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I-Optimal design of poly(lactic-co-glycolic) acid/hydroxyapatite three-dimensional scaffolds produced by thermally induced phase separation

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Abstract

In bone tissue engineering, 3D scaffolds are often designed to have adequate modulus while taking into consideration the requirement for a highly porous network for cell seeding and tissue growth. This paper presents the design optimization of 3D scaffolds made of poly(lactic-co-glycolic) acid (PLGA) and nanohydroxyapatite (nHA), produced by thermally induced phase separation (TIPS). Slow cooling at a rate of 1°C/min enabled a uniform temperature and produced porous scaffolds with a relatively uniform pore size. An I-optimal design of experiments (DoE) with 18 experimental runs was used to relate four responses (scaffold thickness, density, porosity, and modulus) to three experimental factors, namely the TIPS temperature (−20°C, −10°C, and 0°C), PLGA concentration (7%, 10%, and 13% w/v), and nHA content (0%, 15%, and 30% w/w). The response surface analysis using JMP[®] software predicted a temperature of −18.3°C, a PLGA concentration of 10.3% w/v, and a nHA content of 30% w/w to achieve a thickness of 3 mm, a porosity of 83%, and a modulus of ~4 MPa. The set of validation scaffolds prepared using the predicted factor levels had a thickness of 3.05 ± 0.37 mm, a porosity of 86.8 ± 0.9 %, and a modulus of 3.57 ± 2.28 MPa.

Keywords

bone tissue engineering; design of experiments (DoE); thermally-induced phase separation

1. INTRODUCTION

The natural healing capacity of bone is often sufficient to repair fractures and common bone injuries [1]. However, in a critical-size bone defect (nonunion), fibrous connective tissue regenerates faster than bone tissue and dominates the bone defect [2]. Bone grafts and substitutes are used in millions of operations worldwide [3]. Nevertheless, these therapies pose the risk of infection, immune response, disease transfer, or stress shielding [3,4]. Tissue

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engineering is an alternative approach for the repair of damaged tissues and organs [5,6]. In bone tissue engineering, 3D scaffolds that mimic the composition and mechanical properties of bone may enable the patient's body to regenerate the damaged tissue without the need for bone graft and substitutes [7–9].

The key requirement for a tissue-engineered bone graft substitute is the ability to integrate with the surrounding host tissue, while offering the capacity for remodeling upon in vivo implantation [10]. However, the size of scaffold-tissue constructs can seriously limit in vivo integration, due to the diffusional constraints of nutrient supply caused by high metabolic activity of bone cells [11]. Therefore, factors influencing scaffold architecture, such as pore size and porosity, have critical influence on bone ingrowth into the scaffold. While the physical structure of a scaffold regulates the transport of oxygen/nutrients and affects cell migration [12], the surface chemistry affects cell adhesion and morphology [13].

Poly(α -hydroxyl acids) such as poly(L-lactic acid) (PLLA) and poly(lactic-co-glycolic acid) (PLGA) have been processed using a broad range of scaffold fabrication techniques to produce porous constructs for tissue engineering [6,8,14]. Among these techniques, thermally induced phase separation (TIPS) has been shown to generate highly porous scaffolds with an interconnected pore network [15]. In the TIPS method, the cooling rate, quenching (TIPS) temperature, and the presence of ceramic particles can influence the pore size and pore interconnectivity [15–17]. The first step in the TIPS method is to make a homogeneous polymer solution. The solvent used in this method is usually 1,4-dioxane as it has a high melting point (11.8°C) comparable to ambient temperature, which makes it convenient for thermally-induced phase separation. The prepared polymer solution is cooled down to the desired quenching (TIPS) temperature by sudden freezing or via a ramp temperature profile [15,18]. Finally, the solvent is removed by freeze-drying or freeze extraction to generate the final porous structure.

Composites made of polymers and ceramic particles have also shown great promise as 3D scaffolds for bone tissue engineering. In general, the main goal of most studies is to mimic the composition of the natural bone to some extent, while enhancing the mechanical properties of the 3D scaffolds by incorporating ceramic particles [4,17]. In bone tissue engineering, it is often hypothesized that 3D scaffolds should have adequate modulus, as mechanoregulatory effects are considered to be the key factor in cellular differentiation and bone tissue regeneration [19,20]. Bone is mainly composed of nanoscale inorganic hydroxyapatite and organic compounds (e.g., collagen). The nanometer size of the inorganic hydroxyapatite in native bone is considered to be an important factor affecting the mechanical properties of the bone [17,21].

This paper presents an I-optimal design of composite 3D scaffolds made of poly(lactic-co-glycolic) acid (PLGA) and nanohydroxyapatite (nHA) produced by thermally induced phase separation (TIPS). The present study looks into the three main factors affecting the scaffold microporous structure by the TIPS method (namely, PLGA%, nHA%, and the TIPS temperature). The I-optimal design of experiments (DoE) for scaffold design was chosen to minimize the average prediction variance across the design space [22,23]. The response surface methodology enabled us to find the relationships between the three experimental

factors and four responses: scaffold thickness, density, porosity, and modulus. In this study, the primary goal was to replicate the thickness (3 mm) and porosity (83%) of a commercial scaffold, CellCeram™, to be used for comparison in our future *in vitro* cell culture trials. The produced scaffolds were characterized by scanning electron microscopy (SEM), micro-computed tomography (micro-CT), and unconfined compression tests.

2. SPLIT-PLOT I-OPTIMAL DESIGN OF EXPERIMENTS

2.1. Theoretical background

Response surface designs have a widespread use in industry, whenever there is an interest in finding a relationship between a response and a set of quantitative experimental factors. Hence, the design of experiments has been extensively used for the development and optimization of industrial processes [23]. However, as the number of experimental factors in a factorial design increases, it becomes practically challenging to perform the number of runs required for a full factorial design. To overcome this shortcoming, one can reasonably disregard certain high-order interactions and place the emphasis on the main effects and low-order interactions. Then, the information on the main effects and interactions can be obtained by simply running a fraction of the complete factorial experiment [22].

Many experimental designs fall into the category of completely randomized designs (CRDs). Under certain circumstances, however, it is not practical to completely randomize the order of the runs due to the presence of hard-to-change factors [22]. Split-plot designs are attractive when one needs to deal with hard-to-change vs. easy-to-change factors in an experiment. In the analysis of split-plot design experimental outcomes, one needs to incorporate the “batch” effect, i.e., runs carried out under the same hard-to-change experimental factor. As a drawback that ordinary complete factorial designs suffered from, factorial split-plot designs with three or more factors require a larger number of experimental runs [22]. In light of this, the rationale for the fractional factorial design is further warranted in a split-plot design.

2.2. 3D scaffold design

The goal of this study was to target a scaffold thickness of 3 mm and a porosity of 83% by manipulating the three experimental factors (temperature, PLGA%, and nHA%). While maximizing the compressive modulus is often desirable for a porous scaffold in bone tissue engineering, imposing a lower-limit constraint on scaffold porosity is a common practice as well. This enables a porous network for cell ingrowth and for the transport of oxygen, nutrients and biological waste products. Once a response surface is constructed in the target design region, generating a second-order regression model for each response (i.e., porosity and modulus) will allow estimating the relationship between the factors and responses [4,22,24].

As shown in Table 1, the number of the key factors for scaffold fabrication in this study was $N_F = 3$ (X_1 to X_3); we have chosen three levels for each of these factors ($N_L = 3$), i.e., low, medium, and high, denoted by L, M, and H. A completely randomized factorial design would lead to $N_L^{N_F} = 3^3 = 27$ runs. The fractional factorial split-plot I-optimal design

targeted in this study generated a total of 18 runs, considering temperature (X_3) as the hard-to-change factor. This is because it was less time-consuming to run several experiments simultaneously at the same TIPS temperature. The levels of factors used in the 18-run experiment proposed by the JMP[®] software are listed in Table 2. It should be noted that five scaffold designs had no nHA (“0” listed five times under factor “ X_3 ”). The resulting experimental outcomes were then analyzed with a second-order linear model [22]:

$$\begin{aligned}
 Y = & \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \sum_{i=1}^3 \sum_{j>i}^3 \beta_{ij} x_i x_j + \sum_{i=1}^3 \beta_{ii} x_i^2 \\
 & + \sum_{k=1}^3 \theta_{0k} Z_k + \\
 & \sum_{i=1}^3 \sum_{k=1}^3 \theta_{ik} Z_k x_i + \sum_{i=1}^3 \sum_{k=1}^3 \theta_{iik} Z_k x_i^2 \\
 & + \sum_{i=1}^3 \sum_{j>i}^3 \sum_{k=1}^3 \theta_{ijk} Z_k x_i x_j + \epsilon
 \end{aligned} \quad (1)$$

Where $\beta_0 - \beta_3$ are the regression coefficients associated with the first order predictors, β_{ij} are the second order coefficients, β_{ij} are the interaction coefficients, Z_k is the group of dummy variables indicating which batch the measures are from, and $\theta_{0k} - \theta_{3k}$, θ_{ik} , θ_{iik} and θ_{ijk} are the parameters measuring the “batch” effect of the same batch of runs sharing the same temperature. Note that here only the estimates of β s are of interest for the optimization of the experimental conditions. The lower-case factors $x_1 - x_3$ in Eq. 1 are related to the experimental factors in Table 1 ($X_1 - X_3$) and take the values of -1, 0, and 1 based on the following expressions:

$$x_1 = (X_1 - 10)/3 \quad (2)$$

$$x_2 = (X_2 - 15)/15 \quad (3)$$

$$x_3 = (X_3 + 10)/10 \quad (4)$$

As listed in Table 1, X_1 is the PLGA concentration (% w/v), X_2 is the nHA content (% w/w), and X_3 is the TIPS temperature (°C). JMP[®] software was used in the analysis to estimate the set of optimal levels of factors for designing the 3D scaffolds.

3. MATERIALS

PLGA (RESOMER LG 824 S; I.V. = 1.7–2.6 dl/g; with a 79:21–85:15 molar ratio of l-lactide:glycolide) was purchased from Evonik Biomaterials. Ethanol and 1,4-dioxane were purchased from Sigma-Aldrich. Initially, nanohydroxyapatite from Berkeley Advanced Biomaterials (BABI-HAP-N100) with 20–550 nm particle size range, denoted here as nHA-B, was used in the preliminary experiments. Subsequently, nanohydroxyapatite nanoparticles from Sigma-Aldrich was used for comparison (< 200 nm particle size, denoted as nHA). The 18-run DoE experiments used nHA from Sigma-Aldrich. CellCeram[™] commercial scaffolds with a diameter of ~10 mm and height of ~3 mm were purchased from Sigma-Aldrich.

CellCeramTM is made of 60% hydroxyapatite (HA) and 40% β -tri-calcium phosphate (β -TCP), and has an average porosity of 83%.

3.1. Scaffold fabrication

Figure 1 shows the schematics of scaffold fabrication using the TIPS method, featuring a ramp temperature profile. To prepare the mixtures, the hydroxyapatite powder (0%, 15%, and 30% w/w) was blended with dry PLGA inside a 50-ml beaker, and then 7 ml of 1,4-dioxane was added to the blend to prepare solutions with the target polymer concentrations (7%, 10%, and 13% w/v), as listed in Table 2. The mixture was heated at 60°C for 2.5 hours inside a water bath. To obtain a homogeneous solution, the mixture was sonicated before, during and after heating (3 times, 10 min each, amplitude of 20 μ m) using a model 50 Sonic DismembratorTM (Fisherbrand). Then, the beaker was covered with parafilm and placed inside an environmental chamber (Cincinnati Sub-Zero). An Erlenmeyer flask containing ethanol was also transferred to the chamber. The chamber was heated to 40°C at a rate of 1°C/min, maintained at this temperature for 10 min, and then cooled down at a rate of 1°C/min to the target temperatures specified by the I-optimal design (Table 2). The solidified mixture was maintained at the target temperature for 1 hour, and then the chilled ethanol was poured into the beaker for 1,4-dioxane extraction. The beaker was kept inside the chamber for an additional 67 hours to fully extract 1,4-dioxane and produce micropores. Ethanol was refreshed 2.5 hours before removing the scaffolds from the chamber. The scaffolds were then heated to 20°C at a rate of 1°C/min, air-dried for 30 min, punched into 10-mm diameter disks, and immersed in deionized (DI) water at 40°C for 48 hours. Then, the scaffolds were air-dried for 48 hours and kept inside a refrigerator at 4°C until characterization.

3.2. Solvent evaporation measurements

The scaffold fabrication process exposed the 1,4-dioxane/PLGA solution to a temperature of 60°C inside beakers covered with parafilm. Therefore, 1,4-dioxane evaporation measurements were performed to quantify solvent loss during this process. Moreover, solvent loss during the sonication steps potentially altered the composition of the prepared solution. To quantify solvent evaporation during the fabrication process, we performed experiments using 10-ml beakers containing 5 ml of 1,4-dioxane. Table 3 shows the four set of experiments performed at three different temperatures.

3.3. Differential scanning calorimetry (DSC)

The measurements were performed on PLGA using a TA Instruments Q500 equipment. The sample (~12 mg) was heated from 20°C to 200°C at a rate of 10°C/min. The rate of heat flow (in W/g) and the slope of the heat flow versus temperature (in J/g.°C) was recorded as a function of temperature. The latter, representing the specific heat, was used to identify the glass transition temperature of PLGA.

3.4. Scanning electron microscopy (SEM)

The internal architecture of the scaffolds was analyzed by scanning electron microscopy (SEM). The scaffolds were sputter-coated with 20 nm gold using Denton Desk II Sputter Unit and imaged using a Zeiss Supra 35VP operating at 5 kV.

3.5. Microcomputed tomography (micro-CT)

The scans were performed using a Siemens Inveon Tri-Modal Scanner at 80 kVp, 500 μ A, and 1300 ms exposure time. For data analysis, the Inveon research workplace bone morphology tool software was used. The results were used to compare the density distribution of the scaffolds with different compositions. The micro-CT scanner was calibrated using hydroxyapatite (HA) cylindrical phantoms. The following formula was derived to convert the Hounsfield units (HU) to mg HA/cm³:

$$\frac{\text{mg HA}}{\text{cm}^3} = 0.24(\text{HU}) + 1.7023 \quad (5)$$

3.6. Porosity measurements

The average porosity of the scaffolds was calculated using the following equation:

$$\varphi = \frac{V_a - V_t}{V_a} \times 100 \quad (6)$$

where V_a is the apparent volume based on the thickness and diameter of each punched scaffold, and V_t is the true volume of the scaffold, which is related to the PLGA/nHA matrix density (ρ_m) and scaffold mass (m) through:

$$V_t = m / \rho_m \quad (7)$$

Combining (6) and (7), the scaffold density (ρ) can be related to its porosity (φ) as:

$$\rho = \rho_m(1 - \varphi) \quad (8)$$

The scaffold matrix density (ρ_m) in (7) and (8) for PLGA/nHA matrix was estimated using the rule of mixtures based on the factor level X_2 for each of the 18 runs (see Table 2).

$$\rho_m = \left(1 - \frac{X_2}{100}\right) \times \rho_{PLGA} + \left(\frac{X_2}{100}\right) \times \rho_{nHA} \quad (9)$$

Where $\rho_{PLGA} = 1.3 \text{ g/cm}^3$ and $\rho_{nHA} = 3.16 \text{ g/cm}^3$ [17] represent the PLGA and nHA densities, respectively.

3.7. Mechanical characterization

The scaffolds were characterized using an Instron 3344 single tower compression device, equipped with a 1 kN load cell to measure their compressive modulus. The test was performed under unconfined ramp compression at a displacement rate of 1 mm/min. The modulus was estimated for the linear zone (4–6% strain) after making independent measurements on three scaffolds ($n=3$).

4. RESULTS

4.1. Solvent evaporation experiments

Figure 2 shows the evaporated mass of the solvent over time for the four beakers listed in Table 3. The highest evaporation rate is for beaker 4, the only beaker that was not covered with parafilm. This situation arises during the sonications for a total of 30 minutes, and could lead to ~17% solvent evaporation. Beaker 1 shows the highest rate of solvent evaporation among the three beakers covered with parafilm, which is due to the high temperature of 60°C during this experiment. Based on the linear regression, a solvent loss of ~25% is anticipated during the solution preparation step that takes 150 min at 60°C.

4.2. Differential scanning calorimetry

Figure 3 shows the differential scanning calorimetry (DSC) thermogram for PLGA 824S. The step-like transition around 50°C represents the glass transition temperature (T_g) of the polymer. This observation indicates that the PLGA used in this study was over its T_g during solution preparation at 60°C. A higher free volume expected for polymers over T_g [25] facilitated solvent diffusivity and PLGA dissolution, and enabled a homogeneous PLGA solution in this study. The prepared scaffolds were washed in DI water at 40°C, which is below the T_g of the polymer and does not pose the risk of thermal damage at the glassy state of the polymer.

4.3. Scanning electron microscopy

Scanning electron microscopy (SEM) in Fig. 4a – Fig. 4d revealed that nHA-B impacted the pore formation during the TIPS process, when compared to the nHA grade from Sigma-Aldrich (nHA-S). This was partially due to the nonhomogeneous mixtures obtained using nHA-B. Therefore, nHA-S, denoted as nHA in this paper, was used for the 18-run DoE experiments.

Figure 5 shows the SEM images of 15 different scaffolds created in this study. Note that run 4, 5, and 8 have been excluded from this figure, as they were replicates of run 3. The SEM images in Fig. 5a–5e show the pore morphologies of the scaffolds produced at –10°C using different PLGA concentrations (7, 10, and 13% w/v) and nHA levels (0, 15, and 30% w/w). Among these scaffolds, run 1 and run 6 do not contain nHA and show a circular pore structure. The scaffolds containing nHA have elongated pores with irregular microstructures (run 2–5). The pore morphologies of the scaffolds produced at a low temperature of –20°C (run 9 – 12, Fig. 5f–5i) show a similar trend. The scaffold produced in run 10 reveals a dense structure due to its high PLGA concentration (13% w/v) and nHA content (30% w/w), whereas a well-defined pore network is evident for the scaffold with a lower PLGA concentration (10% w/v) and the same nHA content (30% w/w). Finally, Fig. 5j–5o show the scaffolds produced at 0°C (run 13 – 18). When compared with the previous SEM images, the scaffolds prepared at 0°C lack a well-defined porous structure. This could be attributed to the high fabrication temperature, which is only ~12°C below the solidification temperature of 1,4-dioxane. In particular, the dense structure for the scaffold produced in run 14 is due to a high PLGA concentration (13% w/v) in the presence of 30% w/w

4.4. Microcomputed tomography

Figure 6a-6o shows the micro-CT images of the scaffolds. Run 4, 5, and 8 have been excluded from this figure, as they are replicates of run 3. Looking at the color scale bar, it can be seen that the 18-run I-optimal design generated a broad range of scaffold densities. The scaffolds produced at -20°C (run 1 – 8) and -10°C (run 9 – 12) show fairly uniform densities even at a high PLGA and nHA concentration (13% and 30%, respectively), with the exception of one scaffold generated in run 7. The highly viscous PLGA solution may have negatively affected the uniformity of this particular scaffold. On the other hand, all the scaffolds produced at 0°C using a high PLGA concentration of 13% (run 14 and run 17) and/or a high nHA content of 30% (run 14 and 16) show a non-homogeneous density due to non-uniform dispersion of nHA within these scaffolds. Hence, it appears that lower TIPS temperatures were beneficial in terms of homogeneity and reproducibility of the scaffolds. This is consistent with the SEM results, which suggested improved pore formation at TIPS temperatures of -20°C and -10°C .

Figure 6p and 6q show the density distribution for two scaffolds produced at -20°C with 10% w/v of PLGA and nHA contents of 0% and 30% w/w (run 12 and run 9, respectively). Higher values of the CT unit (HU) on the x-axis represent a higher density. Bone and hydroxyapatite are often identified within -17 to 1802 HU, whereas the range for soft tissues and polymers is -590 to -370 HU [26]. The peaks of the histograms for the two scaffolds in Fig. 6p and 6q correspond to -793 and -130 HU, respectively, while the mean HU for the latter is 75 . This value corresponds to ~ 20 mg HA/cm³ for the scaffold produced in run 9, according to the micro-CT calibration formula.

4.5. Scaffold thickness, density and porosity

Table 4 gives the results of scaffold characterization for the 18-run split-plot design. The measured scaffold thickness, diameter, and weight were used to calculate the scaffolds density and porosity. Figure 7a-7c show the scaffold thickness, density, and porosity, respectively. As these results indicate, the scaffold thickness varies between $2.16 - 3.31$ mm for the 18 DoE runs. The scaffold density and porosity vary between $0.17 - 0.46$ g/cm³ and $71 - 89\%$, respectively. The formulations with higher PLGA and nHA concentrations tend to generate thicker/denser scaffolds, whereas higher process temperatures appear to reduce the scaffold thickness. As for CellCeramTM scaffold, it has a reported average porosity of $\sim 83\%$, an average pore size range of $200 - 400$ μm , with an overall range of $100 - 800$ μm (Sigma-Aldrich).

4.6. Scaffold modulus

The compressive modulus measured for these scaffolds revealed a pronounced variability for the 18 runs ($0.21 - 4.6$ MPa). However, the magnitude of the error bars in Fig. 7d points to the lack of statistical significance for the majority of these scaffolds. The geometrical deviation of the scaffolds from a uniform disk considerably affected the reproducibility of the mechanical testing for these porous constructs. While the bottom surface of these scaffolds are often flat due to being in contact with the beaker, their upper surface is often nonuniform.

4.7. Statistical analysis results

The experimental data from the split-plot design (as shown in Fig. 7) were analyzed with a linear model. The parameters of the fitted effects from the models listed in Table 5 were used to construct the pairwise prediction-factor plots. The analysis considers both the first order and second order terms, as well as interaction effects. Insignificant terms (most second order and interactions) were discarded in this study and only the significant terms were retained and later used in the prediction of responses. Note that all main effects (PLGA concentration, nHA content, and the TIPS temperature) were retained regardless of their statistical significance.

According to the predictions (Fig. 8), the scaffold thickness decreased with increasing the TIPS temperature, whereas the PLGA concentration and nHA content had less pronounced effects on the thickness (Fig. 8a). As for the density, it increased with increasing the level of all three experimental factors (Fig. 8b). Increasing the temperature up to -7.3°C reduced the porosity, whereas the nHA content had a minimal effect on the porosity (Fig. 8c). Increasing PLGA concentration had the most pronounced effect by significantly reducing the porosity of the scaffolds. As Fig. 8d indicates, the effects of the three factors on the scaffold modulus were consistent with the corresponding effects on the scaffold density (Fig. 8b). Overall, we found that a PLGA concentration of 10.3% w/v, a nHA content of 30% w/w, and a TIPS temperature of -18.3°C generated scaffolds with the target thickness of 3 mm and the target porosity of 83%. A scaffold density of 0.32 g/cm^3 was predicted in Fig. 8b for this set of factors, which resulted in a modulus of $\sim 3.96\text{ MPa}$ for the optimal scaffold (Fig. 8d). The predicted factor levels ($X_1 = 10.3\%$ w/v, $X_2 = 30\%$ w/w, and $X_{13} = -18.3^{\circ}\text{C}$) correspond to the transformed factor levels of $x_1 = 0.105$, $x_2 = 1.0$, and $x_3 = -0.826$, as shown in Fig. 8.

The quality of the fitted models has been visualized by comparing the experimental and predicted responses (Fig. 9). The model predictions and experimental results for the thickness showed a moderate agreement ($R^2 = 0.61$), whereas both the density and porosity models show strong agreements with their respective experimental results ($R^2 = 0.94$ and $R^2 = 0.90$, respectively). The model predictions and experimental results for the modulus revealed a poor agreement ($R^2 = 0.48$), which is primarily due to the poor reproducibility of the mechanical measurements.

4.8. Validation scaffolds

A set of validation scaffolds were prepared to validate the predictions of the JMP[®] software (Fig. 10a). The factor levels predicted by JMP[®] were used in these experiments (*e.g.*, $X_1 = 10.3\%$ w/v, $X_2 = 30\%$ w/w, and $X_3 = -18.3^{\circ}\text{C}$). Figure 10b-10d compare the predicted and measured thickness, porosity and modulus. While the predictions for the thickness, porosity, and modulus were 3.0 mm, 0.83, and 3.96 MPa, respectively, the validation scaffolds had a thickness of $3.05 \pm 0.37\text{ mm}$, a porosity of $86.8 \pm 0.9\%$, and a modulus of $3.57 \pm 2.28\text{ MPa}$. Hence, the absolute percentage error between the predictions and validations were 1.5% for the thickness and 4.4% for the porosity, whereas the error was somewhat higher for the modulus (10.9%).

5. Discussion

The pore morphology of the 3D scaffolds produced by thermally-induced phase separation (TIPS) depends on the type of polymer and solvent used, concentration of the polymer solution, crystallinity of the polymer, phase separation temperature, cooling rate, as well as the presence of inorganic additives [12–14]. When the temperature of the polymer solution falls below the freezing point of 1,4-dioxane (11.8°C), solvent crystallization expels the polymer phase from the crystallization front [27]. Sudden quenching is often used in scaffold fabrication using the TIPS method. Low quenching temperatures cause a higher cooling rate and a shorter time for solvent nucleation and crystal growth, and ultimately lead to a smaller pore size in the scaffold. As expected, higher quenching temperatures favor large crystals of 1,4-dioxane and lead to larger pores [28].

In this study, a ramp temperature profile was used to slowly cool the polymer solution to the TIPS temperatures of –20, –10, and 0°C at a rate of 1°C/min. This was meant to enable a uniform temperature within the polymer solution and generate a uniform pore size upon solvent extraction. In light of this, the time needed for the solution to reach the desired TIPS temperature had a major influence on the pore size of the scaffolds. This may explain the denser scaffolds produced at a high temperature of 0°C (Fig. 5j–5o), when compared to –10°C (5a–5e) and –20°C (5f–5i). The corresponding drop in the scaffold porosity with increasing the TIPS temperature (Fig. 8c) also supports this observation. Nevertheless, the range of the TIPS temperature in this study (factor X_3 in Table 1) was not broad enough to show a significant variation in the pore size as seen by SEM (Fig. 5).

Nanohydroxyapatite (nHA) has been extensively used for producing polymer/ceramic scaffolds by different fabrication techniques. The pore structure of a scaffold produced by TIPS may vary depending on the nHA concentration. In the absence of nHA or at low concentrations, a ladder-like structure with regular channels has been reported, whereas increasing the nHA concentration has been shown to generate an irregular pore structure [17]. The irregular pore network observed in this study is consistent with these findings (e.g., Fig. 5b vs. Fig. 5d, and Fig. 5e vs. Fig. 5a). As for the effect of PLGA, increasing the PLGA concentration from 7% to 13% reduced the pore size (Fig. 5a vs. 5d) while reducing the scaffold porosity (Fig. 8c). The change in the pore size appears to be consistent with the previous studies, reporting a reduction in the pore size by increasing the polymer concentration [15,17]. Nevertheless, the scaffolds produced at a TIPS temperature of 0°C using a high PLGA concentration of 13% (Fig. 6k and Fig. 6n) and/or a high nHA content of 30% (Fig. 6k and Fig. 6m) showed a non-homogeneous density due to non-uniform dispersion of nHA within these scaffolds.

This study explored a split-plot I-optimal design to find the relationships between the four responses (scaffold thickness, density, porosity, and modulus) with the three experimental factors affecting the scaffold microporous architecture (PLGA%, nHA%, and the TIPS temperature). In our previous study [29], we developed hierarchical PLGA scaffolds by a hybrid method that combined 3D-plotting (3DP) and thermally-induced phase separation (TIPS). The interconnected channels produced by 3DP provided an ideal environment to guide bone ingrowth. The matrix surrounding the channels had micropores generated by

TIPS, where the pore size can be controlled by manipulating the experimental factors [29,30]. These scaffolds supported the growth of MC3T3-E1 osteoblastic cells in vitro [29]. Once combined with nanohydroxyapatite (nHA), the new scaffolds are anticipated to offer a favorable environment for cell adhesion and osteogenic differentiation. The presence of these macrochannels is expected to better satisfy the transport of oxygen, nutrients, and metabolites in vivo, while playing a key role in cell migration and angiogenesis [31,32].

CONCLUSIONS

The primary goal of this study was to replicate the thickness and porosity of a commercial scaffold, CellCeramTM, to be used for comparison in our future *in vitro* cell culture trials. We found that a TIPS temperature of -18.3°C , a PLGA concentration of 10.3% w/v, and a nHA content of 30% w/w generated scaffolds with the target thickness (3 mm) and a porosity (83%). The predicted modulus for this set of experimental factors was ~ 3.17 MPa. A set of validation scaffolds prepared using the predicted factor levels. These scaffolds had a thickness of 3.05 ± 0.37 mm, a porosity of $86.8 \pm 0.9\%$, and a modulus of 3.57 ± 2.82 MPa. The absolute percentage error between the predictions and validations indicated that JMP[®] predictions were satisfactory for the thickness and porosity (1.5% and 4.4%, respectively), whereas it showed a somewhat higher error for the modulus (10.9%). Further optimization of the scaffold architecture by generating interconnected macrochannels is anticipated to provide an ideal environment for cell migration and in vivo vascularization.

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Fig. 1. Schematics of the scaffold fabrication process using thermally induced phase separation, featuring a ramp temperature profile.

Fig. 2.
The Evaporated mass of the solvent over time for beakers 1 – 4 (Table 3).

Fig. 3.
The DSC thermogram for PLGA showing a glass transition temperature around 50°C.

Fig. 4. SEM images comparing the TIPS scaffolds made with 30% nHA-B or 30% nHA-S. These scaffolds contained 7% PLGA (a-b) or 13% PLGA (c-d) and were prepared at -20°C or -10°C , respectively.

Fig. 5. SEM images comparing the 15 TIPS scaffolds made in this study. Run 4, 5, and 8 have been excluded from this figure, as these are replicates of run 3.

Fig. 6.

(a-o) Micro-CT images comparing the 15 TIPS scaffolds made in this study. Run 4, 5, and 8 have been excluded from this figure, as these scaffolds were replicates of run 3. (p-q) The density distribution for two scaffolds produced at -20°C containing 10% w/v of PLGA and two different nHA contents: 0% w/w (p) and 30% w/w (q), produced in run 12 and run 9, respectively.

Fig. 7.

The summary of the measured responses for the 18 DoE runs: a) thickness, b) density, c) porosity, and d) modulus.

Fig. 8. Contour plots produced by JMP® response surface analysis of scaffold thickness (a), density (b), porosity (c), and modulus (d) based on the three experimental factors (PLGA%, nHA%, and the TIPS temperature).

Fig. 9. Plots of the experimental versus predicted responses for (a) thickness, (b) density, (c) porosity, and (d) modulus.

Fig. 10.

(a) Top and side views of a validation scaffold, (b-d) comparison between the predicted and measured thickness, porosity, and modulus for the validation scaffolds, respectively.

Table 1.

The range of the experimental factors in this study.

Factors	L	M	H
PLGA Concentration (X_1)	7%	10%	13%
nHA Concentration (X_2)	0%	15%	30%
Temperature (X_3)	−20°C	−10°C	0°C

Table 2.

The 18-run split-plot I-optimal design.

Run	X_1 (%)	X_2 (%)	X_3 (°C) ^a
1	13	0	−10
2	7	30	−10
3	10	15	−10
4	10	15	−10
5	10	15	−10
6	7	0	−10
7	13	30	−10
8	10	15	−10
9	10	30	−20
10	13	30	−20
11	7	15	−20
12	10	0	−20
13	10	0	0
14	13	30	0
15	7	15	0
16	10	30	0
17	13	15	0
18	7	0	0

^aThe hard-to-change factor

Table 3.

Solvent evaporation experiments performed at three different temperatures.

Beaker	Temperature (°C)	Covered with parafilm	Initial mass of the solvent (g)
1	60	Yes	5.23
2	40	Yes	5.39
3	20	Yes	4.79
4	20	No	4.82

Table 4.

The levels of the three factors and the measured responses for the split-plot design in this study.

Run	PLGA (% w/v)	nHA (% w/w)	Temperature (°C)	Thickness (mm)	Density (g/cm ³)	Porosity (%)	Modulus (MPa)
1	13	0	−10	2.85	0.376	71.0	3.21
2	7	30	−10	2.69	0.299	83.9	3.87
3	10	15	−10	2.48	0.349	77.9	1.66
4	10	15	−10	2.32	0.331	79.0	3.05
5	10	15	−10	2.79	0.343	78.3	0.32
6	7	0	−10	2.41	0.248	80.9	0.97
7	13	30	−10	2.71	0.478	74.3	4.60
8	10	15	−10	2.34	0.406	74.3	2.38
9	10	30	−20	3.15	0.313	83.2	3.26
10	13	30	−20	3.31	0.349	81.2	2.81
11	7	15	−20	3.04	0.168	89.4	0.78
12	10	0	−20	2.59	0.208	84.0	0.21
13	10	0	0	2.32	0.336	74.2	1.41
14	13	30	0	2.44	0.459	75.3	4.02
15	7	15	0	2.16	0.216	86.3	0.92
16	10	30	0	2.45	0.401	78.4	0.55
17	13	15	0	2.90	0.456	71.1	3.69
18	7	0	0	2.29	0.231	82.2	1.22

Table 5. Fitted linear models used for the construction of the response-factor plots (coefficient of 0 indicates a removed term).

Polynomial equation: $\beta_0 + \beta_1x_1 + \beta_2x_2 + \beta_3x_3 + \beta_4x_1x_2 + \beta_5x_1x_3 + \beta_6x_1x_2x_3 + \beta_7x_1^2 + \beta_8x_2^2 + \beta_9x_3^2 + \sum_{k=1}^3 \theta_{0k}Z_k + \sum_{i=1}^3 \sum_{k=1}^3 \theta_{ik}Z_kx_i + \sum_{i=1}^3 \sum_{k=1}^3 \theta_{iik}Z_kx_i^2 + \sum_{i=1}^3 \sum_{j>i}^3 \sum_{k=1}^3 \theta_{ijk}Z_kx_ix_j$

Response	R ²	β_0	β_1	β_2	β_3	β_4	β_5	β_6	β_7	β_8	β_9
Thickness	0.61	2.652	0.158	0.110	-0.268	0	0	0	0	0	0
Density	0.94	0.355	0.0815	0.0349	0.0427	0	0	0	0	0	-0.0610
Porosity	0.90	0.774	-0.0531	0.0136	-0.0254	0	0	0	0	0	0.0400
Modulus	0.48	3.83	1.37	0.736	0.630	0	-0.899	0	0.796	0	-1.42

* See Eq. 2 – 4 for the relationships between $x_1 - x_3$ and $X_1 - X_3$, where X_1 is the PLGA % (w/v), X_2 is the nHA % (w/w), and X_3 is the TIPS temperature (°C).