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DEVELOPMENT OF A RAPID CONTINUOUS FLOW SALT LEACHING KIT FOR FABRICATION OF POLY(3-HYDROXYBUTYRIC ACID-CO-3-HYDROXYVALERATE) (PHBV) POROUS 3-D SCAFFOLD

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Graphical abstract
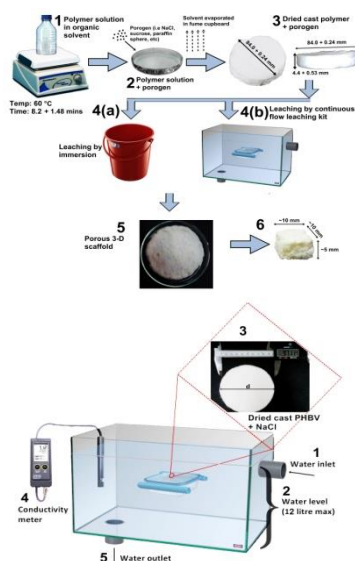
Abstract

Polyhydroxyalkanoates (PHAs) that are synthesized from bacteria that are predominantly produced by microbial fermentation processes on organic waste, such as palm oil mill effluent (POME), olive oil and kitchen waste, contribute to a sustainable waste management. A great variety of materials from this family can be produced, however the application of PHAs in the production of scaffolds in tissue engineering has been mainly constrained to poly(hydroxybutyrate-co-valerate) (PHBV) due to its highly adjustable physico-chemical properties. One of the common methods in making the 3-D scaffolds is by performing solvent-casting particulate-leaching (SCPL) process, but this process requires a long period of soaking in water to extract the entire salt particle in the 3-D scaffolds. Therefore, the objective of this study is to develop a new method to the conventional method of salt leaching process via a highly efficient continuous flow leaching kit. The salt leaching process was carried out by (1) immersing the 3-D porous scaffolds in a fabricated static container containing tap water and (2) by allowing a pre-setting continuous flow rate of water. The concentration of sodium chloride (NaCl) was calculated periodically for both processes based on the salt standard calibration curve. The results showed that the exhaustive salt leaching of the conventional process occurred at 48 ± 5 hrs with the needs of changing the water twice a day. In contrast, the exhaustive salt leaching process via continuous flow leaching kit occurred at 40 ± 5 mins, 72 times faster than the conventional method ($p<0.05$). Therefore, the salt leaching process using continuous flow leaching kit can be considered a highly efficient and time saving procedure as compared to the conventional method.

Keywords: 3-D scaffold, PHBV, salt leaching, continuous flow, salt leaching kit

Abstrak

Polyhydroxyalkanoates (PHA) yang disintesis dari bakteria dihasilkan melalui proses penapaian mikrob sisa organik seperti sisa buangan dari kilang minyak sawit (POME), minyak zaitun dan juga sisa dapur yang mana mampu menyumbang kepada pengurusan sisa buangan secara mampan. Pelbagai jenis bahan boleh dihasilkan dari keluarga PHA, namun aplikasi PHA dalam kejuruteraan tisu, khasnya dalam penghasilan struktur perancah lebih tertumpu pada poly(hydroxybutyrate-co-valerate) (PHBV) kerana sifat fisiko-kimianya yang mudah diubah suai. Salah satu kaedah yang biasa digunakan dalam penghasilan struktur perancah 3-D ialah menggunakan kaedah pembentuk-

pelarut lesap larut-partikel (SCPL), tetapi proses ini memerlukan tempoh masa pengekstrakan yang lama untuk pelarutan garam di dalam struktur perancah 3-D. Oleh itu, objektif kajian ini adalah untuk membangunkan satu kaedah baru untuk proses lesap larut garam konvensional melalui aliran berterusan kit lesap larut. Proses lesap larut dijalankan dengan menggunakan 1) teknik rendam struktur perancah 3-D di dalam takungan yang mengandungi air statik dan 2) pengaliran air berterusan yang telah ditetapkan kadar alirnya. Kepekatan natrium klorida (NaCl) diukur secara berkala dengan merekodkan jumlah air yang telah digunakan dalam kedua-dua proses. Keputusan menunjukkan bahawa proses lesap larut menggunakan teknik rendaman yang biasa mengambil masa selama 48 ± 5 jam bagi melengkapkan proses lesap larut, dengan keperluan penukaran air sebanyak dua kali sehari. Sebaliknya, kit lesap larut aliran berterusan mengambil masa selama 40 ± 5 minit, iaitu 72 kali lebih cepat daripada kaedah konvensional (p<0.05). Kesimpulannya, proses lesap larut menggunakan kit lesap larut aliran berterusan adalah lebih efisyen dan menjimatkan masa berbanding kaedah rendaman biasa.

Kata kunci: Struktur perancah 3-D, PHBV, lesap larut, aliran berterusan, kit lesap larut

1.0 INTRODUCTION

The production of biodegradable polymers is seen to be a viable alternative to replace conventional plastics, since the usage of conventional plastics has resulted in environmental degradation [1]. One of the examples of biodegradable polymers is polyhydroxyalkanoates (PHAs). This polymer is mainly produced by microbial fermentation processes, and a major challenge is to reduce the production costs [2]. Thus, several methods have been investigated to produce PHAs by microbial fermentation on organic waste such as palm oil mill effluent (POME), olive oil and kitchen waste. The production of biodegradable polymers from oil palm industry can be seen as beneficial to the environment as well as contributing to sustainable development [3, 4]. According to the industrial standard, the milling process produces wastewater in the range 0.44-1.18 m^3/tonne fresh fruit bunches (FFB) with the average figure of 0.87 m^3/tonne FFB. It is estimated that for each tonne of CPO that is produced, 5-7.5 tonnes of water are required, and more than 50% of this water ends up as palm oil mill effluent (POME) [5]. POME is an oily wastewater generated by palm oil processing mills and consists of various suspended components. By utilizing the POME and empty palm oil fibre bunch (EPFB) as carbon sources and support matrix, the disposal of POME that needed further treatment could be reduced.

Conventionally presented in complicated forms, POME cannot be directly reused by PHA-producing species such as *Ralstonia eutropha*, a representative bacterium for PHA synthesis [3]. Since the production cost using POME is practically high, Salmiati proposed an anaerobic treatment of POME coupled with PHA production using heterotrophic bacteria [6]. However, to ensure only acetic and propionic acids to be produced and not formic acid and biogas (PHAs inhibitor compound), it was crucial to maintain the pH at 7 in this anaerobic treatment of POME sludge in the first stage of the process [7, 8]. In order to overcome the problem, Md. Din *et al.* proposed the suitability of using mixed cultures to produce PHA in POME, and it shows different types of PHA-constituents could be obtained [9]. The harvesting of these PHA-constituents was more reliable for use as biodegradable plastics material as opposed to a single PHA-constituent.

Furthermore, PHAs were used for tissue engineering of 3-D cell culture system due to its acceptable biodegradability, biocompatibility and resemblance to polypropylene (PP) which offers a wide range of mechanical properties [10, 11, 12]. PHAs have already been studied to some extent for tissue engineering applications mainly for scaffolds materials in combination with ceramic materials as a vehicle for drug delivery and also as a material for cardiac tissue engineering [13, 14, 15]. The potential use of PHAs in tissue engineering is illustrated in Figure 1.

There are vast methods in manufacturing scaffolds such as solvent casting and particulate leaching (SCPL), thermally induced phase separation (TIPS), gas-foaming, fiber bonding, solid free form and electrospinning. However, due to a simple and fairly reproducible method which does not require sophisticated apparatus, solvent casting and particulate leaching (SPCL) method is the best way in manufacturing 3-D porous scaffolds. This method consists of dissolving a polymer in a solvent and then adding particles of a leachable porogen such as salt particles, glucose, and paraffin spheres [16]. Thick paste formed from the mixture is then left to dry in air or under vacuum until the solvent has completely evaporated. The porogen is then leached out and leaves behind a network of interconnected pores. This method can also be used in manufacturing composite scaffolds, where second material (e.g., hydroxyapatite) is added with the porogen and remains within the structure after the porogen is leached out [17, 18]. Unfortunately, thickness limitations intrinsic to the particle leaching process, limited mechanical properties and argument of the

homogeneity and interconnection of the pores in the scaffolds, as well as the presence of residual porogen and solvent, make this solvent casting and particulate leaching processes out of favor [19]. According to Zubairi, the process of leaching using immersion of both PHB and PHBV scaffolds take 2 days prior to salt leaching processes is time consuming [20]. However, the efficiency of end product showed that the scaffolds could retain its mass of porous material throughout the leaching process. Besides, there are still a few other studies which have also shown some concern about the porogen residual [16, 21, 22]. Due to this major drawback, the objective of this study is to develop a new time saving method of salt leaching process via a highly efficient continuous flow leaching kit.
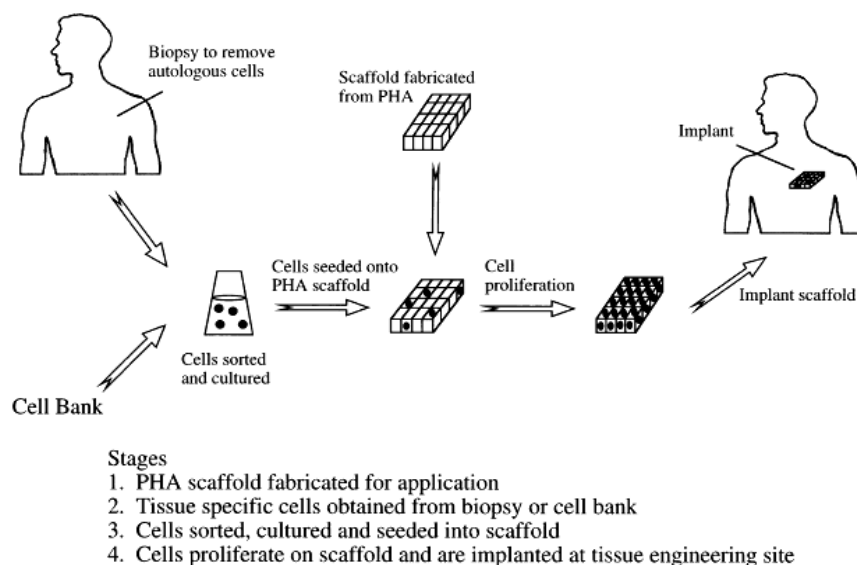

Figure 1 Role of PHAs in Tissue Engineering [12]

2.0 EXPERIMENTAL

2.1 Materials

Poly(3-hydroxybutyric acid-co-3-hydroxyvaleric acid) (PHBV; M_w = 680,000 g/mol with 12% (w/w) polyhydroxyvalerate (PHV) content; CAS No. 80181-31-3) and cell culture grade sodium chloride (particles size: 212 μm to 850 μm) were purchased from Sigma-Aldrich® (Dorset-United Kingdom) [23, 24]. Chloroform 99.9% in purity (AnalaR®) was obtained from VWR International (Leicestershire, United Kingdom). The portable leaching kit was assembled using materials obtained from local hardware.

2.2 Fabrication of PHBV porous 3-D scaffolds

Polymer PHBV weighted 2.4 gram containing 12% (w/w) polyhydroxyvalerate (PHV) was stirred in 60 ml boiled reflux chloroform (99.9% purity) at 60 °C for 8.2 ± 1.48 mins in a fume cupboard (Figure 2). The polymer solutions were cooled down for 10 mins prior to the commixing with sodium chloride (NaCl), which act as leachable porogen. Once cooled, the polymer solutions were then integrated evenly over a coated aluminum foil glass Petri-dish (internal diameter, 8.8 cm × height, 1.8 cm). About 172.31 ± 0.18 g of sodium chloride crystals with a salt weight fraction of 99% (w/w) were then poured and stirred evenly into the polymer solutions with perpetual stirring until the polymer-solvent solution became pasty, thick and packed. The fully filled pasty Petri-dish was tapped a few times so that the content became compact and even. This procedure was conducted in the fume cupboard. Subsequently, the packed-in pasty were put immediately inside the desiccators (to minimize rapid phase disseverment, which could result in an etched surface) and air-dried for 2 days to consummate solvent evaporation.

2.3 Conventional Salt Leaching Process

The dried cast polymers were peeled carefully from the aluminum foils and then dialyzed with 10 liters of tap water at 25 ± 0.79°C for 2 days to remove all sodium chloride crystals. The water was changed twice a day. The porous material floated on top of the water, indicating that the sodium chloride crystal was fully leached out. Then, the wet porous materials were left to dry at room temperature. The process was repeated 4 times (n = 4).

2.4 Salt Leaching via Continuous Flow Leaching Kit

The setup for the continuous flow leaching kit is shown in Figure 3. Firstly, the conductivities (κ) of standard sodium chloride solution (for standard calibration curve) and solution with leached sodium

chloride from the dried cast polymers were measured by using HANNA Instruments™ HI99300 conductivity meter. Two electrodes with an applied AC voltage were placed in the solution. This created a current dependent upon the conductive nature of the solution. The meter read this current and displayed it as conductivity (EC). The sodium chloride solutions were first prepared on the serial concentration (0.2, 0.4, 0.6, 0.8 and 1 mg/ml) and the conductivities (µS/cm) at $25 \pm 0.79°C$ were plotted against its respective concentration to establish a standard calibration curve. Polymer concentrations of 4% (w/v) for PHBV containing 12% (w/w) polyhydroxyvalerate (PHV) were used in preparing dried cast polymers as described in the above protocol. The dried cast of polymer was placed in the leaching kit and the tap water was turned on to remove all sodium chloride crystals. The water flow rate was constantly set to 1.4 ± 0.04 liter/mins ($n = 3$) for both inlet and outlet conduit, thus maintaining the 12 liter volume of water. The kinetic of salt conductivity was measured until it reached down to 218 ± 5.38 µS/cm (conductivity of tap water at $25 \pm 0.79°C$). Then, the total concentration and mass of sodium chloride were determined from the standard curve, based on the sums of conductivities. The amounts of sodium chloride that had been leached out from the dried cast polymers were determined in 4 replications ($n = 4$). Meanwhile, a control sample with the amount of 172.31 ± 0.18 g of sodium chloride was prepared into a small kitbag with fine pores size. The same procedures and measurements were employed as described above.
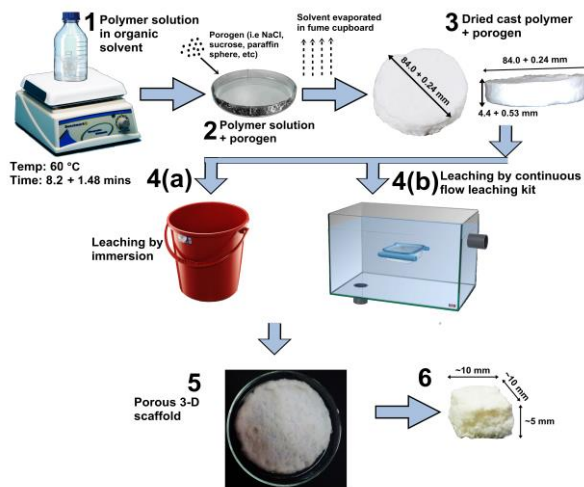

Figure 2 Schematic of SCPL process [20, 25]. The process comprises (1) mixing of polymer in organic solvent at 60 °C for 8.20 ± 1.48 mins; (2) adding porogen to the polymer solution into a Petri-dish and then incubated in the fume cupboard to avoid the development of etching surfaces; (3) dried cast of polymer with porogen after organic solvent was fully evaporated; (4) leaching processes by (a) conventional leaching, (b) continuous flow leaching kit; (5) lyophilized porous 3-D scaffolds with the thickness greater than 1 mm; (6) A rectangular size of ~10 mm x ~10 mm x ~5 mm porous 3-D scaffolds was excised prior to the physico-chemical analysis
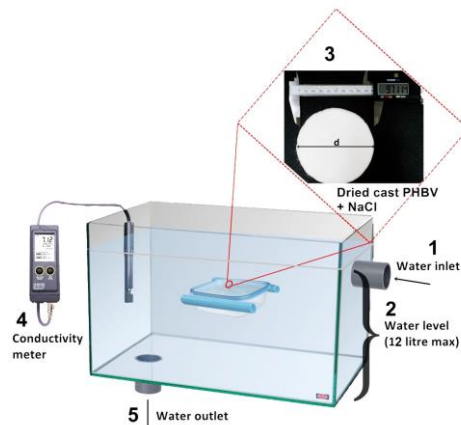

Figure 3 Schematic apparatus of a rapid continuous flow salt leaching kit

2.5 Determination of Salt Leaching Efficiency

A gravimetric analysis was conducted after the lyophilization process to determine any potential of polymer weight loss during the salt-leaching process [20]. The efficiency (%) of salt leaching process was measured via ion conductivity and gravimetric analysis after the lyophilization process was determined based on the Equation 1, Equation 2 and Equation 3 respectively.

Efficiency of salt-leaching measured via ion conductivity (%) = [$M_{NaCl \text{ in the dried cast}}$ − $M_{NaCl \text{ dissolved in the tap water}}$]/$M_{NaCl \text{ in the dried cast}} \times 100\%$ (1)

Efficiency of salt-leaching via gravimetric analysis (%) = [$M_{NaCl \text{ in the dried cast}}$ − $M_{NaCl \text{ remains inside the lyophilized scaffolds}}$]/$M_{NaCl \text{ in the dried cast}} \times 100\%$ (2)

Weight of NaCl remains inside the lyophilized 3-D scaffolds (g) = $M_{Lyophilized \text{ porous 3-D scaffolds}}$ − 2.4 g (weight of polymer at 4%, (w/v))   (3)

2.6 Statistical Analysis

The data was presented as means ± standard deviation (SD) of mean values. Statistical comparison was performed using Students *t*-test (SPSS version 19.0 IBM co.) for salt leaching efficiency study. Statistical analysis of data for sodium chloride residual effect was carried out using one-way analysis of variance (ANOVA) to determine the presence of any significant difference among sample means of the groups, which later was followed by Tukey's test (SPSS version 19.0 IBM co.) for multiple comparisons to determine the values that were significantly different. The $p < 0.05$ was considered statistically significant.

3.0 RESULTS AND DISCUSSION

3.1 Salt Leaching Efficiency

The conventional salt leaching process takes about 48 ± 5 hrs in order to complete the exhaustive leaching process [20]. In fact, the process requires changing the salty water twice a day. This is considered a tedious and time consuming procedure. On the contrary, the salt leaching process via continuous flow leaching kit could only last for 40 ± 5 mins ($p<0.05$) to complete the salt removal as compared to the conventional method (72 times faster than the conventional method ($p<0.05$)). The available salt in the porous 3-D structure (Figure 4) was assumed to have been exhaustively removed as calculated via material balance (Table 1) and standard calibration curve (Figure 5).
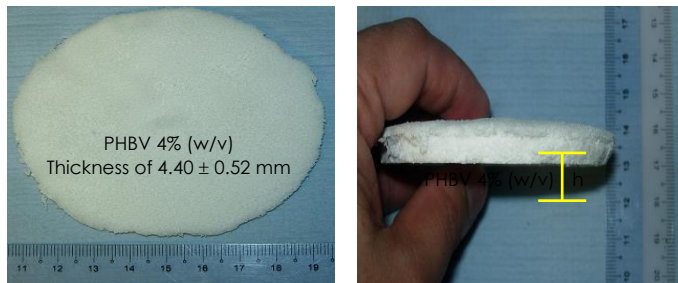

Figure 4 Morphology of polymeric porous 3-D scaffolds in a cylindrical shape with an approximate thickness (h) of 5 mm: (a) Aerial view of PHBV (4%, w/v) and (b) Side view of PHBV (4%, w/v)

Table 1 Material balance and efficiency (%) of the control and 3-D scaffolds continuous salt leaching process

Independent/dependent variables	Control		3-D scaffolds	
	Pre-leaching	Post-leaching	Pre-leaching	Post-leaching
Mass of polymer (g)	-	ND	2.42 ± 0.04	*1.82 ± 0.01 [a]
Total conductivities (µS/cm)	-	2572.02 ± 152.20	-	2554.30 ± 25.20
Total volume of salty water (ml)	-	141.70 ± 19.10	-	148.80 ± 10.50
Final concentration (mg/ml)	-	1.28 ± 0.08	-	1.27 ± 0.01
Mass of NaCl (g)	172.31 ± 0.18	*181.40 ± 35.56	172.31 ± 0.18	*189.10 ± 12.03 [b]
Efficiency of salt leaching measured via ion conductivity (%) [c]	-	113.90 ± 13.64 [c] [d]	-	109.7 ± 7.02 [c] [d]
Efficiency of salt leaching measured via gravimetric analysis (%) [c]	-	100 [e]	-	100 [e]
Weight of NaCl remains inside the lyophilized 3-D scaffolds (g) [c]	-	0	-	0

[a] Mass of porous 3-D scaffolds post lyophilization process. There was an approximately 20% (w/w) of polymer material loss throughout the fabrication and leaching process. [b] The value was calculated based on the NaCl standard calibration curve (Figure 5) and the total amount of collected salty water. [c] The calculations were conducted based on the formula shown in Equation 1, 2 and 3 [20]. [d]

Greater than 100% - This might be due to the fluctuation of the ambient temperature which could strongly affect the sensitivity of the conductivity probe and several other reasons as mentioned above. [e] The efficiency of the salt leaching process was assumed to be 100%. There might be some traces of salt remnants available in the 3-D scaffolds (further analysis is required to confirm this assumption). *$p<0.05$ - Besides, there was a significant difference between pre- and post-leaching process ($n = 4$).
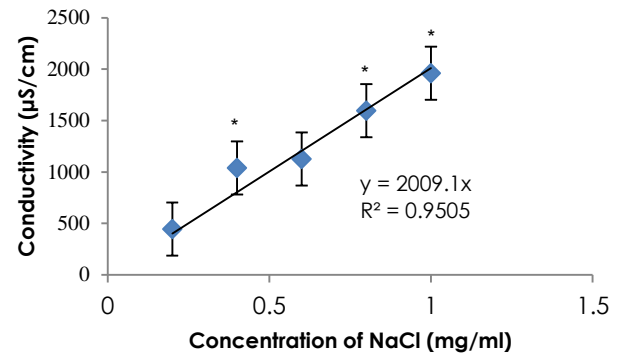

Figure 5 Relationship between conductivity of sodium chloride (Sigma-Aldrich®) solution with respect to its different concentration (mg/ml). The values were normalized by subtracting the conductivity (κ) of tap water which was 218.25 ± 5.38 µS/cm at 25 ± 0.79 °C. It was a linear correlation between conductivity (µS/cm) and the concentration of sodium chloride (mg/ml); y = 2009.1x; R^2 = 0.9505 ($n = 4$). *There was a significant difference with $p<0.05$ between the points

Meanwhile, the kinetic equilibrium of continuous salt leaching process was constructed for both control (Figure 6) and 3-D scaffolds (Figure 7) to corroborate the rapid solubility of salt via continuous flow of water. As the conductivity reached the tap water value (218.25 ± 5.38 µS/cm), the continuous salt leaching process was considered exhaustive (100% salt removal) and then set for a vacuum drying process (lyophilization). Both control and 3-D scaffolds produced comparable salt leaching kinetic profile even though 3-D scaffolds salt leaching process ends faster than the control (fine pores kitbag prolonged the salt solubility) ($p<0.05$). However, there was a significant amount of polymeric material absent from the dried 3-D scaffolds with an approximately 20% (w/w) of polymeric material losses after lyophilization process ($p<0.05$) (Table 1). This phenomenon occurred possibly due to the spilling of polymer solution whilst homogenizing the solution with salt in the Petri dish. In addition, there were some small apparent polymeric materials floating on the water surface during the salt leaching process. The high porosity (Table 2) of PHBV had possibly allowed a huge amount of water to absorb deep into the porous structure via capillary action [20, 26, 27]. Thus, as the water absorption capacity became saturated, the structure started to swell immediately (visual assessment). Part of the scaffolds became too soft as the structure could not

possibly bear the weight of the up regulated water content. Since the leaching process was in the continuous flow, the turbulence inside the container could easily rupture the soft part and eventually bring up the small shredded materials on the water surface. For that reason, a small modification is planned on both containers by installing and covering a honeycomb-like structure to minimize the turbulence and swirl level.

Table 2 Physical properties of PHBV (4%, w/v) porous 3-D scaffolds.

Physical properties	Polymeric porous 3-D scaffolds
	PHBV (4%, w/v)
BET surface area, A_s, m^2 g^{-1}[a]	0.82 ± 0.03
Geometrical bulk density, g cm^{-3}	0.072 ± 0.28
Skeletal density, g cm^{-3}[b]	0.92 ± 0.14
Porosity, %	92.17 ± 0.73

D = interconnected pore window diameter (D_{mode}: one of a range of values that has the highest frequency). [a] BET surface area (m^2 g^{-1}) = total skeletal surface area (m^2)/skeletal mass (g). [b] ρ_s is the skeletal density of the crushed scaffolds, which was determined from helium pycnometry.

Furthermore, based on the material balance as shown in Table 1, there was a significant amount of salt content increase in the total collection of leached salty water ($p<0.05$). The extra amount of salt remnants (calculated) was considered to have affected the material balance of the salt leaching process. However, the calculated values had been normalized with the conductivity value of the tap water (218.25 ± 5.38 µS/cm), inclusive of other available foreign compounds [28]. Therefore, the discrepancy of the measured and calculated values of the total salt content between pre- and post-leaching process on both control and 3-D scaffolds was possibly due to the fluctuation of the ambient temperature which strongly affected the sensitivity of the conductivity probe [29]. Moreover, the same efficiency (%) of both control and 3-D scaffolds ($p>0.05$) has proven that the above mentioned problem does affect conductivity (µS/cm). Surprisingly, the instrument was not equipped with the automatic temperature control (ATC) to compensate any temperature fluctuation of the salty solution. Even though the efficiency via gravimetric analysis showed an exhaustive removal of salt (100%), there might be some salt remnants available inside the 3-D scaffolds which could not be determined via material balance. The calculated amount of the material losses (an approximately 20% (w/w)) could possibly be only from the polymers themselves but not from the salt. Therefore, spectroscopy analysis (e.g., Nuclear Magnetic Resonance Spectroscopy, NMR) should be implemented to analyse the polymer

molecular composition and verify the amount of contaminated salt (if any) in the 3-D scaffolds.
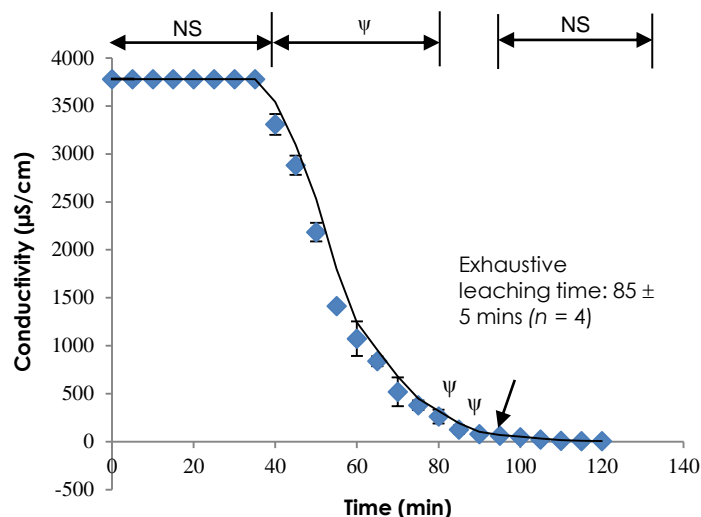
Figure 6 Kinetic equilibrium of sodium chloride (Sigma-Aldrich®) conductivity via continuous salt leaching process (control). Control: The absence of 3-D polymeric material - The same amount of salt was used in the making of 3-D scaffolds). The values were normalized by subtracting the conductivity (κ) of tap water which was 218.25 ± 5.38 µS/cm at 25 ± 0.79 °C.). (ψ) $p<0.05$ comparisons were made of the previous values. NS indicated no significant difference as compared with previous value ($n = 4$)
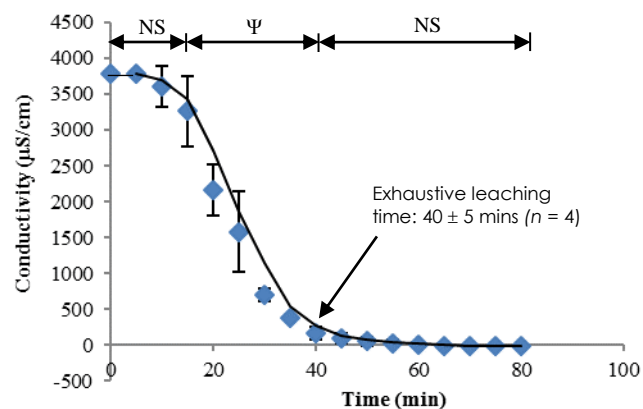
Figure 7 Kinetic equilibrium of sodium chloride (Sigma-Aldrich®) conductivity via continuous salt leaching process (3-D scaffolds). The values were normalized by subtracting the conductivity (κ) of tap water which was 218.25 ± 5.38 µS/cm at 25 ± 0.79 °C. The salt leaching process was considered exhaustive as the conductivity value reached 218.25 ± 5.38 µS/cm (conductivity of tap water at 25 ± 0.79 °C). (ψ) $p<0.05$ comparisons were made of the previous values. NS indicated no significant difference as compared with the previous value ($n = 4$).

4.0 CONCLUSION

A new alternative and efficient way of salt leaching process was successfully attained through the usage

of a highly efficient continuous flow leaching kit. The process took only 40 ± 5 mins to achieve an exhaustive salt leaching process in a 3-D porous structure. Even though the newly developed method had a notable loss of polymeric material and some sensitivity issues, the rigidity of the dried 3-D scaffolds were uncompromised prior to the 3-D cell culture study. In conclusion, the salt leaching process using a highly efficient continuous flow leaching kit is considered the best method to remove salt remnants exhaustively in a 3-D porous structure. In fact, it saves a significant amount of time as compared to the conventional method (approximately 2 days of completion) whilst maintaining the rigidity of the 3-D structure.

Acknowledgement

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