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Development of 3D Printed PLGA/TiO₂ Nanocomposite Scaffolds for Bone Tissue Engineering Applications

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Abstract

Porous scaffolds were 3D-printed using poly lactic-co-glycolic acid (PLGA)/TiO₂ composite (10:1 weight ratio) for bone tissue engineering applications. Addition of TiO₂ nanoparticles improved the compressive modulus of scaffolds. Differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA) revealed an increase in both glass transition temperature and thermal decomposition onset of the composite compared to pure PLGA. Furthermore, addition of TiO₂ was found to enhance the wettability of the surface evidenced by reducing the contact angle from 90.5 ± 3.2 to 79.8 ± 2.4 which in favor of cellular attachment and activity. The obtained results revealed that PLGA/TiO₂ scaffolds significantly improved osteoblast proliferation compared to pure PLGA ($P < 0.05$). Furthermore, osteoblasts cultured on PLGA/TiO₂ nanocomposite represented significantly higher ALP activity and improved calcium secretion compared to pure PLGA scaffolds ($p < 0.05$).

Keywords

3D printing; Nanocomposite scaffold; PLGA; TiO₂; Tissue engineering

1. Introduction

Bone tissue engineering (BTE) proposes an alternative approach to the repair and regeneration of damaged bone tissue [1, 2]. The principle involved is the regeneration of living tissue, where critically-sized bone defects emanated from infection, tumor resection, or traumatic fractures cannot be healed spontaneously, and external interventions are needed in most cases to regenerate new bone to maintain or improve its function [3]. One approach is to incorporate osteoblast cells onto three dimensional (3D) scaffolds that can physically support them. The structure and properties of the scaffold is therefore very important for both in vitro and in vivo functioning.

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There are many requirements and limitation for developments of an efficient 3D scaffold [4]. The biocompatibility and bioactivity of BTE scaffolds impact the initial steps of cell attachment, prior to cell proliferation and differentiation. The macroscopic and microscopic porosity of scaffolds can simultaneously improve cell growth and delivery of nutrients and oxygen to the cells. Moreover, controlling the mechanical properties of scaffolds and providing an appropriate media for final tissue growth are considered as another important characteristic of scaffolds in tissue engineering [5–7]. Degradation of porous scaffolds also plays a critical role in tissue engineering due to its importance in cell viability and growth [8].

Different methods have been employed for the fabrication of 3D-scaffolds including microsphere sintering [9, 10], freeze-drying [11], porogen leaching [12], 3D-printing [13] etc. among which 3D printing is becoming popular progressively owing to its precise control over the pore size and architecture of the prototype [14].

Owing to advances in computer-aided design (CAD) and fabrication technologies, progress in the field of 3D-printing has accelerated in recent years thereby the development of biocompatible systems for tissue engineering applications has been facilitated [15]. Different techniques of 3D-printing including laminated object manufacturing (LOM) [15], selective laser sintering/melting (SLS/SLM) [16], stereolithography (SLA) [17] and fused deposition modeling (FDM) [18] have been used to process various materials among which the latter is mainly used for printing thermoplastic polymers such as polycaprolactone (PCL), polylactic acid (PLA) and poly(lactic-co-glycolic acid) (PLGA) [19, 20]. Favorable degradation characteristics of PLGA owing to adjustable ratio of lactide to glycolide [21] has encouraged the researchers to focus on 3D-printing of porous scaffolds made of PLGA and PLGA containing formulations. Vozzi et al. reported fabrication procedure of PLGA scaffolds using soft lithography and microsyringe deposition [22]. Guo et al. investigated the effect of lactic acid: glycolic acid (LA:GA) molecular weight ratios and printing parameters on print resolution and developed a model to predict precision under different combinations of printing conditions and material compositions [20]. 3D-printed scaffolds of pure PLGA or in combination with other materials have been evaluated in vivo too. Ge et al. evaluated PLGA scaffolds for bone regeneration within the intraperiosteum and iliac bone defects in a rabbit model. Histological analysis showed that despite initial tissue ingrowth within the scaffolds at 4 weeks post-surgery, the implanted scaffolds facilitated new bone tissue formation and maturation over the time course of 24 weeks in both defect models [23]. In another study, Shim et al. 3D-printed a guided bone regeneration membrane using PCL/PLGA/ β -TCP and loaded the membrane with rhBMP-2 and achieved almost entire healing of rabbit calvaria defects within 8 weeks [24].

Despite the favorable properties of PLGA, chemical hydrolysis reaction of ester bonds on its backbone triggers the production of carboxylic acid functional groups which causes an acidic milieu and reduces mechanical properties of the scaffold [25]. The composite scaffolds in turn demonstrate higher mechanical properties and improved cell behavior in comparison with conventional composites [26]. In this regard, it has been reported that ceramic particles such as bioglass and calcium phosphates [27] have been chiefly used as additives to enhance the poor mechanical properties of PLGA. Such PLGA-based composite

scaffolds can be employed as the bone substitute materials and injectable pastes to fill defects because of their fascinating biocompatibility and osteoconductivity properties [28]. Recently, nanocomposites of TiO_2 have drawn considerable attention in biomedical applications because of their effect on mechanical properties and bioactivity [29–31]. It has been reported that PLGA composites containing 20 wt% of TiO_2 nanoparticle could enhance the formation of hydroxyapatite (HA) after 21 days when exposed to simulated body fluid (SBF) [32]. Furthermore, Salarian et al. reported that the incorporation of TiO_2 nanofibers into polymer matrix could increase the tensile and flexural properties of bone cement composites [33]. Furthermore, addition of Titania to PLGA microspheres has been shown improve both mechanical properties and osteoblast proliferation [34].

In the present study, PLGA/ TiO_2 scaffolds were fabricated through 3D printing. The effect of the addition of TiO_2 to PLGA on the mechanical properties 3D-printed scaffolds was investigated. Finally, osteoblast proliferation, alkaline phosphatase activity and calcium secretion were evaluated for PLGA/ TiO_2 scaffolds compared to PLGA ones.

2. Experimental details

2.1. Materials

Poly(D,L-lactide-co-glycolide) (PLGA) with lactide to glycolide ratio of 50:50 (PURASORB PDLG 5004) was purchased from Corbion (Netherlands). TiO_2 (rutile) was obtained from Strem Chemicals Inc. (USA). All the solvents were of analytical grade.

2.2. Composite preparation and characterization

The PLGA/titania nanocomposite to be printed was prepared through solvent casting method. Briefly, 1 g of PLGA was dissolved in 20 ml dichloromethane followed by addition of 100 mg TiO_2 powder. The mixture was vigorously agitated to obtain a uniform suspension and dried at room temperature for 24 h. Subsequently, the obtained film was transferred to a vacuum oven and kept for an extra 24 h to completely remove the organic solvent.

A differential scanning calorimeter (DSC 404 F1, NETZSCH, Germany) was used to determine the effect of addition of TiO_2 on glass transition temperature and thermal degradation of the polymer thereby the interaction of TiO_2 and polymer chains at the interface can be investigated. The PLGA and PLGA/ TiO_2 nanocomposite were heated from room temperature to 80 °C and 350 °C for glass transition temperature and degradation tests, respectively, at a heating rate of 10 °C/min in aluminum pans while argon was used as the purge gas. The resulting DSC curves were analyzed to determine the glass transition (T_g) temperature and onset of thermal degradation.

Thermogravimetric analysis (TGA) of PLGA and PLGA/ TiO_2 composite was performed using a SDT650 TGA/DTA (TA Instruments) under nitrogen atmosphere. Approximately 15 mg of samples was placed in designated crucibles and heated at a 10 °C/min rate or underwent the following program: heated to 280 °C at 20 °C/min, then to 390 °C at 2 °C/min and finally to 460 °C at 10 °C/min.

The wettability of pure PLGA and composite films was evaluated through contact angle measurement. A 5 μ l drop of DI water was placed on the samples, and the image was captured using a Dino-lite digital microscope camera. Each image was then processed using ImageJ software to determine the contact angle.

2.3. Scaffold preparation via 3D-printing

The composite film was chopped and fed into the 3D-printer reservoir. A 3D-Bioplotter (Envision TEC, Germany) was used to deposit the struts of the scaffolds at high temperature mode. The printing parameters have been tabulated in Table 1.

2.4. Scaffold characterization

The surface characteristics and morphology of scaffolds were studied by 3D laser scanning digital microscopy (Olympus LEXT OLS 4000, Japan). The porosity of the scaffolds was measured through a liquid displacement (LDM) method [35]. Briefly, after measuring the weight of a scaffold (W_i), it was immersed in a certain volume of ethanol (V_1) at 25 °C. Each scaffold was kept in ethanol for 5 minutes to ensure complete penetration of the solvent into the pores. Then, the volume of the ethanol after immersion of the scaffold was recorded (V_2). The change in the ethanol volume ($V_2 - V_1$) represents the volume of the scaffold. Using the initial (W_i) and final (W_f) weights of the scaffolds after soaking in ethanol, the pore volume of scaffolds can be achieved as $(W_f - W_i)/\rho_{\text{ethanol}}$ ($\rho_{\text{ethanol}} = 0.789 \text{ g/cm}^3$) and the porosity can be calculated using the following equation:

$$\text{Porosity} = \frac{(W_f - W_i)/\rho_{\text{ethanol}}}{V_2 - V_1} \quad (1)$$

The compressive strength and modulus of the scaffolds were measured using mechanical testing machine (AGS-X, Shimadzu, Japan) with a 5 kN load cell and cross head speed of 1 mm/min.

2.5. Cell culture

The Seeding efficiency on the scaffolds was determined using (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) assay and calculated according to equation (2). Human osteoblasts (HOB, Cell Applications, USA) were seeded onto PLGA and PLGA/TiO₂ scaffolds as well as the wells of 24-well plate as the control groups at a density of 3×10^4 cells/well and were incubated under standard culturing conditions. Cells were allowed to attach for 24 and then the scaffolds were transferred to new well plates. The number of remaining cells attached to the bottom of each plate after transferring the scaffolds was quantified through MTT assay. Briefly, 100 μ L MTT solution (5 mg/ml) was added to each well and incubated for 3 h. Then, the mixture of cell culture media and MTT dye was replaced by 1 ml DMSO and shaken for 10 min to dissolve formazan crystals. Finally, the optical density (OD) was recorded at 570 nm using a Multi-Mode Reader (Synergy HTX, BioTEK) and the seeding efficiency was calculated using the following equation:

$$\text{Seeding efficiency} = \left(1 - \frac{\text{OD of remaining wells after transferring the scaffold}}{\text{OD of control wells}} \right) \times 100 \quad (2)$$

Cell attachment was investigated using scanning electron microscopy (SEM) (JEOL JSM 6510, Japan) and laser microscopy (LEXT OLS 4000, Olympus, Japan). The osteoblast cells were seeded onto the scaffolds and allowed to attach. After 24 h, samples were washed using copious amount of phosphate buffered saline (PBS). Afterwards, the cells were fixed using Karnovsky's fixative method (composed of Paraformaldehyde-Glutaraldehyde) for 2 h. Then, the samples were rinsed with PBS and post-fixed utilizing 1% Osmium Tetroxide solution for 1.5 h. Subsequently, the scaffolds were washed again and dehydrated by the means of ascending ethanol series (50, 70, 80, 95 and 100 v/v%). Finally, the samples were chemically dried using 50 and 100 v/v% Hexamethyldisilazane (HMDS) each for 10 min and left at room temperature for complete drying. The imaging was conducted after sputter-coating with gold utilizing secondary electron modes at different magnifications. The same procedure was conducted for laser microscopy imaging except the gold coating step.

Alamar Blue assay as a fluorometric indicator of cell metabolic activity was employed to determine viability and proliferation of normal human osteoblasts (HOB). The cell-scaffold constructs were washed with PBS after removing the cell culture media and then refed with 1.8 ml of medium and 0.2 ml of the Alamar Blue dye and incubated for 4 h. The resulting solution was pipetted out of the wells and the fluorescence measurement was conducted at room temperature by using a Multi-Mode Reader at the excitation/emission wavelengths of 540/590 nm.

Alkaline phosphatase (ALP) activity (n= 3) was assessed utilizing an ALP assay kit (G-Biosciences, St Louis, MO, USA). The cells were digested, collected, and lysed after 7 and 21 days. Subsequently, the lysates were reacted with p-nitrophenyl phosphate (p-NPP), and the absorbance of p-nitrophenol was measured at the wavelength of 405 nm using a Multi-Mode Reader.

Alizarin Red staining, a common histochemical technique, was used to detect and quantify calcium deposit on the scaffolds after 3 days of osteoblast culture. Positive alizarin red staining for calcium has been demonstrated to represent calcium phosphate by energy-dispersive spectroscopy (EDS) [36]. The scaffolds were washed with deionized water three times, and stained with 40 mM Alizarin Red S solution (pH = 5.5) for 20 min at room temperature while being shaken. Finally, the excess dye was removed and the samples were washed with distilled water, air-dried and observed under light microscope. For quantitative analysis, alizarin red was eluted with 10% acetic acid, under mild shaking for 15 min and optical density was measured at 405 nm. The obtained values were normalized to seeding efficiency and reported as the ratio of calcium content for each scaffold to calcium content for control (well plate). The precipitated calcium on the samples was also detected using Energy-dispersive spectroscopy (EDS, Thermo Fisher Scientific).

2.6. Statistical analysis

The statistical analysis was performed using one-way ANOVA followed by two-tailed t-test as post-hoc analysis. Differences were considered significant for p-values < 0.05.

3. Results and Discussion

3.1. Composite characterization

DSC is widely used to determine transition temperatures such as glass transitions, melting, denaturation, cross-linking reactions, purity and decomposition rate [37–39]. The thermal properties of PLGA/TiO₂ nanocomposite as well as pure PLGA were examined by DSC and shown in Figure 1. As clearly seen, the DSC measurements show the effect of TiO₂ particles on PLGA matrix regarding both glass transition and decomposition temperature. Figure 1A and B illustrate the glass transition temperature (T_g) of pure PLGA and PLGA/TiO₂ nanocomposite, respectively. The glass transition temperature increased about 2°C by addition of about 10% TiO₂. The increase in T_g might be a results of attractive polymer–filler interfacial interactions that cause the local dynamics of the polymer to slow down [40].

The effect of nanoscale confinement through addition of fillers on the glass transition temperature (T_g) of polymers, is still controversial [41]. Some studies have reported increase in T_g upon addition of different fillers, reasonably expected due to physical adsorption or chemical attachment of polymer chains to rigid particles and consequently slowing down the polymer dynamics [42], while others report no change or even decrease in T_g [43]. Such disparate results can be explained by taking into account the nature of the interfacial interactions between the polymer and particles [44, 45]. In a study by Yim et al. [46], the increase in T_g of different composites compared to the unfilled polymer was correlated with the polymer–filler interaction energies measured by the heats of adsorption of the model compounds of the polymers on the filler surfaces. Torres et al. found that while lower TiO₂ content in PLGA-TiO₂ composite foams shifts the T_g toward higher temperatures, higher contents causes reduction in T_g [47]. Nanofiller clustering observed at high concentrations not only leads to the loss of interphase volume but also hinders the formation of a percolating interphase network in the nanocomposite and results in a decrease of T_g [48].

According to Figure 1C and D, the major endothermic events, onset of which can be observed at 246.5°C and 269.75°C, correspond to the decomposition of pure PLGA and PLGA/TiO₂ composite, respectively. The incorporation of TiO₂ into the polymer matrix shifts the decomposition endotherms towards higher values. It has been reported that addition of uniformly dispersed fillers, that do not have reactive sites that could catalyze degradation reactions, to PLGA can improve mechanical stability of the copolymer. The filler is suggested to effectively block the transport of decomposition products at the initial stages of decomposition thereby the decomposition progress is delayed to some degree [49].

The thermal degradation of pure PLGA and PLGA/TiO₂ composite was also investigate using TGA/DTA as seen in Figure 1E–H. The onset temperature of weight loss was found to be around 10 degrees higher for PLGA/TiO₂ compared to PLGA regardless of the ramp rate. At heating rate of 10 °C/min, PLGA weight loss began at 342 °C (Figure 1E) while addition of TiO₂ shifted the onset to 352 °C (Figure 1F). When 2 °C/min was employed, both onsets

shifted toward lower temperatures. The weight loss onset temperature was found to be 308 °C and 319 °C for PLGA (Figure 1G) and PLGA/TiO₂ (Figure 1H), respectively. The positive effect of TiO₂ on thermal stability of polyesters has been reported in other studies too [50]. The mechanisms suggested to explain the observed increase in weight loss temperatures are as follow [50–52]: In the initial stages, a big fraction of thermal energy is absorbed by TiO₂ particles compared to the polymers; it is probable that TiO₂ particles can interact with and retard the outflow of volatile degradation products.

3.2. Scaffold characterization

In this study, 3D-printing was used to fabricate desirable constructs with high resolution for bone tissue engineering. An interconnected porous structure with pore diameter of about 450–500 µm was achieved. Figure 2 depicts the 3D printed PLGA and PLGA/TiO₂ nanocomposite scaffolds. The porous network of the composite scaffolds can be clearly seen in the images taken using both regular camera and laser microscope. The laser microscope images confirmed the pore size of 450–500 µm for both PLGA (Figure 2C) and PLGA/TiO₂ scaffolds (Figure 2F).

In case of bone tissue engineering, mechanical properties of 3D scaffolds are obviously crucial to their biomechanical performance. Mechanical properties of the scaffolds depend on both composition and porosity. Interestingly, despite having higher porosity, the PLGA/TiO₂ scaffolds exhibited superior compressive modulus compared to the PLGA ones. The results of porosity measurement and compression tests are summarized in Table 2.

As seen in the table, the porosity of PLGA/TiO₂ was found to be higher than pure PLGA that might be a result of change in the viscosity and melting/softening temperature of the polymer through addition of TiO₂. Indeed, interaction of the polymer chains with filler particles not only increases the softening temperature as shown by DSC but also increase the viscosity of the formulation at a certain temperature [53, 54]. Since all the parameter including temperature and pressure used to print pure and composite scaffolds have been the same, a lower material flow rate is expected for the PLGA/TiO₂ formulation. As a result, strands become slightly thinner and porosity increases. The laser microscope images (Figure 2C, F) confirms the thinner strands for PLGA/TiO₂ compared to pure PLGA scaffolds. However, according to Table 1, the values of compressive modulus and strength for composite scaffolds are larger or equal to those of pure PLGA. The superior mechanical properties of PLGA/TiO₂ scaffolds can be attributed to the high surface energy of TiO₂ particles through which the linear chains of PLGA could coil on the surface of such particles [55]. It has been reported that preparation of composite scaffolds through incorporation of TiO₂ particles into PLGA matrix gives rise to similar mechanical properties within the range of cancellous bone (compressive modulus: 50–500 MPa, compressive strength: 2–12 MPa) [34]. The fabricated scaffolds in this study present slightly better mechanical properties than the mentioned range and hence, can be reliably used as an appropriate candidate for cancellous bone repair. Compared to the scaffolds prepared via microsphere sintering method that is well known for notable mechanical properties, the compressive modulus of PLGA and PLGA/TiO₂ nanocomposite scaffolds prepared in this study were significantly higher (489.22 ± 27.83 MPa vs. 111.18 ± 19.89 MPa for PLGA and 541.23 ± 11.80 MPa vs.

222.33 ± 20.16 MPa for PLGA/TiO₂) [34]. It highlights one of the aspects of superiority of 3D printing method over other methods of fabrications such as microsphere sintering.

3.3. Cell culture

The seeding efficiency of PLGA and PLGA/TiO₂ nanocomposite scaffolds was investigated using MTT assay and the results are tabulated in Table 3. No significant difference ($p > 0.05$) was observed between two groups in terms of seeding efficiency representing the consistency of seeding procedure and pertaining parameters.

The laser microscope images of the cells attached to the scaffolds are shown in Figure 3A. As can be clearly seen, the osteoblasts grew on the printed strands of the scaffold and formed an adherent plexus all over the scaffold. The bioactivity of the scaffold encourages the cells to anchor their cytoskeletal projections between the strands and induces cell migration along and in depth of the scaffold as seen in Figure 3B and C. Osteoblasts were found to be attached to the top 12 layers of the scaffold at different focus planes (Figure 3B and C) revealing a penetration depth of at least 2 mm. The total number of layers was 16 and only the bottom 4 layers was not reached by the cells; in other words, cells have covered around 75% of the depth of the scaffold. This reveals the capability of the 3D-printed scaffold to host a uniform distribution of the cells rather than only superficial accommodation. Accessibility of deep regions of the scaffold for the cells along with efficient diffusion of nutrients toward in-depth regions due to large pore size and channel like facilitates cell immigration. Proper cellular behavior of PLGA/TiO₂ nanocomposite scaffolds could be attributed to the micro-scale surface topography and high specific surface area due to presence of TiO₂ particles which leads to more biocompatible and bioactive surface for cells [56]. The microscale surface topography of composite scaffolds is essential for cell viability and osteoblast function. It has been reported that high specific surface area, micro and nanoscale surface topography can provide available sites for protein absorption which in turn increases the construct-cell interactions [57]. Topography-induced cell behavior has been suggested to be influenced by changes in focal adhesion (FA) arrangement, which in turn alters the actin cytoskeleton (CSK) and mechanotransduction of the cells. As an evidence, nanotopography-induced human mesenchymal stem cell (hMSC) differentiation through cell mechanotransduction has been shown to be modulated by the integrin-activated focal adhesion kinase (FAK) [58].

Focal adhesions, considered as mechanosensors, are sites of focal contact between the cell and the extracellular matrix. They are composed of integrin for physical connection and nonreceptor tyrosine kinases such as Focal adhesion kinase (FAK) for signal transmission to convert mechanical signals from extracellular matrix to cytoplasmic signaling in the cells mechanotransduction in osteoblasts [59–62].

Incorporation of TiO₂ particles in the formulation can enhance protein absorption and subsequent osteoblast adhesion as observed in the current study. It is in great accordance with the results obtained from TiO₂-coated PLGA films used to promote the attachment and proliferation of human dermal fibroblasts and rat cortical neural cells [63]. Furthermore, the presence of TiO₂ particles enhances the hydrophilicity of composite scaffolds which plays a pivotal role in attachment of HOB cells on PLGA/TiO₂ scaffolds [64]. To investigate the

hydrophilicity of composite compared to pure PLGA, contact angle measurement was conducted. Our results showed that addition of TiO_2 to PLGA can decrease the contact angle from 90.5 to 79.8 degrees as seen in Figure 4. Moreover, TiO_2 nanoparticles have been reported to improve osteogenic differentiation of adipose derived stem cells (hASCs) [65].

Brønsted acid site (Ti-OH) can form on the surface of TiO_2 particles as a results of water absorption [29, 66]. It has been suggested that Ti-OH groups incorporate the calcium ions to form calcium titanate. This positively charged intermediate can both incorporate the negatively charged phosphate ions in the solution to form an amorphous calcium phosphate (ACP) which later crystallize into bonelike apatite [67, 68] or directly act as a bioactive platform for cell adhesion. It has been reported that incorporation of calcium titanate into a composite containing hydroxyapatite/tricalcium phosphate can result in over 4 times higher osteoblast adhesion compared to pure hydroxyapatite possibly due to increasing shrinkage in the unit lattice parameters and decreasing grain size [69].

Application of some of the conventional methods for determining the cell number and viability is restricted because of their destructive nature. Recently, resazurin-based assays such as Alamar Blue and Prestoblue have been widely used to overcome this issue due to their nondestructive essence. Resazurin could be irreversibly reduced to the pink-colored and red fluorescent resorufin through the metabolic activity of living cells [70]. As presented in Figure 5a, the presence of TiO_2 particles significantly increases the proliferation rate and the number of viable cells on the composite scaffolds compared to pure PLGA ($p < 0.05$) at both time points. Even though the number of viable cells on both scaffolds increased over time, the difference became more significant. Taking approximately the same seeding efficiency for both scaffolds into account, the difference becomes even more evident. The improved cell viability and proliferation on PLGA/ TiO_2 nanocomposite scaffolds can be attributed to the synergistic effect of PLGA and TiO_2 particles which can provide more bioactive receptor binding sites for the attachment of filopodia of human osteoblast cells [71].

ALP activity is an important factor in bone mineral formation and osteoblastic differentiation on biomaterials *in vitro* [33]. Figure 5B demonstrates ALP activity of PLGA and PLGA/ TiO_2 nanocomposite scaffolds after incubation for 7 and 14 days. The ALP activity was significantly higher for PLGA/ TiO_2 nanocomposite scaffolds compared to pure PLGA at both time points ($P < 0.05$) suggesting the facilitated osteogenesis. Again, one should note the same seeding efficiency for both scaffolds at the beginning of the experiment. It has been suggested that the excellent biocompatibility of TiO_2 -based composites is mainly due to their exceptional bioactivity and the surface morphological characteristics [72].

Figure 6 shows the Alizarin Red stained PLGA and PLGA/ TiO_2 scaffolds after 3 days of osteoblast culture. It should be noted that in this figure only the calcified inorganic phase is stained in red color. Based on quantitative assessments, it was found that the amount of calcium precipitated on PLGA/ TiO_2 nanocomposite scaffold was significantly higher than that of pure PLGA scaffold ($p < 0.05$) (Table 3) confirming the results obtained from EDS analysis (Table 4).

The interaction of negatively charged units of TiO₂ dissociated from the Ti-OH groups on the surface of TiO₂ particles with the positively charged calcium ions is suggested to trigger the nucleation through precipitation of Ca²⁺ cations and incorporation of the phosphate ions onto the consequent positively charged surface. Higher chloride content compared to phosphate is a result of high charge density and high concentration of this anion in the media. The obtained results are in agreement with the outcome of our previous study on the bioactivity of TiO₂ coated scaffolds [29].

One may suggest increasing TiO₂ content in order to obtain better mechanical properties and improved biological performance. However, it should be noted that despite improvement in mechanical and biological aspects, higher TiO₂ content may bring about difficulties in printing due to increased viscosity [53, 54] and complications in handleability due to accelerated hydrolytic degradation [73]. The latter occurs at the interface between the polymer matrix and the filler particles and is influenced by the content and dispersion of the filler particles [73].

4. Conclusion

The 3D Printed PLGA/TiO₂ nanocomposite scaffold was introduced in this study as a potential candidate for bone tissue engineering. The composite scaffolds fabricated by 3D printing method presented favorable mechanical and biological features supporting cell viability and increasing ALP activity which are necessary for bone tissue regeneration. The contact angle as a measure of surface wettability decreased around 10 degrees when TiO₂ was added to PLGA. The incorporation of TiO₂ particles into the structure of the PLGA scaffold can be introduced as an efficient model of 3D Printed scaffolds for bone tissue engineering.

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Highlights

- PLGA-TiO₂ composite scaffolds were successfully 3D-printed.
- The effect of addition of TiO₂ to PLGA on thermal and mechanical properties was investigated.
- Addition of TiO₂ improved mechanical properties and wettability.
- PLGA-TiO₂ composite scaffold had better biological performance compared to pure PLGA.

Figure 1.

Glass transition temperature of PLGA (A), PLGA/TiO₂ (B) and decomposition onset temperature of PLGA (C) and PLGA/TiO₂ (D) measured using DSC. Thermal degradation behavior of PLGA (E) and PLGA/TiO₂ (F) when a 10 °C/min ramp rate was applied. Thermal degradation behavior of PLGA (G) and PLGA/TiO₂ (H) when a 2 °C/min ramp rate was applied.

Figure 2.
The morphology of 3D printed scaffolds including pure PLGA (A, B and C) and PLGA/
TiO₂ nanocomposite (D, E and F) in different magnification

Figure 3.

Laser microscope images of osteoblasts cultured on PLGA/TiO₂ nanocomposite scaffold at different magnification (A). Cell penetration depth in two different focal planes detected by laser microscopy (B and C)

Figure 4.
Contact angle of PLGA (A) vs. PLGA/TiO₂ (B)

Figure 5.
Cell proliferation (A) and ALP activity (B) of human osteoblast cells on PLGA and PLGA/TiO₂ nanocomposite scaffolds after incubation for 7 and 21 days (* pvalue>0.05)

Figure 6.
Alizarin red results of (a) PLGA and (b) PLGA/TiO₂ nanocomposite scaffold with precipitated calcium via osteoblast culture

Table 1.

different parameters used to print both PLGA and PLGA/TiO₂ scaffolds

Printing parameter	Value
Temperature	160 °C
Pressure	1.2–2 bar
Plotting speed	2–4 mm/s
needle diameter	0.7 mm
distance between strands	0.8 mm
slicing thickness	0.3 mm
pre-flow delay	0.5 s

Table 2.Mechanical properties of PLGA and PLGA/TiO₂ nanocomposite scaffolds

Scaffold	Porosity	Compressive modulus (MPa)	Compressive Strength (MPa)
PLGA	37.44 ± 1.60	489.22 ± 27.83	29.87 ± 3.10
PLGA/TiO ₂	45.10 ± 9.51	541.23 ± 11.80	29.9 ± 3.16

Table 3.

The seeding efficiency results of PLGA and PLGA/TiO₂ nanocomposite scaffolds and the calcium content of each scaffold compared to control group after 3 days of culture

Material	Seeding efficiency	Scaffold calcium content/control calcium content
PLGA	47 ± 13 %	2.23 ± 0.18
PLGA/TiO ₂	51 ± 18 %	3.57 ± 0.57

Table 4.

EDS analysis of PLGA and PLGA/TiO₂ nanocomposite scaffolds used to detect the quantity of calcium precipitated on each scaffold during osteoblast culture

Element	PLGA (wt%)	PLGA/TiO ₂ (wt%)
P	0	0.75 ± 0.14
Cl	5.70 ± 0.32	1.87 ± 0.19
Ca	0.19 ± 0.09	1.40 ± 0.22