Porous Three Dimensional (3-D) Scaffolds of Poly(3-Hydroxybutyric Acid) (PHB) and Poly(3-Hydroxybutyric-co-3-Hydroxyvaleric Acid) (PHBV): Determination of Salt Leaching Efficiency of Solvent-Casting Particulate-Leaching (SCPL)

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**ABSTRACT**

As a family of biodegradable polyesters, polyhydroxyalkanoates (PHAs) have attracted increasing interest as tissue engineering materials due to their general biodegradability, good biocompatibility, together with other adjustable mechanical properties. Among PHA, polyhydroxybutyrate (PHB) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) were selected for the fabrication of porous 3-D scaffolds. The scaffolds of poly(3-hydroxybutyric acid) (PHB) and poly(3-hydroxybutyric acid-co-3-hydroxyvaleric acid) (PHBV) with an improved thickness greater than 1 mm were fabricated using a conventional method of solvent-casting particulate-leaching (SCPL). The polymeric porous 3-D scaffolds were fabricated using an ideal polymer concentration of 4% (w/v). Even since the technique introduced in the 90’s, to our knowledge, there was no studies have been conducted to measure the efficiency of the salt leaching process. The objectives of this study are to determine the amount of salt remains inside the porous 3-D scaffolds and the effect of salt remnants on cell growth media electrolytes content by using the conductivity (κ) measurement. Based on these findings, both polymeric porous 3-D scaffolds with an improved thickness did not possess any effects to the electrolytes balance inside the cell growth media which could possibly lead to cellular damage (e.g., necrosis). Therefore, it has been proven that this method was undoubtedly the suitable method to construct porous 3-D scaffolds with an improved thickness greater than 1 mm without perturbing to any contaminants (e.g., porogen and solvents) that could affect the in vitro cell proliferation and tissue growth.

INTRODUCTION

Polyhydroxyalkanoates (PHA) are aliphatic polyesters that are synthesized by microorganisms under unbalanced growth conditions [1,2]. They are generally biocompatible, biodegradable (via microbial degradation and enzymatic hydrolysis) and thermoprocessable, making them attractive as biomaterials for applications in medical devices and tissue engineering and regenerative medicine (TERM). Over the past years, PHA, particularly poly-3-hydroxybutyrate (PHB), copolymers of 3-hydroxybutyrate and 3-hydroxyvalerate (PHBV), poly-4-hydroxybutyrate (P4HB), copolymers of 3-hydroxybutyrate and 3-hydroxyhexanoate (PHBHHx) and poly-3-hydroxyoctanoate (PHOH) were demonstrated to be suitable for tissue engineering applications [3].

The most common type is the polyhydroxybutyrate (PHB) which comes from the polymerization of 3-hydroxybutyrate monomer. Its physico-chemical features in addition to mechanical properties are similar to those of polypropylene (PP) and polylactic-co-glycolic acids (PLGA) but inferior in its rigidity [1,4]. The major function of PHB in microorganisms is to serve as an intracellular energy and carbon storage product in much the same mode as glycogen in mammalian tissue. In fact, this polymer accumulates in isolated, membrane bound granules in the bacterial cell [5-7] from which it can be extracted directly with the usage of chlorinated organic solvents (i.e., chlorform and dichloromethane) [8,9] or by membrane rupturing techniques (i.e., mechanical, chemical and enzymatic disruption of cell walls). Besides PHB, its copolymers with diverse ratios of hydroxyvalerate (HV) which are known as polyhydroxybutyrate-valerate (PHBV) are also extensively used. These copolymers are less crystalline, more flexible and more readily processable than PHB itself [10].
Meanwhile, scaffold design and fabrication are one of the major areas of biomaterial research and they are also important subjects for tissue engineering and regenerative medicine research. Briefly, the principal function of a scaffold is to maneuver cell behavior such as migration, proliferation, differentiation, maintenance of phenotype and apoptosis by facilitating sensing and responding to the environment via cell-matrix and cell-cell communications [11]. Furthermore, scaffold provides the necessary support for cells to proliferate and maintains their differentiated functions, and its architecture defines the ultimate shape of a new organ [12]. Therefore, many methods to prepare porous three-dimensional biodegradable scaffolds have been developed in tissue engineering including gas forming, fiber extrusion and bonding, three-dimensional printing, phase separation, emulsion freeze-drying and porogen leaching [5,6,13,14,15]. One of the principle methods behind tissue engineering involves growing the relevant cells in vitro into the required three-dimensional (3-D) organ or tissue. But cells lack the ability to grow in favored 3-D orientations and thus define the anatomical shape of the tissue. Instead, they randomly migrate to form a two dimensional (2-D) layer of cells [16]. However, 3-D tissues are required and this is achieved by seeding the cells onto porous matrices, to which the cells attach and colonize. According to Liu and Cao [17], an ideal scaffold should possess the following characteristics to bring about the desired biological response: (1) the scaffold should possess inter-connecting pores of appropriate scale to favor tissue integration and vascularisation, (2) be made from material with controlled biodegradability or bio-resorbability, (3) appropriate surface chemistry to favor cellular attachment, differentiation and proliferation, (4) possess adequate mechanical properties to match the intended site of implantation and handling, (5) should not induce any adverse response and (6) be easily fabricated into a variety of shapes and sizes.

One of the prominent methods of making a porous 3-D scaffolds is the solvent-casting particulate leaching (SCPL). This method was first developed in the early 1990s by Mikos et al., [18] for polylactic acid (PLA) and polyglycolic acid (PGA) polymers. In principal, it consists of dissolving a polymer in a solvent and then adding particles of a leachable porogen (i.e., salt particles, glucose and paraffin spheres). The mixture forms a thick paste which is left to dry in air or under vacuum until the solvent has evaporated completely. The porogen is then leached out and leaves behind a network of interconnected pores [18,19]. In the case of composites, the second phase is added with the porogen and remains within the structure after the porogen is leached out. The advantages of the solvent casting method are that it is a simple and fairly reproducible method which does not require sophisticated apparatus, controlled porosity and interconnectivity [20]. The disadvantages include thickness limitations, structures generally isotropic, the use of hazardous solvent [21,22] and limited mechanical properties. Some researchers question the homogeneity and interconnectivity of the pores in the scaffolds as well as the presence of residual porogen and solvent [22]. On top of that, it works only for fabricating the thin membranes or very thin 3-D specimens. For that reason, we aim to produce a 3-D porous scaffold with an improved thickness greater than 1 mm. However, the efficiency of the salt leaching process needs to be examined beforehand so that the 3-D scaffold will not produce any negative effect to the observed cells.

MATERIALS AND METHODS

Materials:
Poly(3-hydroxybutyric acid) (PHB; $M_w = 300,000$ gmol$^{-1}$), poly(3-hydroxybutyric acid-co-3-hydroxyvaleric acid) (PHBV; $M_w = 680,000$ gmol$^{-1}$ with 12% (w/w) polyhydroxyvalerate (PHV) content) (Deng et al. 2002) and cell culture grade sodium chloride (particles size: 212 to 850 μm) were purchased from Sigma-Aldrich® (Dorset-United Kingdom). Chloroform 99.9% in purity (AnalR®) was obtained from VWR International (Leicestershire, United Kingdom). Gibco® Iscove's Modified Dulbecco's Medium (IMDM) and Gibco® Phosphate Buffered Saline (PBS) were purchased from Invitrogen™ Ltd, United Kingdom.

Fabrication of PHB and PHBV Porous 3-D Scaffolds:
The 3-D porous scaffolds were fabricated using solvent-casting particulate-leaching method. A cell culture grade sodium chloride (particles size: 212 μm to 850 μm, Sigma-Aldrich™ (Dorset-United Kingdom)) was used as porogen. Polymer concentrations of 4% (w/v) for PHB and PHBV were prepared in 60 ml of boiled reflux chloroform (99.9% purity) at 60 °C. Once cooled, the polymer solutions were added evenly over an aluminum foil-coated glass Petri-dish. Sodium chloride crystals were then poured evenly and mixed into the polymer solutions with continuous stirring until the polymer-solvent solution became pasty, thick and packed. Subsequently, the packed-in pasty were put immediately inside a lyophilization flask (to avoid the etching effect) and air-dried for 2 days for complete solvent evaporation. The dried-casting polymers were peeled carefully from the aluminum foil and then dialyzed with 10 liters of cold deionized water (DIW) at 21 °C for each polymer for 2 days to remove all sodium chloride crystals. Later, they were frozen at -70 °C for 3 hrs and then transferred into a cryostat bath containing ethylene glycol (-15 °C) to lyophilize the remaining deionized water and chloroform via the sublimation process. Prior to salt residual analysis, scaffolds were cut into cuboids with an approximate size of 10 mm × 10 mm × 5 mm.
Salt Leaching Efficiency Study:
Polymer concentrations of 4% (w/v) for PHB and PHBV containing 12% by weight of polyhydroxyvalerate (PHV) were used for preparing polymer-solvent cast as described in the above protocol. The conductivities (κ) of sodium chloride solution and leachable sodium chloride solution from the polymer-solvent cast were measured by using the OAKTON™ Con 11 conductivity meter (Automatic Temperature Compensation (ATC) function was activated to compensate to reference temperature of 25 °C). The sodium chloride solutions were first prepared on the serial concentration (5, 10, 20, 30 mg/ml) and the conductivities (mS/cm) at 21 °C (measured by the conductivity meter) were plotted against its respective concentration to establish a standard calibration curve. The amounts of sodium chloride (M = mg) that have been leached out from polymer-solvent cast were determined in 10 replications (n = 10) by measuring conductivities of 10 liters leached sodium chloride solution twice a day (deionized water was changed twice a day) until the conductivity of the solution reduced to 2.73 μS/cm (conductivity of deionized water at 21 °C). Then, the total concentration (mg/ml) and mass of sodium chloride (mg) were determined from the standard curve based on their sums of conductivities. The efficiency (%) of salt leaching and end product of SCPL (after lyophilization process) were determined and the equations are shown below:

% Efficiency of salt leaching process = \[ \frac{M_{NaCl} \text{ in the cast} - M_{NaCl} \text{ in the DIW}}{M_{NaCl} \text{ in the cast}} \times 100\% \]  

% Efficiency of SCPL end product = \[ \left( \frac{M_{NaCl} \text{ in the cast} - M_{NaCl} \text{ remains after the lyophilization process}}{M_{NaCl} \text{ in the cast}} \right) \times 100\% \]

Mass of sodium chloride remains in the lyophilized 3-D scaffolds (g) = \( \frac{M_{3-D \text{ scaffolds after lyophilization process}} - 2.4 \text{ g}}{3} \)

Residual Effect of Sodium Chloride on Cell Growth Media:
Polymer concentrations of 4% (w/v) for PHB and PHBV containing 12% by weight of polyhydroxyvalerate (PHV) were used for preparing polymeric porous 3-D scaffolds as described in the above protocol. The cubes were put into 50 ml centrifuge tubes containing cell growth media of 90% (w/v) Iscove’s Modified Dulbecco’s Medium (IMDM) + 10% (w/v) Fetal Bovine Serum (FBS) + 1% (w/v) Penicillin-Streptomycin (PS) (V = 30 ml, pH 7.2) and incubated at 37 °C for 7 days. The conductivity (κ) of cell growth media was recorded at daily basis at temperature of 21 °C. Three replications were prepared for each polymers (n = 3). All measurements were expressed as mean ± standard deviation (SD) and statistical analysis were computed for statistically differences between conductivity (κ) of cell growth media immersed with porous 3-D scaffolds and control (cell growth media without a scaffold).

Statistical Analysis:
Data was presented as means ± standard deviation (SD) of mean values. Statistical comparison was performed using Students t-test (SPSS version 16.0 IBM co.) for salt leaching efficiency study. Statistical analysis of data for sodium chloride residual effect was carried out by one-way analysis of variance (ANOVA) to determine the presence of any significant difference among sample means of the groups, followed by Tukey’s test (SPSS version 16.0 IBM co.) for multiple comparisons to determine the values that were significantly different. The p<0.05 were considered statistically significant.

RESULTS AND DISCUSSION
Salt Leaching Efficiency Study:
One of the disadvantages using solvent-casting particulate-leaching (SCPL) technique is the existence of porogen residual. None of the studies that use SCPL method in fabricating porous 3-D scaffolds have explained on its efficiency or the amount of porogen remnants inside the fabricated porous 3-D scaffolds. Only a few studies have shown some concern about the porogen residual [16,23] but none of them have ever conducted any independent experimental works to validate such claims. Only one study [24] has used other method which was by using the titration of 1 mol/l silver nitrate (AgNO₃). Alas, they were just observing until no further white precipitates (AgCl) to appear in water during the titration, which suggested that the concentration of the Cl ions in the deionized water was less than 1.8 × 10⁻¹⁰ mol/l, as the solubility product equilibrium constants (K_{sp}) of the AgCl is 1.8 × 10⁻¹⁰.

In this study, we aim to validate such claims by determining the mass balance of the sodium chloride after the sodium chloride-DIW leaching process and after the lyophilization process of removing excess deionized water from the porous 3-D scaffolds. Initially, the calibration curve of sodium chloride (Sigma-Aldrich) cell culture grade solution of 5, 10, 20 and 30 (mg/ml) were prepared and plotted against its respective electrical
conductance (Fig. 1). The reason for this is that when salt dissolves into water, it releases Na and Cl ions, which are electrically charged atoms. These enable electricity to flow through the water. Once the conductivity of the water has been measured, the number can be easily converted into concentration and the mass of sodium chloride or total dissolved solids (TDS) that has been leached out from the polymer-solvent cast can be obtained by multiplying with a known volume in milliters or liters.

![Graph](image)

**Fig. 1:** Relationship between conductivity of sodium chloride (Sigma-Aldrich®) solution with respect to its different concentration (mg/ml). Conductivity (κ) of deionized water was 2.73 μS/cm at 21 °C. It was a linear correlation between conductivity (mS/cm) and the concentration of sodium chloride (mg/ml); Y = 2.8475x + 8.5027; r² = 0.9999 (n = 3).

It was observed that the salt-leaching efficiency (%) of PHB porous 3-D scaffolds was significantly higher than PHBV (p<0.05) (Fig. 2). It showed that salt-leaching process of PHB porous 3-D scaffolds was much efficient than PHBV. Moreover, it has been expected that PHB will leach NaCl much efficient than PHBV due to the fact that PHB is much hydrophilic that PHBV (data not shown) wherein allowing much water to penetrate deep inside the porous structure and extract the NaCl particles from its pores.

![Bar chart](image)

**Fig. 2:** Verification of salt-leaching efficiency with SCPL post treatment (after lyophilization process) for PHB and PHBV (4%, w/v). *Significant difference with p<0.05 between the samples was highlighted by the lines (n = 10).

Meanwhile, the efficiency (%) of SCPL end product showed that both polymeric porous 3-D scaffolds retain its mass of porous material (2.4 g) throughout the salt-leaching process. There was less than ~0.5 g of extra weight on the dried cylindrical shape of porous 3-D scaffolds (Fig. 3) which indicates that salt remnants were still trapped inside the porous structure. In addition, the visible observation during the salt leaching process further proof that there was no apparent rupture structure floating on the deionized water. This indicates that there was no loss of porous material mass (2.4 g) during the whole leaching process. However, given the fact
that both efficacies demonstrate less than 100%, it shows that there was a significant amount of salt remains inside the porous 3-D scaffolds. As for that reason, another dependent experimental work was conducted to study the effect of salt remnants against cell growth media by measuring its conductivity ($\kappa$).

**Fig. 3:** Morphology of the polymeric porous 3-D scaffolds in a cylindrical shape prepared using the solvent-casting particulate-leaching (SCPL) technique. They were later cut into a rectangular shape with an approximate size of 5 mm $\times$ 10 mm $\times$ 10 mm: (a) Aerial view of the PHB 4% (w/v) porous 3-D scaffold; (b) Side view of the PHB 4% (w/v) porous 3-D scaffold with the thickness of 5.25 $\pm$ 0.36 mm; (c) Aerial view of the PHBV 4% (w/v) porous 3-D scaffold; (d) Side view of the PHBV 4% (w/v) porous 3-D scaffold with the thickness of 4.40 $\pm$ 0.52 mm. $d$: thickness.

**Residual Effect of Sodium Chloride on Cell Growth Media:**

As related to the above mentioned findings, the porous 3-D scaffolds of 4% (w/v) PHB and PHBV were subjected to 7 days incubation at 37 °C and 5% CO$_2$ in the cell growth media to observe any significant conductivity ($\kappa$) changes within that period of treatment. No media changes were conducted until day 7. The cell growth media were prepared according to the cell provider recommendation and the initial pH was adjusted to 7.2. All cell growth media contains several essential inorganic salts such as potassium chloride (KCl), magnesium sulfate (MgSO$_4$), sodium chloride (NaCl), sodium phosphate (NaH$_2$PO$_4$) and etc. Thus, the conductivity ($\kappa$) of the cell growth media (control) was measured with value of 20.77 mS/cm at 21 °C. It was observed that as time of incubation increases up to 7 days, there were insignificant differences of conductivity ($\kappa$) between both polymers in comparison with the control ($p>0.05$) (Fig. 4). This indicates that the amount of NaCl left inside the porous 3-D scaffolds were irrelevant in affecting the electrolytes balance of cell growth media. In fact, the amount of sodium chloride left inside the porous 3-D scaffolds might be considered as too small to produce any adverse effect to cell growth due to the electrolytes imbalance from hypertonic media solution (excessive inorganic salts).

Meanwhile, one of the most concerning issues regarding the SCPL technique is the amount of porogen left over inside the fabricated scaffolds. Previous study [25] has reported that the presence of residual organic solvent and porogen is the most significant problem facing these techniques due to the risks of toxicity and carcinogenicity it poses to the cells. Based on our recent finding, it was contrary with the above statement where there were insignificant differences of conductivity ($\kappa$) in the cell growth media for PHB and PHBV porous 3-D scaffolds as compared to the control (cell growth media without scaffolds). This indicates that both polymeric
porous 3-D scaffolds did not give any significant electrolytes imbalance (inorganic salts) inside the cell growth media due to the excessive amount of sodium chloride.

![Conductivity (κ) of cell growth media as a function of time at 20 ± 1 °C. The polymeric porous 3-D scaffolds were submerged in cell growth media (90% IMDM + 10% FBS + 1% PS) and incubated at 37 °C, and 5% CO₂ for 7 days. NS indicates no significant difference as compared with the control (n = 3).](image)

The finding was also consistent with several previous studies [4,20,26] indicating that such amount of solvent and porogen that left inside the scaffolds were to too small (less than ~1% (w/w) of porogen) to be considered as toxic to the cell and affect the cell behaviour (i.e., attachment, migration, differentiation and apoptosis) entirely. Besides that, even though the efficiency (%) of salt leaching and SCPL end product for both polymers did not even close to 100%, those porous 3-D scaffolds can actually be considered as 100% free from porogen and solvent residual if the above mentioned results (the effect of sodium chloride residual on cell growth media) were taken into account. Moreover, both polymers were also undergone for several pre-treatments prior to cell culture study. For instance, the sublimation process (lyophilization) was run for 48 hrs under vacuum pressure of 2 mbar to remove all remaining deionized water (DIW) inside the porous 3-D scaffolds after the salt leaching process. Thus, this process will absolutely remove all the solvent residual inside the porous structure. Then, followed by the sterilization process which involves several times of soaking and washing with phosphate buffered saline (PBS) and cell growth media (more than 3 hrs). All of those pre-treatments could possibly be relevant enough to verify that all remnants have been sufficiently removed and diluted from the porous 3-D scaffolds.

**Conclusion:**

In conclusion, the insignificant differences of conductivity (κ) for both polymeric porous 3-D scaffolds treated in the cell growth media in comparison with control demonstrated that the salt remnant were irrelevant in affecting the electrolytes balance inside the cell growth media which could lead to cellular damage such as necrosis. Therefore, it was suggested that this method was by far the convenient method to produce porous 3-D scaffolds with an improved thickness greater than 1 mm without perturbing to any contaminants (e.g., porogen and solvents) that could hinder or affect the in vitro cell proliferation. Previous studies have shown that by modifying several parameters such as polymer concentration, porogen weight fraction, type of solvent, and porogen particle shape could actually promote significant changes on the stability, morphology and surface chemistry of the polymeric porous 3-D scaffolds. Therefore, it would be suggested that by modifying its surface chemistries, both polymeric porous 3-D scaffolds would perhaps to allow better cell attachment and proliferation due to the functionalization with oxygen-containing groups into hydrophilic surfaces. However, further evaluations are still required to confirm such preliminary results.

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