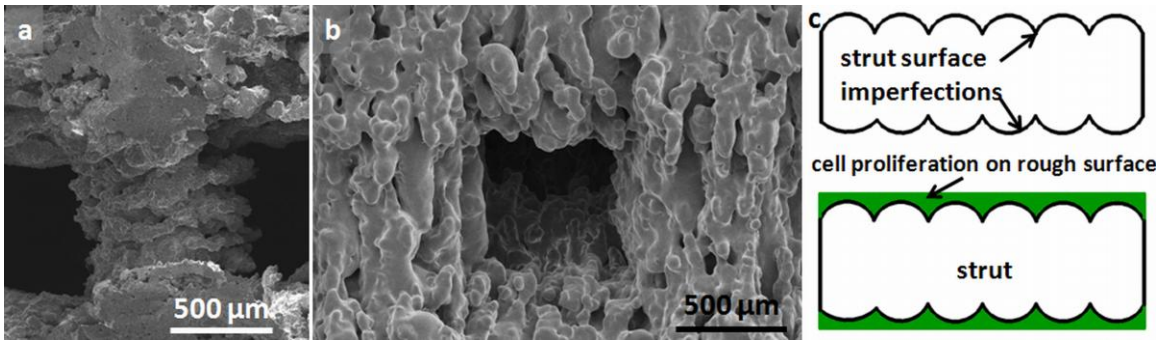

Figure 5. (a) The surface of the strut exhibiting layered fabrication in the SLS process, (b) a typical surface of the SLS scaffold, (c) schematic showing the proliferation of cells on the rough surface during the initial incubation period.

The effect of pore geometry on tissue growth and the biochemical signals between cells, as well as how the cells react to the radius of curvature of the scaffold, is still a subject of investigation to the scientific community [15, 23, 24]. While some studies have reported that tissue growth relates to curvature, other studies found no effect of curvature on tissue growth; and most of the studies are speculative in nature. In order to comprehend the differences in cell proliferation among the scaffolds in this study, the internal surface area per unit volume was calculated for the scaffolds with different pore geometries, based on their CAD files, since a higher surface area translates to more area for cells to proliferate on the surface. The value for the X architecture (0.7 mm^{-1}) was much lower in comparison to the other architectures (1.4 for cubic, 1.9 for gyroid, and 1.5 for spherical and diamond), and it reflects in the cell proliferation results as the X scaffold offered no significant cell growth (Figure 3). However, the internal surface area per volume alone does not explain the differences in the cell proliferation among the scaffolds. Though the scaffolds with spherical and diamond architecture have the same value of internal surface area per volume, the cell proliferation results are in sharp contrast in that the cells seeded on the scaffolds with the diamond architecture proliferate significantly more in comparison to the spherical scaffolds. Therefore, the perimeter of a unit cell is plotted with respect to the thickness (height) of the unit cell to understand the changes in the surface area of a unit cell rather than the overall value. Figure 6 shows the variation in the perimeter of the cross section of a unit cell along the thickness, for all the five architectures. As the length of unit cell is not uniform among the different architectures, the z height is divided by the unit cell length to provide the z value, which varies from 0 to 1 corresponding to the start and finish of the unit cell thickness. Similarly, the perimeter values are also converted to ratios based on the maximum perimeter value for the corresponding unit cell. The curvature of the internal surface could be understood from the variation of the perimeter of the unit cell for different architectures. Among all the scaffolds, the perimeter profile of the diamond architecture resembles a trigonometrical function (sine/cosine) with more frequent changes in sign (positive to negative and vice versa) to the slope of the curve whereas the changes to the slope of the curve are not drastic for the spherical architecture. Such frequent and drastic changes to the slope of the perimeter profile suggest a larger curvature to the unit cell, which offers a higher cell proliferation as observed from our results (Figure 3). A relatively slow change in slope or a constant slope of the perimeter profile would suggest small or no curvature which appears to be less beneficial for cell proliferation, especially in a static cell culture condition where the nutrient flow is limited and the only external stimulus for the cells to proliferate could be the curvature. In the case of diamond and gyroid architectures, a larger surface area, together with a larger surface curvature, might allow for better cell proliferation in the static cell culture conditions. Therefore, it can be stated that a preferable porous scaffold for tissue engineering applications should provide the necessary surface roughness for the initial cell attachment, and a larger surface area and surface curvature for improved cell proliferation.

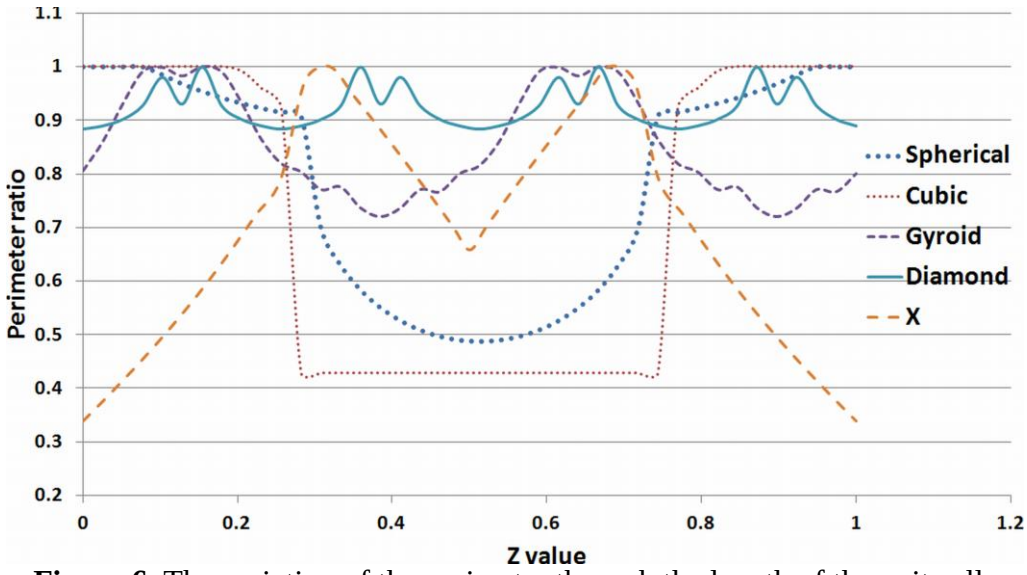

Figure 6. The variation of the perimeter through the length of the unit cell.

4. Conclusions

The *in vitro* assessment in this study indicates that bioactive glass scaffolds fabricated by the selective laser sintering process provides the necessary surface roughness for the initial phase of incubation for cell attachment. The cell proliferation study shows the effectiveness of SLS produced, gyroid and diamond architectures in providing sustained cell proliferation for 6 days of incubation compared to other scaffold architectures (cubic, spherical, and X architectures) which did not provide significant cell growth after 4 days of incubation. Our experimental results and analysis indicate that a larger surface area per unit volume of the scaffold, combined with a larger surface curvature, allows for improved cell proliferation *in vitro* for the scaffold architecture.

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