

Biological and mechanical response of Graphene Oxide surface-treated Polylactic Acid 3D-printed bone scaffolds: Experimental and Numerical approaches

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Abstract

Employing 3D printing bone scaffolds with various polymers is growing due to their biocompatibility, biodegradability, and good mechanical properties. However, their biological properties need modification to have fewer difficulties in clinical experiments. Herein, the fused-deposition modeling technique is used to design triply-periodic-minimal-surfaces polylactic-acid scaffolds and evaluate their biological response under static and dynamic cell culture conditions. To enhance the biological response of 3D-printed bone scaffolds, graphene-oxide (GO) is coated on the surface of the scaffolds. Fourier-transform infrared spectroscopy, X-ray diffraction, and energy-dispersion X-ray analysis are conducted to check the GO presence and its effects. Also, computational fluid dynamics analysis is implemented to investigate the shear stress on the scaffold, which is a critical parameter for cell proliferation under dynamic cell culture conditions. Compression tests and contact-angle measurements are performed to assess the GO effect on mechanical properties and wettability, respectively. Also, it was shown that surface-treated scaffolds have lower mechanical properties and higher wettability than uncoated scaffolds. A perfusion bioreactor is used to study cell culture. Also, field-emission-scanning-electron-microscope and 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl-tetrazolium-bromide (MTT) assay analyses are conducted to observe cell viability and cell attachment. An increase of up to 220% in viability was achieved with GO and dynamic cell culture.

Keywords: 3D printing, Tissue engineering, Bone scaffolds, Dynamic culture, Mechanical properties

Abbreviations

AM	Additive manufacturing
CAD	Computer-aided design
DMSO	Dimethyl sulfoxide
ECM	Extracellular matrix
EDX	Energy dispersion X-ray

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FDM	Fused deposition modeling
FE-SEM	Field Emission Scanning Electron Microscope
FTIR	Fourier-transform infrared spectroscopy
GO	Graphene oxide
OoC	Organ-on-a-chip
PBS	Polybutylene succinate
PLA	Polylactic acid
SEM	Scanning electron microscope
TE	Tissue engineering
TPMS	Triply periodic minimal surfaces
XRD	X-ray diffraction
CFD	Computational fluid dynamics

1- Introduction

Bones in the body continuously rebuild themselves under physiological conditions and have a high regeneration ability. However, in some cases, such as physical trauma, congenital abnormalities, removing tumors, chronic joint disease, or infections, the bone repair mechanism can be slowed and may not lead to complete regeneration [1]. It is essential to follow medical guidelines in such circumstances when the injury outweighs the body's healing capacity. After blood, autologous bone grafts are the most commonly transplanted tissue type worldwide [2]. Allo- or autografts of bone are the most common treatment choices for substantial bone defects, with metallic prostheses as a secondary therapy option [3]. These scaffolds are osteoinductive and osteoconductive due to the presence of growth factors, bone material, and osteogenic cells in the structure [4]. However, it has several drawbacks, including infection, a limited supply of donor bone tissue, hemorrhage, donor site morbidity, second operation site for bone graft harvest, allogenic origin, persistent discomfort, and long-term mechanical failure. Despite the fact that they are accessible in a variety of shapes and sizes and are devoid of morbidity concerns, allografts have the potential to transmit infectious diseases and trigger immunological rejection [5]. Additionally, the sterilizing process negatively impacts the biological and mechanical properties of the graft.

By employing Tissue engineering (T.E.), many Organ-on-a-chips are developed [6], and new treatments and regeneration strategies are developed for bone tissue. T.E. is a rapidly growing field that seeks to restore the function of damaged organs by starting with a pool of regenerative cells that can self-renew and differentiate other cell types and then improve those functions [7], [8]. A crucial stage of tissue Engineering is the design and fabrication of a scaffold that provides further cell-biomaterial interaction, biodegradability, adequate transport of gases, nutrients, and regulatory factors

for cell survival and proliferation, as well as low inflammatory and toxic effects, as shown Figure 1 [9], [10]. Numerous studies have developed and manufactured scaffolds for optimizing these characteristics. Considering that patients' bone morphology and defects vary significantly in size and shape and anatomical factors must be addressed during graft fixation and design to optimize graft osseointegration with host tissue. Additive manufacturing (AM) technology and computer-aided design (CAD) can provide complex bone grafts or scaffolds based on the patients' medical images such as magnetic resonance imaging and computed tomography [11]. Another significant feature of AM is that it allows for the construction of scaffolds or artificial constructions with predetermined micro and macrostructures [12]. Selectivity in microstructure is helpful as the micro-scale pore size, orientation, and surface chemistry control regulate the amount and nature of vascular and bone tissue regeneration [13]. Moreover, different printing methods may be used in fabricating scaffolds and prostheses. Fused deposition modeling (FDM), stereolithography (SLA), powder 3D printing, selective laser sintering/melting (SLS and SLM), and 3D-bioprinting are all possible using techniques for *in-vitro* and *in-vivo* applications [14], [15]. The additive manufacturing technique enables researchers to overcome the low complexity and poor similarity of the scaffolds to human bones. In the last years, many models and mathematical shapes have been presented to make scaffolds much quite close to the natural tissues. A wide range of materials can be chosen based on the 3D printing technology and application. A few are thermoplastics and polymers (PCL, PLA, ABS, PLLA, PLGA, and PVA) [16], Photopolymer resins [17], temperature-sensitive polymers [18], ceramics [19], hydrogels [20], metals [16], and composites [21]. TE commonly employs static cell culture due to its simplicity. However, it has some deficiencies, including limitations in transporting waste and nutrient materials which lead to the non-formation of extracellular matrix (ECM) in the core of the scaffold [22]. These deficiencies can be solved by employing dynamic cell culture conditions such as perfusion bioreactor, which facilitate mass transport through the scaffold [23]. Besides these advantages, such a system can apply mechanical stimulation to the scaffold and promote forming ECM. Also, since the nature of such mechanical stimulation is from fluid flow shear stress, the amount of this mechanical loading can be changed easily by altering the flow rate [24]. It should be mentioned that recently many microfluidic systems have been developed to assist efficient scaffold preparation [25].

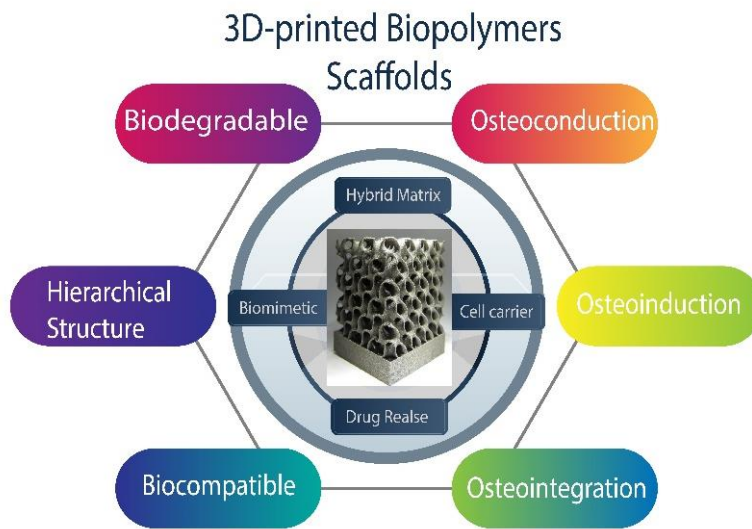


Figure 1. Requirement of 3D-printed bone tissue scaffolds

Researchers use various approaches to generate complicated geometries. Triply periodic minimal surfaces (TPMS) are a type of function-based structure that have previously received considerable attention because of their distinctive combination of features such as a high surface-to-volume ratio, stiffness-to-weight ratio, and cellular interconnectivity [26], [27]. Many researchers employed TPMS structures to produce bone tissue with various materials such as Ti6Al4V and polylactic acid (PLA) [28]. The critical parameters in TPMS characterization are mechanical properties, osteointegration capacity, pore size, and interconnectivity [29], which significantly impact the differentiation of pre-osteoblastic cell lines and suitability for bone regeneration [30]. Despite advances in 3D printing bone scaffolds with mimicked geometry, some challenges should be addressed. The surface morphology of the bone scaffold has remarkable effects on the biological scaffold's performance. This is because the appropriate surface morphology can increase the attachment of surrounding cells and facilitate tissue regeneration [31]. Many researchers have investigated the effects of surface treatment and nanoparticle coating on surfaces. They found that these procedures can stimulate bioactivity, including cell proliferation, tissue compatibility, and biodegradability[32]. Graphene oxide (GO) has been more popular in recent years because of its advantageous features, including the capacity to carry a high charge, high specific surface area, antimicrobial capabilities, and outstanding optical and mechanical properties [33], [34]. Under various conditions, GO promotes the adhesion and proliferation of osteoblast and mesenchymal stem cells. It has also been proved that GO may promote

the differentiation of stem cells [35]. Due to the remarkable physicochemical properties of GO, such as large surface area, high dispersibility, and hydrophilicity, this material has significant potential to stimulate tissue regeneration, whether incorporated into scaffolds or coated [36].

Numerous studies have been conducted to fabricate efficient bone scaffolds which is a challenging part of TE. In this article, the effect of graphene oxide coating on the physicochemical properties of different TPMS-based bone scaffolds was investigated. In this regard, a function-based method was employed to generate PLA-based 3D porous scaffolds with the FDM 3D printing method. The results of static and dynamic cell culture are compared to determine the scaffold modification effect. The CFD analysis is used to determine the shear stress on the scaffolds. Furthermore, X-ray diffraction (XRD), Fourier-transform infrared spectroscopy (FTIR), Energy dispersion X-ray (EDX), Scanning electron microscope (SEM), MTT assay, compression test, and contact angle measurement results are performed to determine the various aspects of GO effects on the physicochemical properties of the bone scaffold.

2- Materials and Methods

2.1. Materials

Calcium chloride-Dihydrate was obtained from Merck, Dulbecco's Modified Eagle Medium (DMEM), and Fetal Bovine Serum (FBS) were purchased from GIBCO. Graphene oxide, Glutaraldehyde, Chloroform and osmium tetroxide were obtained from Sigma Aldrich, and polylactic acid filament (PLA) was purchased from Youso.

2.2. Design of 3D Printing Scaffolds and Fabrication

2.2.1. Design of Scaffolds

The 3D printing method was employed to fabricate bone scaffolds. So, the FDM process was used to print TPMS structures. Since the TPMS structure is a scaffold-based implicit function, an in-house code was generated to design the TPMS scaffold by preparing its STL file for printing. All the scaffolds were fabricated in a 20 mm diameter and 5 mm height cylindrical shape. Eq. Figure 1 illustrates the implicit function of the Gyroid surface that has been used to design and print bone scaffolds. By changing the c and s , different porosities and geometries can be obtained.

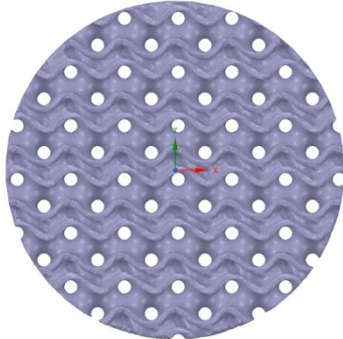
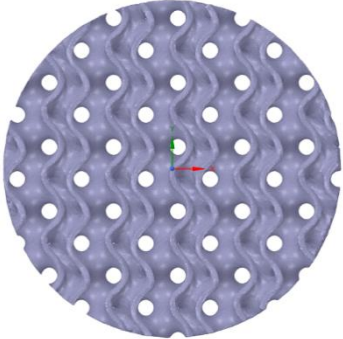
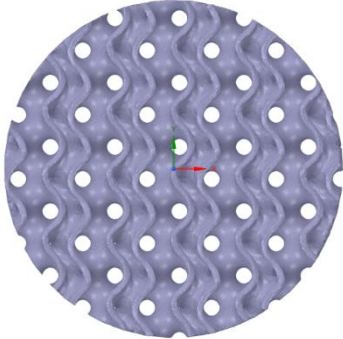
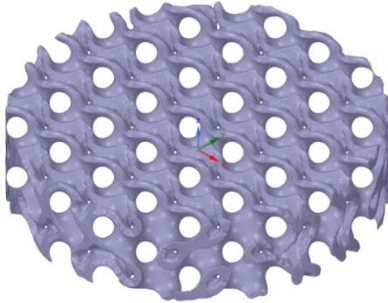
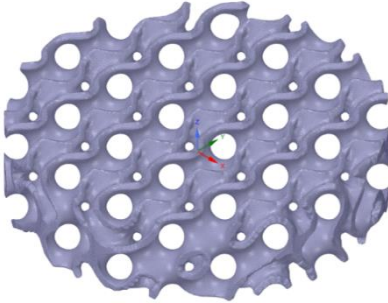
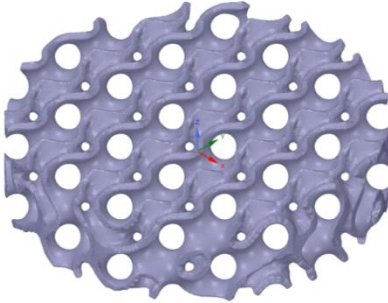
$$\sin\left(\frac{x}{s}\right)\cos\left(\frac{y}{s}\right) + \sin\left(\frac{y}{s}\right)\cos\left(\frac{z}{s}\right) + \sin\left(\frac{z}{s}\right)\cos\left(\frac{x}{s}\right) \quad (1)$$

$$\rho^* = \left(1 - \frac{\rho}{\rho_o}\right) \times 100 \quad (2)$$

ρ and ρ_o are the density of a scaffold and bulk material, respectively.

The biological and mechanical properties of bone scaffolds significantly depend on the scaffold's porosity, which rely on S and C parameters in Eq. 2. Three scaffolds with different permeability are designed with constant $C=0$ and variable S , as presented in Table 1.

Table 1. Three different scaffolds with variable S

	Scaffold (1)	Scaffold (2)	Scaffold (3)
S	0.5	0.6	0.75
Front view			
Isometric view			

2.2.3. Coating graphene oxide on printed scaffolds

To coat GO on printed scaffolds, the dip coating method was employed. So, to obtain a uniform GO suspension, a 0.1 mg/mL solution of the GO in chloroform was sonicated using an ultrasonic probe at 60% amplitude for 50 min. After obtaining a homogenous solution, the printed scaffolds were immersed in GO-chloroform solution, and then the scaffolds were pulled out of the solution.

2.3. Characterization of scaffolds

2.3.1. CFD Simulations

The CFD modeling was conducted with ANSYS Fluent (Ansys Inc., USA). Continuity. Momentum equations for incompressible laminar flow are as following [31]:

$$\rho \cdot \nabla \cdot U = 0 \quad (3)$$

$$\rho [U \cdot \nabla \cdot U] = -\nabla P + \mu \cdot \nabla^2 \cdot U \quad (4)$$

Inlet flow rate and Zero pressure outlet is considered for the inlet and outlet boundary conditions, respectively. No slip boundary condition was applied on walls, as well. Figure 2 shows the cross-sectional and 3D schematic view of the bioreactor for modeling with the boundary conditions.

In the geometry preparation, Ansys Space claim Module was used to produce high-precision geometry from imported STL format of TPMS file [31]. Then, the domain of 3D model was extracted and prepared for the meshing. Fluent Meshing was used to constructing the 3D domain.

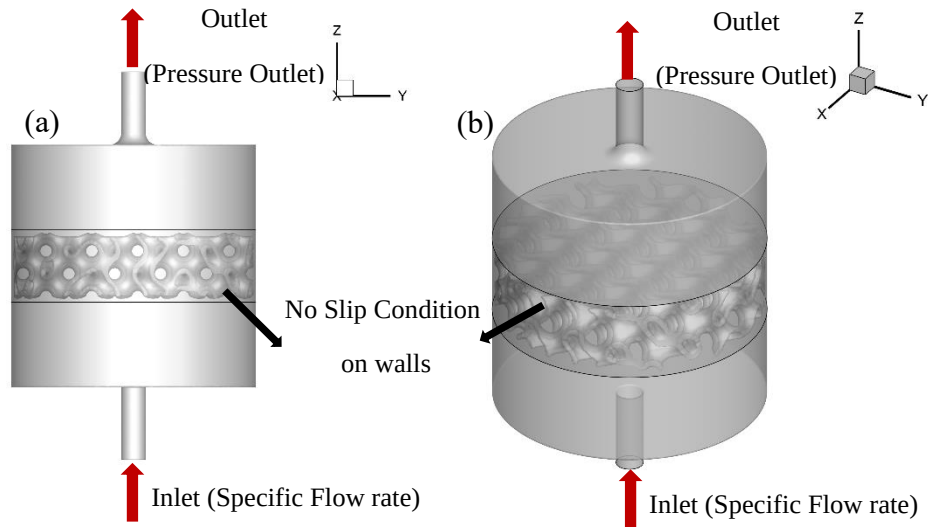


Figure 2. (a) cross-sectional and (b) 3D schematic view of the bioreactor

2.3.2. Mechanical Properties Evaluation

The mechanical properties of bone scaffolds play a significant role in their biological performances. Therefore, the compression test evaluated the mechanical scaffolds' performance (SANTAM universal testing machine). The test was performed at 0.5 mm/min speed in the temperature room. In addition, the compression tests were duplicated three times for both coated and uncoated scaffolds.

2.3.3. Biodegradability Measurement

Degradation test is investigated by immersing scaffolds in PBS solution (PH=7.4, 37°C) and incubating for 6 weeks. Scaffolds were washed with deionized water, freeze dried and weighted weekly. Biodegradability of the scaffolds are calculated as the following:

$$\text{Biodegradability Index \%} = \frac{W_i - W_n}{W_i} \quad (5)$$

Where W_i is the initial mass and W_t is the mass after n weeks.

2.3.4. FESEM Structure Characterization

The microstructures of the printed scaffold and cells attachment of PLA-cell and PLA-GO-cell scaffold samples were analyzed using FESEM-EDX (FESEM-EDX, JEOL, JSM 6701F). In this vein, cell-seeded scaffolds were immersed in a 2.5% glutaraldehyde mixture for 24 hrs. In the following, the scaffolds were rinsed with polybutylene succinate (PBS) and were immersed in 1% osmium tetroxide in 0.1 M sodium cacodylate for two hours. Then, using a series of ethanol solutions, scaffolds were dried out. Next, the scaffolds were cut to coat the cells and scaffold with Au.

2.3.5. XRD Analysis

The prepared samples' crystal structure and phase identification were determined by the X-ray diffraction (XRD) technique using an INEL EQUINOX 3000 diffractor and CuK α lamp ($\lambda = 1.5416$ Å). The XRD peaks for PLA-GO-cell were measured in the range of $2\theta = 10^\circ - 35^\circ$ and were plotted in the range of $2\theta = 10^\circ - 35^\circ$ using Origin-Pro software.

2.3.6. FTIR Analysis

FT-IR analysis was performed to characterize different functional groups of GO by using Shimadzu FT-IR 8000 series Fourier Transform – Infrared spectrophotometer.

2.3.7. Measurement of the Contact Angle

In order to determine the surface wettability of the PLA and PLA-GO, the contact angle was measured. In this regard, $2.5 \mu\text{L}$ of distilled water was dropped on the surface, and the image was captured with a Dino2 digital microscope, and its software was used to measure the contact angle. All the tests were repeated five times, and the results of contact angle were presented as means with standard deviation.

2.4. Dynamic vs. Static Cell Culture

According to the previous sections, dynamic cell culture can enhance mass transfer across the scaffold and stimulate cell growth by applying mechanical force. Therefore, the perfusion bioreactor was designed. This perfusion bioreactor setup consisted of a peristaltic pump, silicone pipes, a culture medium reservoir, and the scaffold chamber. The culture chamber was designed in a cylindrical shape so that the radius of the chamber was equal to the scaffold's radius, which can be fixed in the bioreactor with tangential force and not moved during the fluid flow. The cylindrical shape of the bioreactor was 20 mm in diameter and 5 mm in height. Figure 1 presents the designed and fabricated perfusion bioreactor setup. Also, the inlet and outlet of the bioreactor chamber were considered to be 2 mm. The flow rate of the bioreactor was determined to be 0.6 mL/min based on the previous research. Both the dynamic and static scaffolds were incubated at 37 °C and 5% CO₂.

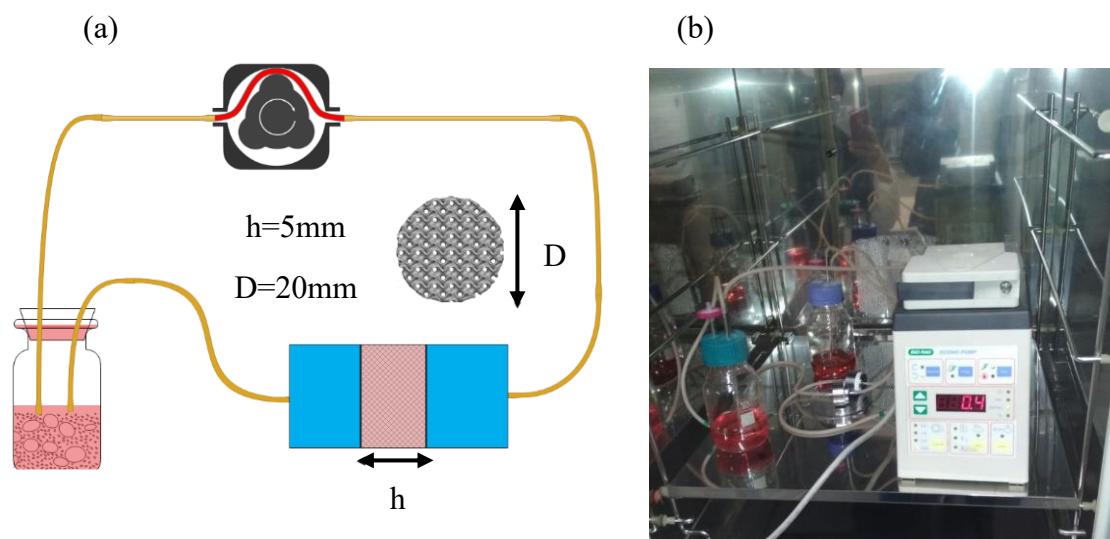


Figure 3. (a) Schematic and (b) experimental setup of the bioreactor

2.5. Evaluation of Cells Proliferation by MTT Assay

Before cell seeding, all the scaffolds were sterilized by immersing in ethanol for 2 hours, washed three times in PBS, and exposed to ultraviolet (U.V.) light for half an hour. The L929 cell line with the density of 2×10^6 cells per scaffold was chosen for cell seeding. The cell seeding was performed so that 2×10^6 cells were suspended in 1 ml culture media, and 15 μ l of culture media-cells solution were seeded in the scaffolds every 15 minutes and incubated for 15 minutes. After seven days, The MTT test was performed to specify the cell viability in both dynamic and static cell cultures. First, the scaffolds were rinsed with PBS and then placed into a 6-wells plate including the ratio of 5:1 culture medium and MTT (with 5 mg/ml concentration in PBS), and then were incubated for 3 hours at 37 ° C. Following, 500 μ l of DMSO was added to the wells, and the scaffolds were washed entirely, and the ELISA user read the absorbance at 570 nm wavelength.

2.6. Statistical Analysis

Biological data are represented as mean $\pm$ standard deviation. The statistical analysis was conducted by one-way analysis of variance (ANOVA) using SPSS software. Statistical significance was accepted as $P < 0.05$.

3- Results

As mentioned before, a function-based method was employed to generate scaffold geometry. The optimal scaffold was fabricated after a pre-analysis with computational methods. Simulating the scaffolds prior to fabrication would aid in selecting the desire case and obtaining the best results in the experimental section.

3.1. CFD Simulation

A CFD analysis was performed to investigate the pressure drop, fluid velocity, and wall shear stress on different TPMS scaffolds. Three geometries were produced by selecting $S=0.5$, 0.6 , and 0.75 and three flow rates were simulated for each geometry: $Q=0.4$, 0.6 and 0.8 ml/min. Figure 4 shows the velocity contour and stream lines for the bioreactor. As can be seen, the flow has a high velocity at the inlet and loses momentum upon entering the bioreactor. Then, fluid flows through the pores and exits the bioreactor. Velocity contours in the scaffold demonstrate that size of the pores gets smaller by decreasing S and flow has larger magnitude in the scaffolds. Larger velocity in the pores expose higher skin frictions and leads to larger shear rates and pressure drop in the system.

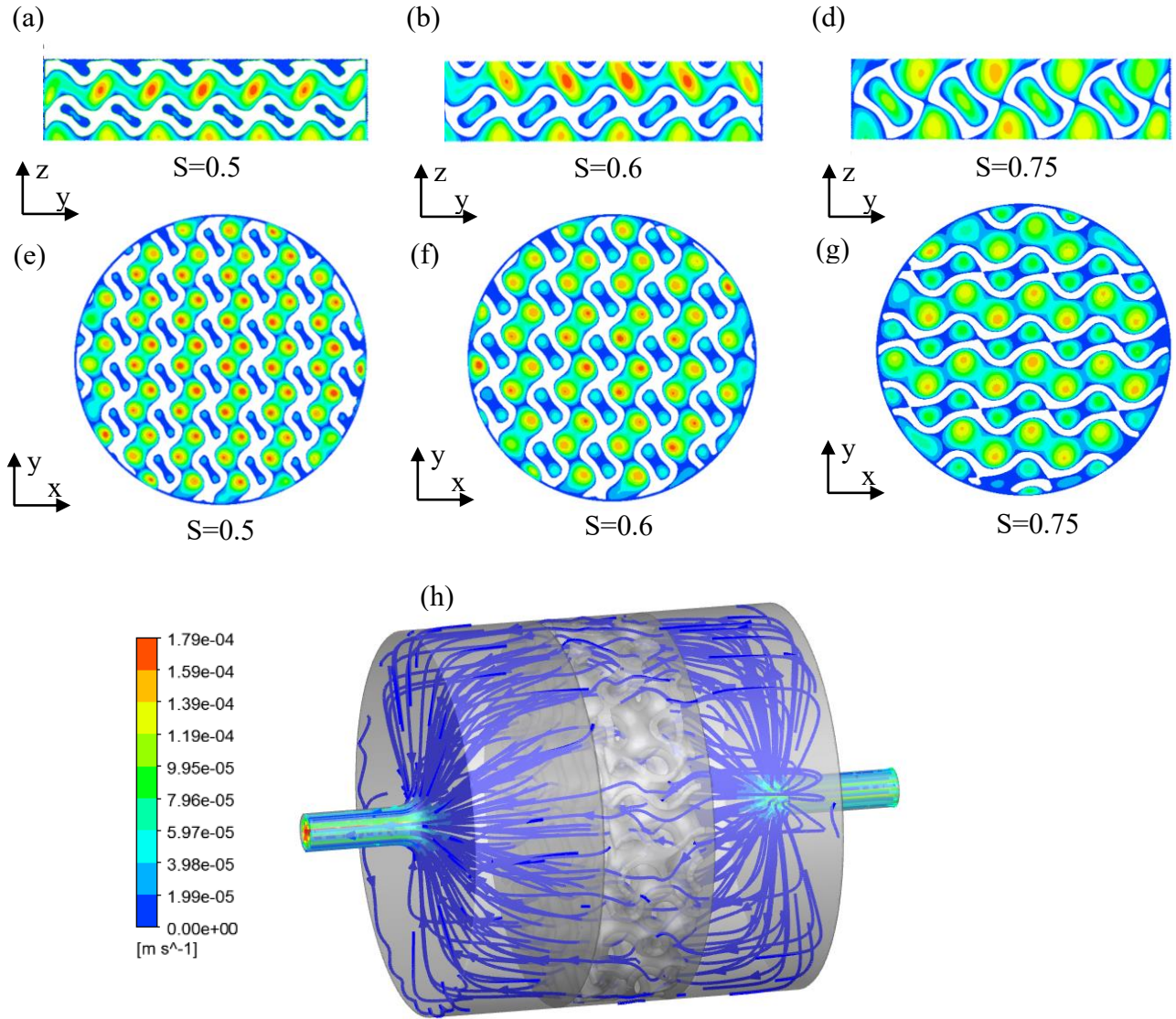


Figure 4. velocity contours and fluid streamlines in the bioreactor

Figures 5a, b, and c depict the local pressure contour for TPMS scaffolds. As can be seen, scaffolds with a lower S expose larger pressure drops to the bioreactor, and scaffold (1) has the maximum pressure drop as it has the smallest pore sizes. Due to the larger shear rates and frictional losses, larger pressure drops can be seen at higher flow rates.

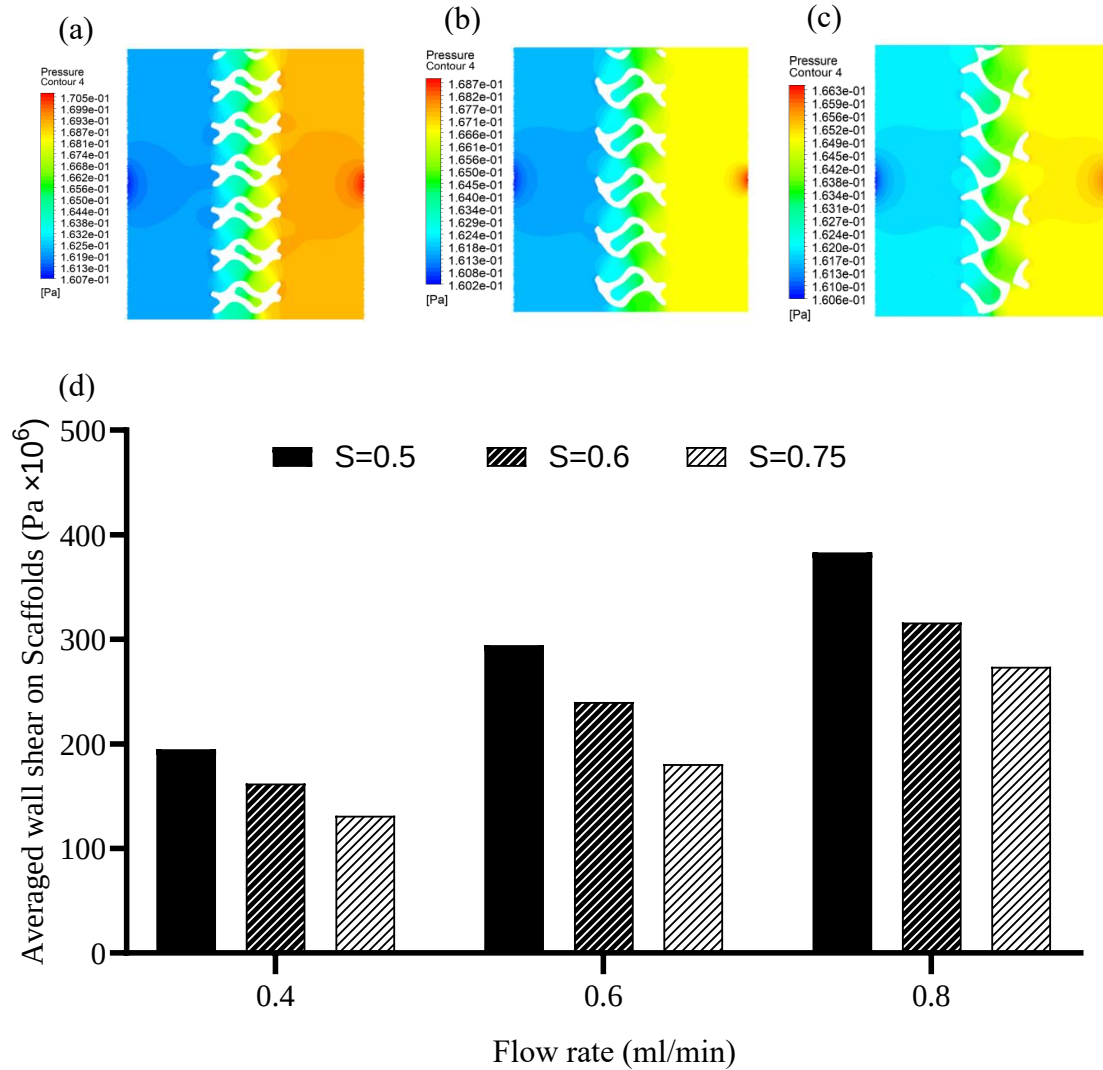


Figure 5 – (a) to (c) local pressure contour at S=0.5, 0.6 and 0.75; (d) mean wall shear stress in scaffolds

The effects of TPMS geometry on wall shear stress (WSS) were also investigated using CFD analysis. Figure 5d shows the mean WSS for all scaffolds. It shows that mean WSS increases at higher flow rates, and increasing the S parameter changes its value. Smaller pore size and higher fluid velocity in the cavities at S=0.5 result in a larger WSS compared to the higher S parameter. Previous research reported that higher shear rates on the scaffolds and cells have positive effects on the cell culture [37]. Figure 5d shows that scaffold (1) has the maximum WSS at all the flow rates and can be chosen for the desired case for fabrication.

3.2. Mechanical Properties and In Vitro Experiments

Figure 6 shows the designed and printed geometry of the scaffold. Since the FDM 3D printing is a layer-by-layer process, some anomalies can be seen in scaffolds, and the final geometry has differences compared to the designed CAD, which this feature can change the biological properties of scaffolds. Figure 6(a) shows the designed CAD file, which is the input of the 3D print. Figures 6(b) and 6(c) are the images of printed scaffold that were captured by Dino digital microscope and SEM. As can be seen, the scaffold has layer-by-layer nature due to the characteristic of the FDM method. Besides the mechanical properties effects of these layer-by-layer structures, they can significantly affect the biological properties of scaffolds, specifically cell attachment. Figure 6(d) is the SEM image for the PLA-GO scaffold, and the 3D printing layers are faded, as seen. However, the surface of the scaffold has cavities on the surface, which will affect the scaffold properties besides the coated GO. These cavities on the surface appear due to the kinetics of the corrosion because of the evaporating droplets [38]. Moreover, the mechanical properties of scaffolds are shown in Figure 6(e). The compressive test showed that coated scaffolds have a lower mechanical property which is due to the immersion of TPMS-based PLA scaffolds in chloroform. The value of young modules for coated scaffolds is about 15 MPa, and this value for uncoated scaffolds is about 27 MPa. Figure 6(f) demonstrate the biodegradability of the scaffolds. As can be seen, GO effects biodegradability after 6 weeks and has more degradation rate compared to the simple PLA scaffolds. This is because GO with oxygen-containing functional groups attract water molecules and transported them into the interface of GO and PLA matrix. As a result, molecular chains of the PLA break due to the attack of water molecules on ester bonds of PLA [39].

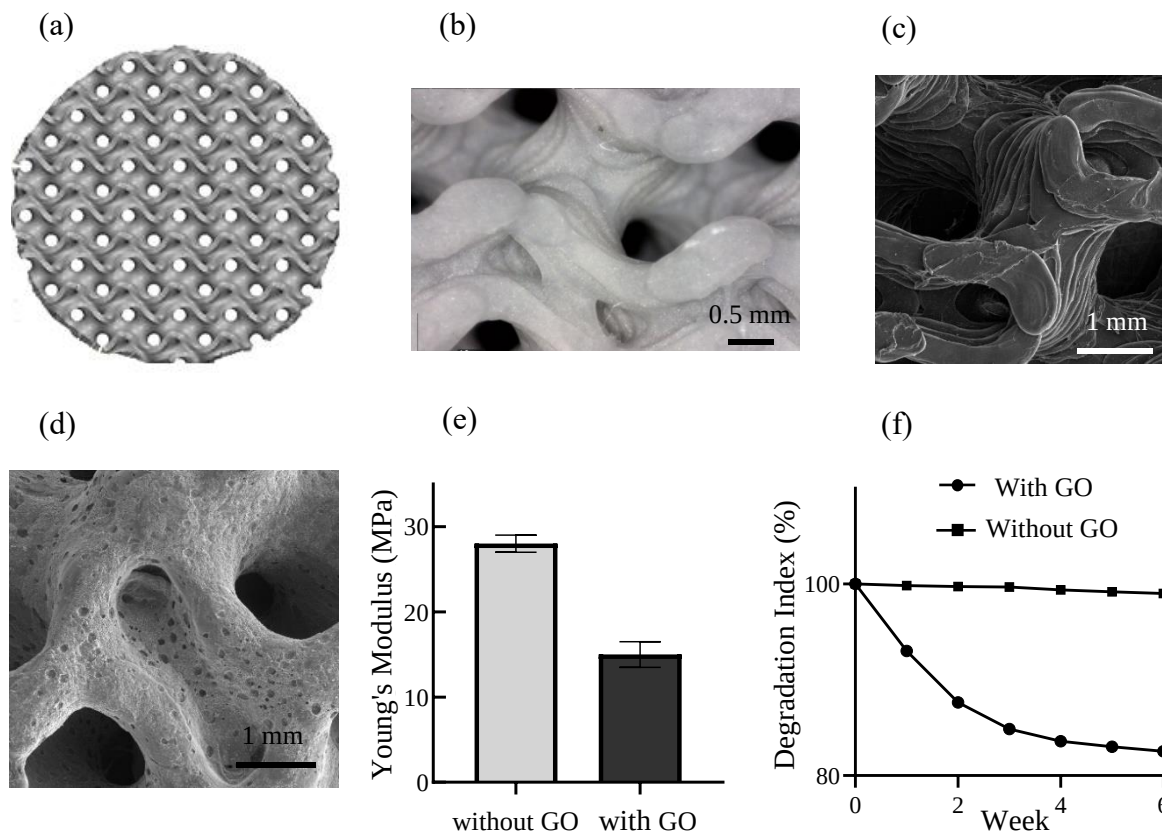


Figure 6. Images of the scaffold. (a) the CAD scaffold. (b) and (c) are the digital microscope and SEM images of the PLA scaffold, which reveals that the scaffolds are layer-by-layer. (d) the SEM images of the PLA-GO scaffold shows the roughness on the surface (e) mechanical properties of scaffolds.

As discussed in Figure 6, deep-coating scaffolds in chloroform change the surface morphology. MTT assay is performed to assess the effects of these cavities on the cell viability. Two surface-treated scaffolds in chloroform and chloroform-GO solution are analyzed and compared with a bare scaffold. Figure 7 shows the MTT results of cell culture for each scaffold under various conditions. Results show that cavities do not have significant effects on cell viability, while PLA-GO scaffold enhances cell viability.

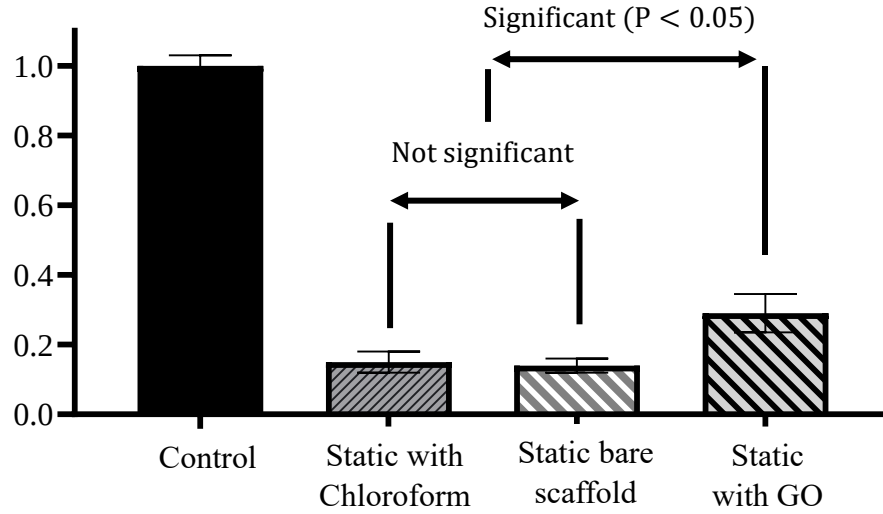


Figure 7. MTT assay results for static cell cultures in the Two surface-treated PLA scaffolds in chloroform and chloroform-GO solution and the bare PLA scaffold

To ensure GO's presence and coating on the PLA surface, FTIR, XRD, and EDX analyses are performed. Figure 8(a) shows the FT-IR spectra. The sharp peak around 2666.67-2904.75 was due to CH and CH₂ stretching. The bond located at 3522.27 was attributed to the presence of the OH group. The bonds located at 1061.15, 1231.1, 1518.68, 1720.1, and 2119.33 cm⁻¹ are attributed to C-O, O-H, C=C, C=O, and C-H functional groups. Therefore, FT-IR results revealed the presence of GO in samples and correspond with the reference article [40]. The XRD patterns for the crystal structure of the PLA-GO-cell scaffold are illustrated in Figure 8(b). It indicates a broad peak around 20 ~ 27°, which is consistent with GO's honeycomb structure. All other peaks are identical to the reference paper, which is presented peaks for PLA [41]. Furthermore, it showed that the coating procedure was effective, and the PLA-GO structure was well established. Energy dispersion X-ray (EDX) analysis was used to determine the structure and chemical characteristics or purity of the sample. The EDX results demonstrate the presence of primary chemical elements on the samples. Figures 8(c) and 8(d) show the results of the EDX analysis for the PLA and PLA- GO bone scaffolds, respectively, which expresses the proportion of elements on the surface of the scaffolds. By comparing Figures 8(c) and 8(d), it can be seen that the percentage of carbon element in the PLA-GO bone scaffold sample is higher than in the PLA bone scaffold sample, indicating the presence of a GO layer on the bone scaffold.

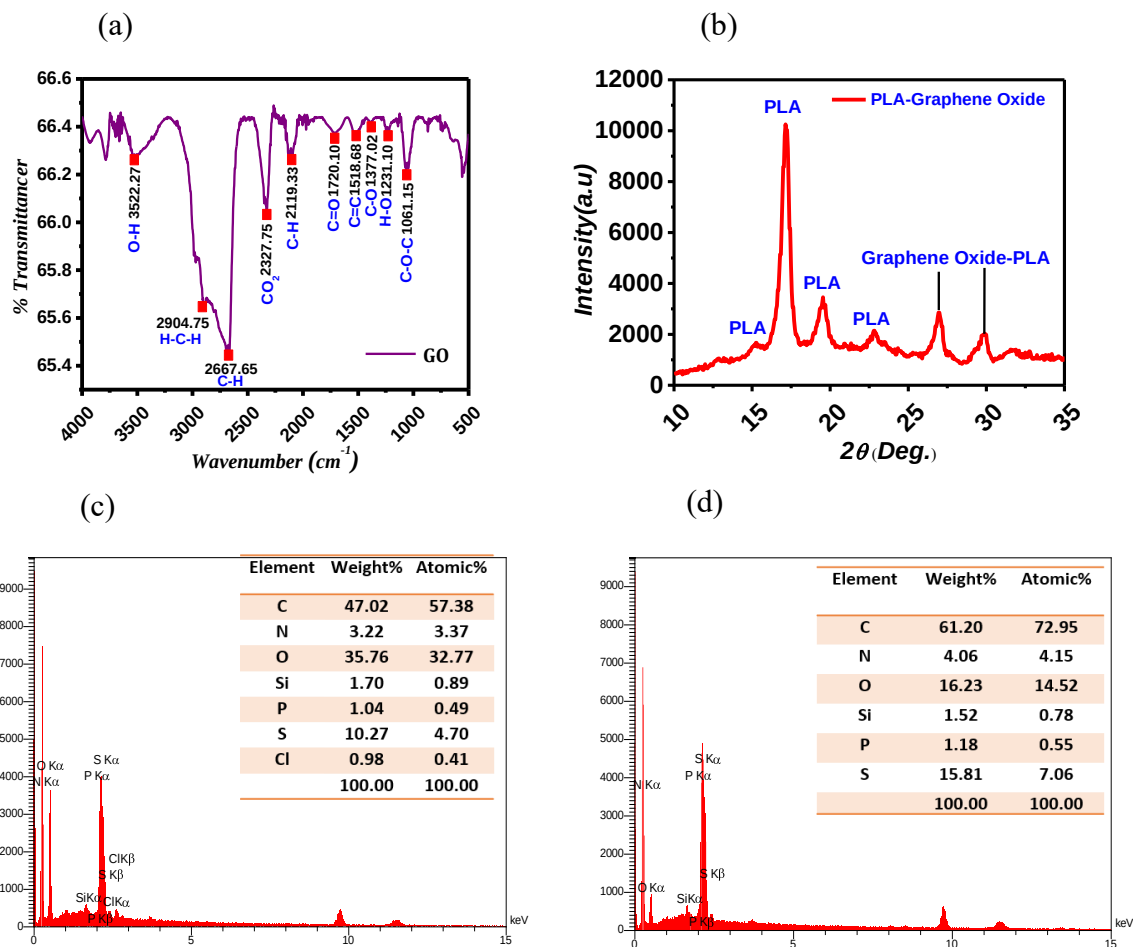


Figure 8. Chemical analysis of PLA-GO scaffold. (a) FTIR results for PLA-GO and the associated bonds for each peak. (b) XRD results for PLA-GO and the associated bonds for PLA and GO on the surface. (c) and (d) are the EDX results for PLA and PLA-GO scaffolds and shows the presence of each component on the surfaces.

FE-SEM images were obtained to analyze the morphology of the samples. Figure 9(a) shows the FE-SEM image of the PLA scaffold after cell culture. The attached cell on the surface is observable and scaffold has a smoother surface compared to the PLA-GO scaffold at Figure 9(b).

FE-SEM images for the PLA-GO-cell scaffold can be seen in Figures 9(b) and 9(c) with a scale-Sbar of 100 μm and 1 μm , respectively. As shown in Figure 9(b), GO nanoparticles are positioned as thin layers on top of PLA surfaces and scaffold has a high roughness surface. Figure 9(c) is a magnified FE-SEM image of a L929 cell on the PLA-GO scaffold and shows a good cell attachment.

After loading the GO on the PLA bone scaffold, a two-component structure is created, which is characteristic of composite GO samples. In the new PLA-GO scaffolds, stronger interactions are observed as a result of the extended surfaces of GO nanostructures, and also surface wettability is

affected not only by surface chemical characteristics but also by topographic parameters such as roughness and texture. For observing the hydrophilicity effect of the GO on the surface, the contact angles of PLA and PLA-GO were measured. As shown in Figure 9(d) and 9(e), the contact angles of PLA and PLA-GO are 68.25° and 51.13° , respectively. It is concluded that the GO had a positive impact on the surface of the PLA and improved wettability, which would result in higher cell adhesion and cell viability.

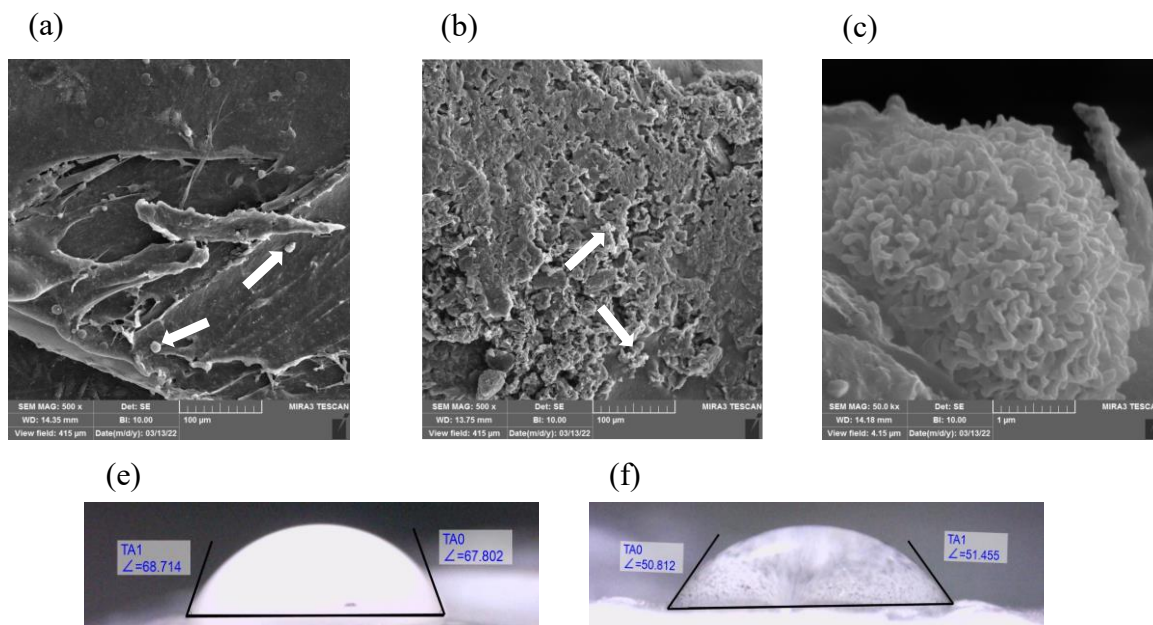


Figure 9. FE-SEM and contact angle images for the scaffolds. Figure (a) is FE-SEM image of the PLA scaffold after cell culture. Figure (b) is FE-SEM image of the PLA-GO scaffold after cell culture and figure (c) is the magnified image of a cell on the surface. Figure (d) and (e) are the images of the drops on the PLA and PLA-GO surfaces for contact angle measurement.

MTT results of cell culture for each scaffold under various conditions are depicted in Figure 10. The 2D-cell culture was employed as a baseline for comparison. According to Figure 10, GO totally improves cell viability and cell attachment. Effects of GO on static MTT results is considerable and shows more significant proliferation compared to the one without GO (up to 76%). Moreover, MTT results indicate that the static cell culture of the PLA-GO scaffold represents the same viability as the dynamic cell culture of the simple scaffold, and it is a notable issue. By comparing static and dynamic cell culture results, the prominent effect of the cell culture method is evident and shows the superiority of dynamic cell culture to static cell culture. Overall, the dynamic cell culture of the GO-contained scaffold in the proposed perfusion bioreactor demonstrated more significant proliferation compared

to the other cases and MTT assay results show improvements up to 134% in comparison to the dynamic cell culture without GO and up to 220% in comparison to the static cell culture without GO.

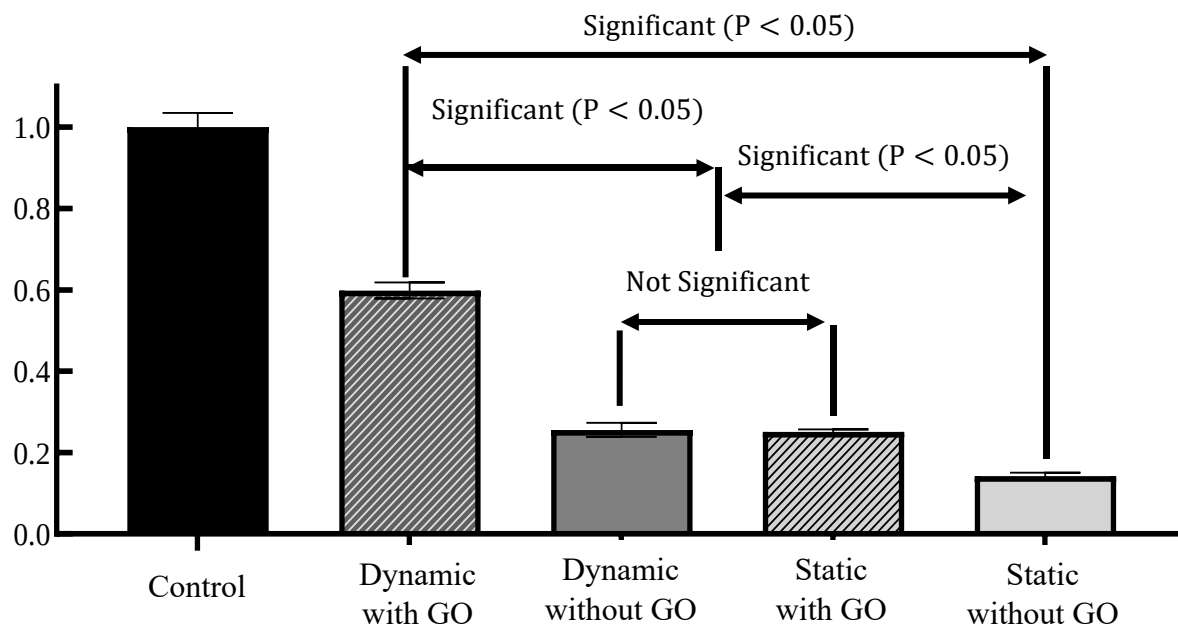


Figure 10. MTT assay results for dynamic and static cell cultures in the PLA and PLA-GO scaffolds.

Discussion

Polymer scaffolds are getting popular recently, and much clinical and academic research was focused on them. These new scaffolds are biocompatible and biodegradable and have good mechanical properties. However, research and explorations with various methods are ongoing to increase the biological and mechanical properties. Here, the PLA TPMS scaffold structure was designed and 3D-printed using the FDM technique. Later, GO is applied to the scaffold's surface by immersing it in a chloroform-GO solution. Then both static and dynamic cell cultures were conducted to study the biological response of bone scaffolds.

TPMS structures have been presented as high-performance structures that can be fabricated using FDM and other 3D printing techniques. These formations resemble the natural microstructure of bone. Several investigators have recently focused on these structures' mechanical characteristics and found them satisfactory candidates for bone regeneration applications due to their high volume-to-surface ratio. Previous researchers examined the impact of graphene and its derivatives on bone tissue and its regeneration capability [42]. Mohammadi et al. [43] investigated the PCL-GO fibers and observed increased protein absorption and higher cell adhesion and proliferation, but no biocompatibility change. Belaid et al. [14] analyzed PLA-GO and showed that this FDM composite scaffold is bioresorbable, biocompatible, biodegradable, and has excellent mechanical properties. However, coating GO with chloroform would decrease the mechanical properties. As the coating would lower the mechanical properties, scaffold design would be an essential part, and employing a well-constructed TPMS structure would compensate for this defect, as it is proved that the TPMS structures would have greater mechanical properties [44]. Also, this type of scaffold can be used alternatively in cases where mechanical properties are not a considerable issue, and it is not under much stress. For example, In the case that we want to use PCL as the scaffold biomaterial, we can use PLA biomaterial coated with GO, with the difference that it has a better biological response. As a result, GO Combination with PLA in a TPMS structure would help the mechanical and biological properties of the scaffold. Here, the PLA TPMS scaffold structure is designed and 3D-printed with the FDM technique. Later, GO was applied to the scaffold's surface by immersing it in a chloroform-GO solution. Coating GO, just on the surface, diminished the risk of toxicity of GO as it has a low concentration in the structure, according to the studies that answered concerns about its potential toxicity and possible residue in vivo [42], [45]. Also, as reported by other studies [46], [47], PLA-GO

scaffolds demonstrate higher hydrophilicity rather than PLA scaffolds, promoting cell adhesion and viability, and this study agrees with those findings.

The cell culture method would have positive effects on cell viability too. Static cell culture is a simple method for reaching satisfactory results, but has some limitations that should be overcome. Dynamic cell culture is preferred in the high thickness scaffolds, and its impact on the peripheral and central cell density is inevitable [48]. In previous research, static and dynamic cell culture and their effects in a TPMS scaffold were investigated and showed that dynamic cell culture can enhance biological response and mass transport through the scaffolds and applies mechanical loading to the cells, which helps cell proliferation [49].

As a result, dynamic cell culture is conducted with static cell cultures to increase the biological response in the designed bioreactor. The MTT assay results show that dynamic cell culture and GO coating provided a suitable scaffold with considerable viability. The effect of static cell culture in the PLA-GO scaffold is comparable with dynamic cell culture; as can be seen, the MTT assay for static cell culture in the PLA-GO scaffold is as good as the pure PLA scaffold in dynamic cell culture. It shows the superior performance of Static and dynamic cell culture in TPMS –based PLA scaffolds in the presence of GO nanoparticles at the surface.

4- Conclusion

One of the medical procedures used to treat bone diseases and disorders is TE. Several strategies are offered in this field to overcome the limitations and deficiencies to make this method more practical. This research presents a surface-treated TPMS-based PLA structure as a scaffold to enhance the mechanical and biological characteristics.

TPMS designs can withstand greater loads, and their porous structure closely resembles bone structure. FDM 3D printing is employed to fabricate the structure. GO is coated on the surface of the TPMS PLA scaffold to promote biological and mechanical properties. The PLA and PLA-GO scaffolds are characterized using FESEM, EDX, FTIR, XRD, contact angle, and compression tests. For a more excellent scaffold, dynamic cell culture is performed and compared with conventional static cell culture. All the cases are analyzed via MTT assay to measure cell viability.

- 1) Comprehensively, dynamic cell culture in the TPMS scaffold demonstrates superior performance and results in significantly increased viability and proliferation.
- 2) The PLA-GO scaffold exhibits greater hydrophilicity and may aid in cell culture.
- 3) There is a decrease in the compressibility of the PLA-GO scaffold. However, if weaker materials than PLA are needed to design scaffolds, such as PCL, Go-coated PLA could be used, as immersing in chloroform can reduce mechanical properties.
- 4) The PLA-GO scaffold has shown high cell viability, and the results of the MTT test indicate that the static cell culture response of the PLA-GO scaffold performed better than the dynamic cell culture on the PLA scaffold.
- 5) CFD analysis shows that the pressure drop, velocity contour and wall shear stress in the scaffolds depend on the geometry of the TPMS structure and flow rate, and CFD analysis can be used to choose the optimum geometry for printing.
- 6) Higher flow rates increase pressure drop, velocity magnitude and wall shear stress in the scaffold.
- 7) Smaller geometrical parameter (S) increase velocity magnitude and wall shear.

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