

## Research Article

# Efficacy Study of Carrageenan as an Alternative Infused Material (Filler) in Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) Porous 3D Scaffold

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Polymeric porous 3D scaffold plays an important role in culturing mammalian cells as *ex vivo* model. However, the scaffold used is ineffective due to its structural and cell acceptability weaknesses. Therefore, this research attempts to overcome the weaknesses by using carrageenan from red seaweed *Kappaphycus alvarezii* as an alternative infused material (filler) of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) porous 3D scaffold. The 3D scaffold was conventionally fabricated using the solvent-casting particulate-leaching (SCPL) method. Carrageenan was later infused into 3D porous scaffolds under vacuum pressure and freeze-drying process. Five carrageenan concentrations were prepared and its physicochemical properties such as pH and viscosity were carried out on each concentration to determine the best solutions to produce a new composite 3D structure. The preliminary result shows that carrageenan concentrations of 2, 4, and 6% (w/v) were considered the best solutions for the infusion process due to its stable rheology properties. The pH and viscosity profiles of three selected carrageenan solutions were exhibited in the range of 9.00–9.20 and 0.047–1.144 Pa·s, respectively. Moreover, the incorporated carrageenan gel fraction was in the range of 4.30% to 14.95% (w/w) which was determined by gravimetric analysis and dye staining method (visual assessment). The well-infused carrageenan 3D scaffold was further characterized based on its internal morphology and degradability study. The vertical cross-sections of the scaffolds revealed homogeneous accumulation of dried gelatinous carrageenan which was covered throughout its pores wall. The degradation rate ( $K$ ) of the carrageenan infused 3D scaffold was between  $0.01 \pm 1.66$  (mg/day) and  $0.03 \pm 3.23$  (mg/day). The higher the carrageenan concentration used, the faster the degradation rate occurring ( $p < 0.05$ ). The 3D infused scaffold of 4% (w/v) carrageenan concentration (S2) produced a moderate degradation rate of  $0.02 \pm 1.55$  (mg/day) with a sustained structural integrity up to 28 days. The carrageenan infused scaffold of 4% (w/v) was demonstrated to be the best 3D structure for a long-term cell culture (>2 weeks). In conclusion, the usage of carrageenan as a composite material exhibits its great potential to be used in tissue engineering application and 3D cell culture model.

## 1. Introduction

Tissue engineering is a field of technology that thrives in the present moment. The construction of body organ through engineering tissues method can be used for helping the generation of any body organ or cell that is not working, in order to be replaced with organs cell or a new one [1]. Construction activities of organ can be developed in laboratory for replacing or helping the growth of tissue cells that are damaged or suffered from illnesses using a combination of biomaterials, living cells, and also the active molecules [2]. The latest methods that are frequently used

to assist in breeding and tissue reconstruction/regenerative include using cell therapy, gene delivery system, and the structure of biological 3D scaffolding [3]. The 3D scaffolds produced have many pores, which are important for cell-to-cell interaction and communication as this facilitates the cell growth tremendously [2]. However, the 3D scaffold pores structure could possibly cause some cells to accidentally flush out during cell culture. Lack of biosignaling compounds and empty voids will eventually reduce the cell density and distribution of cells throughout the cell culture process [4]. Therefore, these pores should be filled with soft material like hydrogel in order to assist the cell growth and at the same time

increase the cells density without losing too much of them in the growth media [5].

According to Zhensheng et al. [4], scaffolds that are infused with hydrogel help increase cell growth up to 89% as compared to the scaffolds that are not infused with hydrogel (36% growth). Natural-based hydrogel like carrageenan has been chosen as the best candidate due to the unique features found in its molecular structures such as unique mechanism of gelation, ability to absorb high water content, abundant negative charges, functional groups that can be easily modified accordingly, and great chemical and proteins delivery for the tissue regeneration [6]. In fact, it is not just good for the infused material but it also has good therapeutic properties such as anticancer, antiviral, anticoagulation, and antihyperlipidaemia properties [5]. Nowadays, carrageenan is often used as the matrix polymer in the production of controlled drug release tablets and stabilizer in the system of microparticles as it extends medicine shelf-life. The use of natural resources which are easily available with inexpensive production makes it the best choices so far for the infused material in the 3D scaffold [7].

Construction of scaffold for certain function should be produced from material which is compatible with the physical and mechanical features of the body. This is to make sure that the body systems will not do any rejection or fatal reaction against foreign materials that enter the body system [8]. In fact, it needs to have similar features as extracellular matrix (ECM) where it can provide three-dimensional environment that could facilitate certain function to cell growth. Theoretically, the structure of the generated scaffold needs to have a wide surface area for cell-to-cell interactions and the material itself must be easy to degrade when reproduction of a new tissue begins to form [9]. Therefore, the objectives of this research were to study the efficacy of carrageenan as an alternative infused material in poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) porous 3D scaffold and to determine the newly infused carrageenan 3D scaffold effective degradability prior to mammalian cell culture studies.

## 2. Material and Methods

**2.1. Materials and Chemicals.** The material for the fabrication of 3D porous scaffold was poly(3-hydroxybutyric acid-co-3-hydroxyvaleric acid) (PHBV) and Dulbecco's Modified Eagle Medium (DMEM) for the degradability study of the infused carrageenan was purchased from Sigma-Aldrich™ (UK). The infused material in 3D scaffolds was the type *K*-carrageenan from red seaweed species, namely, *Kappaphycus alvarezii*. This carrageenan (commercially available) was purchased directly from Tacara Company Sdn. Bhd., Semporna-Sabah, Malaysia.

**2.2. Fabrication of PHBV 3D Porous Scaffolds and Surface Modification.** The PHBV 3D porous scaffolds were fabricated based on the Syazwan and Zubairi [10] method. A total of 2.4 g poly(3-hydroxybutyric acid-co-3-hydroxyvaleric acid) (PHBV) was mixed together with 60 ml chloroform (99.9% purity) into the Scotch bottle and boiled for 10 mins at 60°C. The solution was later poured into a petri dish coated

with aluminum foil. A total of 172.3 g NaCl was added to the polymer solution. The mixture was stirred thoroughly and left in the fume hood for 2 days to allow slow chloroform evaporation. Once the mold hardened, the scaffold underwent the salt particulate-leaching process, where the scaffold was immersed into deionized water for 2 days to exhaustively remove all porogens. The scaffold was later oven-dried at temperature of 60°C overnight. Finally, to increase the efficacy of surface bonding between PHBV and carrageenan (hydrophilicity), the scaffold was immersed into 0.6 M NaOH solution for 60 mins at 20°C. The scaffold was prepared into 4 parts with the approximate size of 4 cm × 3 cm × 1 cm prior to physical properties analysis and degradation study.

**2.3. Preparation of Carrageenan Solution.** The carrageenan white powder was weighed according to the predetermined quantities based on Table 1. Five different formulations were prepared in Scotch bottles with varying solvent (250 ml of deionized water) to solid ratio of 2, 4, 6, 8, and 10 g/ml. The solutions were boiled and well stirred at 80°C for 30 mins to 1 hour. All solutions were cooled down to ambient temperature (28 ± 2°C) prior to 3D scaffold infusion process and after infusion physical properties analysis.

**2.4. pH Analysis of Carrageenan Solutions and In Vitro Degradation Study.** The pH values of all *k*-carrageenan solutions (2%, 4%, 6%, 8%, and 10% (w/v)) and Dulbecco's Modified Eagle Medium (DMEM) postdegradability studies of the infused carrageenan were carried out based on the standard AOAC method. Initially, pH meter (Orion™ Versa Star Pro™ pH Benchtop Meter) was calibrated with buffer solutions with a pH of 4, 7, and 10 prior to measurement. All readings were carried out in triplicate ( $n = 3$ ) for both studies.

**2.5. Carrageenan Solutions Viscosity.** The viscosity (Pa·s) of the prepared carrageenan solutions (2%, 4%, 6%, 8%, and 10% (w/v)) was determined using Anton Paar Physica MCR 301 Rheometer with spinner type CP25-2-SN12504 (diameter 0.051 mm). The spindle rotation was performed for 10 minutes at 60 ± 1°C. All readings were carried out in triplicate ( $n = 3$ ).

**2.6. Experimental Design.** The design of experiment (DOE) used in this study was the completely randomized block design (CRBD), where the PHBV porous 3D scaffold filling process was carried out under vacuum pressure (≈700 mbar) with 5 different concentrations (% (w/v)) of an increased solid loading (g). Table 1 shows five different formulations of carrageenan solution (filler) for the 3D scaffold infusion process.

**2.7. Infusion of Carrageenan (Filler) into PHBV 3D Scaffold.** Previous study used hydrogel from alginate as an ingredient in the production of 3D scaffold [11]. The modification was carried out by using carrageenan (food grade sources) as an alternative material unlike hydrogel to infuse into 3D PHBV scaffold microstructure as an empty porous filler (biomimicking *in vivo* soft tissue 3D structure). Five different

TABLE 1: Five different formulations of carrageenan solution using completely randomized block design (CRBD) method.

Carrageenan formulation	Concentration (% w/v)	Solid loading (g)
S1	2	5
S2	4	10
S3	6	15
S4	8	20
S5	10	25

formulations of carrageenan solution were infused into 4 randomly selected locations of the PHBV 3D scaffold with an approximate size of 4 cm × 3 cm × 1 cm. The carrageenan infusion process was carried out by pouring 20 ml of the hot gel solution onto the 3D porous scaffold at 60–80°C. The hot solution was used as to prevent those solutions from becoming solid prior to infusion. The red dye (5 ml of 40 mM Alizarin Red S; Sigma-Aldrich®) was added to the gel solution prior to the infusion process as it serves as an indicator to observe the absorption activity of carrageenan into 3D scaffold. The infusion process was carried out under vacuum pressure for 30 mins. Then, the infused carrageenan 3D scaffold was left at room temperature for 5 mins for cooling and hardening the solution (at room temperature) prior to freeze drying process at –84°C for 24 hrs to remove water ultimately. The freeze-dried 3D scaffold was subsequently soaked into 1% (w/v) CaCl<sub>2</sub> for 20 mins to strengthen the 3D scaffold surface bonding with dried carrageenan. All samples were rinsed three times using deionized water to remove any excessive CaCl<sub>2</sub>. The scaffolds were then once again being put into freeze dryer for 24 hrs to remove any excess moisture from the washing. All samples were kept in a sterile container prior to analysis. The quantity of carrageenan which has been successfully infused into the 3D scaffold was determined based on

$$\text{Mass of carrageenan infused (g)} = W_f - W_o, \quad (1)$$

where  $W_f$  is mass of scaffold after carrageenan infusion (g) and  $W_o$  is mass of scaffold before carrageenan infusion (g).

### 2.8. Infused Carrageenan 3D Scaffold Water Uptake Analysis.

The infused carrageenan 3D scaffolds were initially soaked in deionized water (DIW) for 48 hrs. Then, the scaffolds were air-dried (until constant weight is achieved) at 50–60°C for 24 hrs to remove DIW exhaustively from the porous scaffold. The total water uptake from the infused carrageenan 3D scaffold was calculated based on (2). All measurements were done in triplicate ( $n = 3$ ) and expressed as mean ± standard deviation (SD).

$$\text{Water uptake (\%, w/w)} = \frac{W_f(\text{g}) - W_b(\text{g})}{W_b(\text{g})} \times 100\%, \quad (2)$$

where  $W_f$  is weight of scaffold after air-drying (g) and  $W_b$  is weight of scaffold before air-drying (g).

**2.9. Surface Morphology Analysis.** To identify the efficacy of the infusion process and structural integrity postdegradability study, the infused carrageenan 3D scaffold surface morphology analysis was carried out using scanning electron microscopy (Tabletop Microscope, TM-1000). Vertical cross-sections of the porous 3D scaffolds (an approximate size of 1 cm × 1 cm × 1 cm) were attained. Once sectioned, the specimens were mounted on aluminum stumps, coated by gold-sputtering in an argon atmosphere for 2 mins at an acceleration current of 20 mA prior to the evaluation by SEM at an acceleration voltage of 20 kV.

**2.10. In Vitro Degradation Study.** The *in vitro* degradation study was carried out on three different types of scaffold of three different carrageenan concentration (Table 1: S1, S2, and S3) and naked 3D scaffold without carrageenan infusion (S0: control). All samples were put into centrifuge tubes containing 20 ml of DMEM complete solution (2 ml of 10% fetal bovine serum and 0.2 ml of 1% antibiotic). Each centrifuge tube contained 3 cuboid size 3D scaffolds with the approximate dimension of 1 cm × 1 cm × 1 cm. All samples were incubated for 7, 14, 21, and 28 days in 5% CO<sub>2</sub> incubator at 37°C. The sample weight and pH media were recorded before and after incubation on a weekly basis. The 1st-order kinetic inversed exponential (3) of the remaining 3D scaffold weight (mg) versus days of incubation was created to determine the degradation rate constant,  $k$  (mg/days), of all samples. The gravimetric and pH measurement was recorded in triplicate ( $n = 3$ ) and mean value was recorded with two decimal places for each analysis.

$$C_t = C_0 e^{-kt}, \quad (3)$$

where  $C_t$  is weight of scaffold remaining (mg) per time (days),  $C_0$  is initial weight of scaffold (mg), and  $k$  is degradation rate constant [12].

**2.11. Statistical Analysis.** All results were statistically analysed using one way-ANOVA and this was followed by Tukey's post hoc *t*-test analysis. A  $p < 0.05$  was considered statistically significant. All values were expressed as mean ± standard deviation (SD).

## 3. Results and Discussion

**3.1. Acidity/Alkalinity Carrageenan Solution.** The results obtained in Figure 1 showed that the alkalinity of all carrageenan concentrations ranging from 2% to 10% (w/v) was in the range of  $9.00 \pm 0.08$  to  $9.20 \pm 0.04$  ( $p > 0.05$ ). These values coincide with the values presented by Chan et al. (2013) [13] in which the value for  $k$ -carrageenan should be in the range of 8 to 11. These results show that  $k$ -carrageenan solution was an alkaline condition which is caused by the availability of functional groups of sulphate (SO<sub>4</sub><sup>2-</sup>) in the disaccharide unit [13]. The alkalinity properties of the carrageenan solution have potentially induced the surface chemistry of the PHBV 3D scaffold to be moderately hydrophobic (data not shown), which is suitable for mammalian cell culture work.

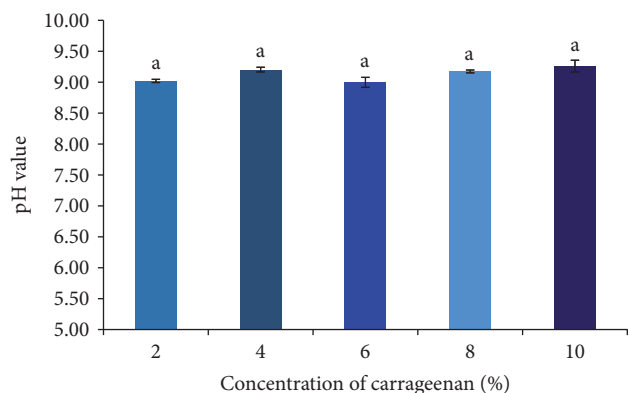


FIGURE 1: pH profiles of different carrageenan concentrations ranging from 2 to 10. The experiments were carried out in triplicate ( $n = 3$ ). Similar alphabet shows insignificant difference ( $p > 0.05$ ) between samples.

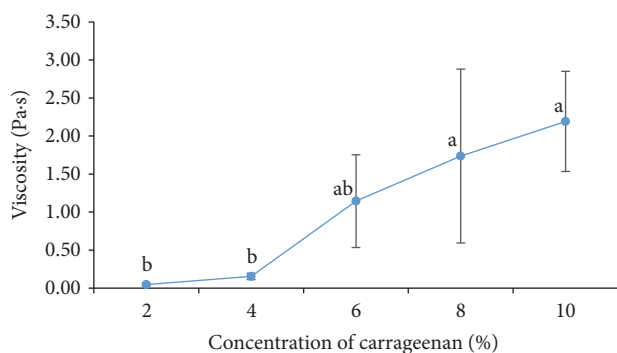


FIGURE 2: Pseudoplastic behavioural viscosity (Pa·s) of different carrageenan concentrations (% w/v). The experiments were carried out in triplicate ( $n = 3$ ). Different alphabet shows significant difference ( $p < 0.05$ ) between samples.

**3.2. Suitability of Carrageenan Solution Viscosity.** Figure 2 shows that the viscosity of *k*-carrageenan solution increased as higher concentrations were used ( $p < 0.05$ ). This pseudoplastic behavioural solution creates thermoreversible gel [13] from 2% to 6% (w/v). As it passes beyond 6% (w/v), the solution can no longer form the thermoreversible gel which became coagulated and difficult to stir for viscosity measurement. This phenomenon was due to the fact that carrageenan solution forms a gel at room temperature (25–37°C). In fact, the formation of carrageenan gel is closely linked to the development of helical bonding that occurs, where the strength of the gel formed on polysaccharide is influenced by the presence of cation and sulphate that are balanced in the solution [14, 15]. This resulted in the solution carrageenan forming a clod gel rapidly at temperature less than 60°C and eventually will make it even difficult to measure viscosity. For that reason, the concentration that was considered to be the best viscosity as a 3D porous scaffold filler was ranging from 2% to 6% (w/v). Those viscosity values of a working concentration of 2% to 6% (w/v) were in the range of 0.005–0.8 Pa·s which is superficially suitable in the

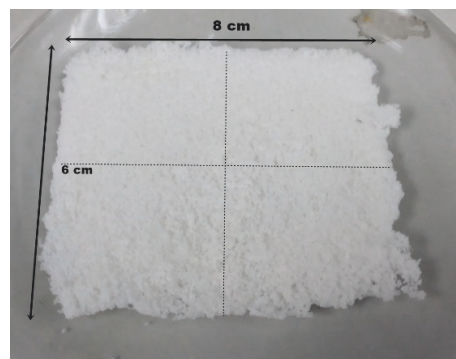


FIGURE 3: Naked porous 3D scaffold with an approximate size of 8 cm × 6 cm × 1 cm (thickness) fabricated via solvent-casting particulate-leaching (SCPL).

pharmaceutical or food products manufacturing industries [16].

**3.3. Naked Porous 3D Scaffold Prior to Infusion.** Fabrication of a 3D scaffold was based on the Syazwan and Zubairi [10, 17] methods via solvent-casting particulate-leaching (SCPL) process and its physicochemical properties were presented in the findings. Briefly, this naked porous 3D scaffold was much likely as a soft sponge and easier rupture if it is not handled in a great care. Figure 3 shows the aerial view of a porous 3D scaffold with an approximate size of 8 cm × 6 cm × 1 cm (thickness) prior to being cut into smaller pieces ( $\approx 1$  cm ×  $\approx 1$  cm ×  $\approx 1$  cm) using a polystyrene hot wire cutter and later carrageenan infusion (filling) process.

**3.4. Infused Carrageenan Gel (Filler) 3D Scaffold.** The 3D scaffolds (Figures 4(b)–4(d)) that have been infused with carrageenan (2, 4, and 6% (w/v)) showed a good permeability throughout the whole porous structure (red dye staining). On the contrary, the use of 8% and 10% (w/v) carrageenan concentration produced a big lump area (unstained from red dye) of impenetrable solution which exhibits a white spot as shown in Figures 4(e) and 4(f). Meanwhile, the amount of dried carrageenan that has been infused into the porous 3D scaffolds was in the range of  $5.49 \pm 1.22\%$  to  $13.79 \pm 1.52\%$  (w/w) of the 2% and 10% (w/v) carrageenan concentrations, respectively. Even though the infusion was carried out under vacuum pressure at 60–80°C, the high solid loading of carrageenan (high in viscosity) has obstructed the solution to be easily infused into the porous structure and eventually leads to a faster gel hardening at the scaffold's peripheral area. The 3D scaffolds that have been infused with carrageenan gel exhibited a good compression testing (data not shown) as compared to the control (naked 3D scaffold) ( $p < 0.05$ ) as the porous structure has been reinforced with solid carrageenan.

**3.5. Infused Carrageenan 3D Scaffold Water Uptake Efficacy.** The water uptake efficacy was carried out to determine the amount of water absorbed into the 3D scaffold as it is essential for cell proliferation. Figure 5 shows that the amount of the

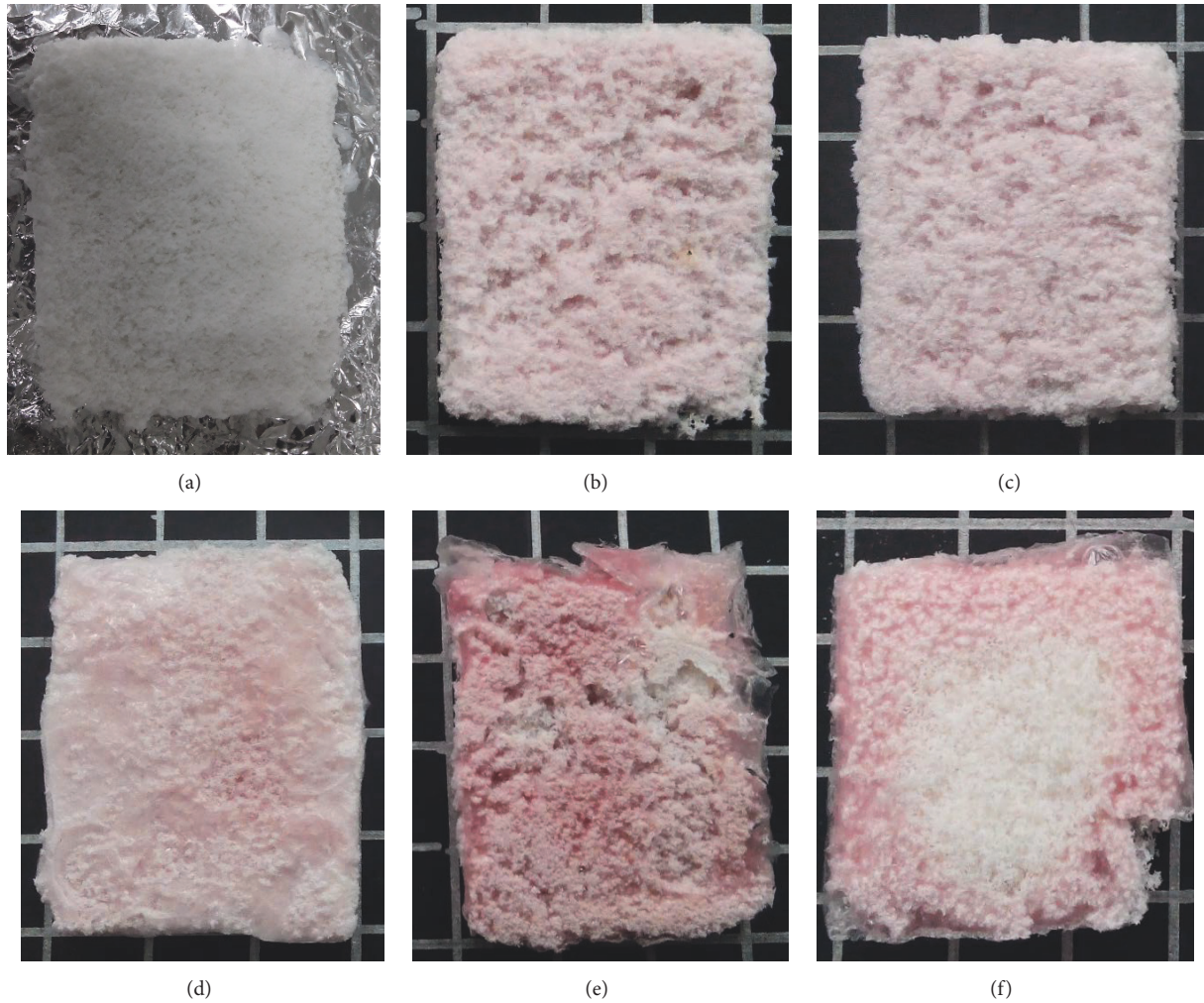


FIGURE 4: Aerial view of the red dye stained 3D scaffolds postinfusion of 5 different carrageenan concentrations: (a) control (naked 3D scaffold); (b) infusion of 2% (w/v) carrageenan solution; (c) infusion of 4% (w/v) carrageenan solution; (d) infusion of 6% (w/v) carrageenan solution; (e) infusion of 8% (w/v) carrageenan solution; and (f) infusion of 10% (w/v) carrageenan solution.

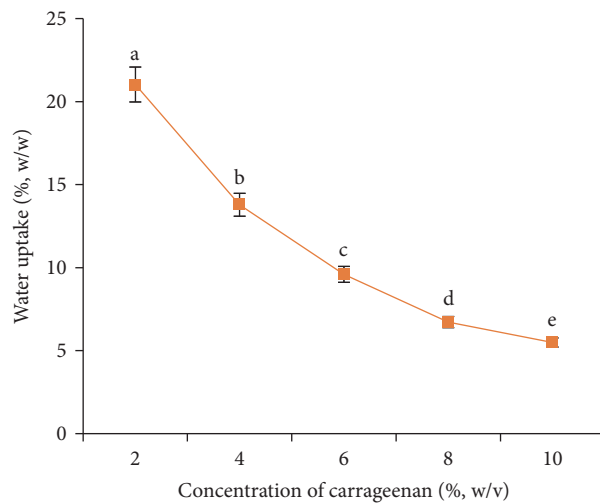


FIGURE 5: Inversely correlated 3D scaffold water absorption capacity (% w/w) versus infused carrageenan concentrations (% w/v). The experiments were carried out in triplicate ( $n = 3$ ). Different alphabet shows significant difference ( $p < 0.05$ ) between samples.

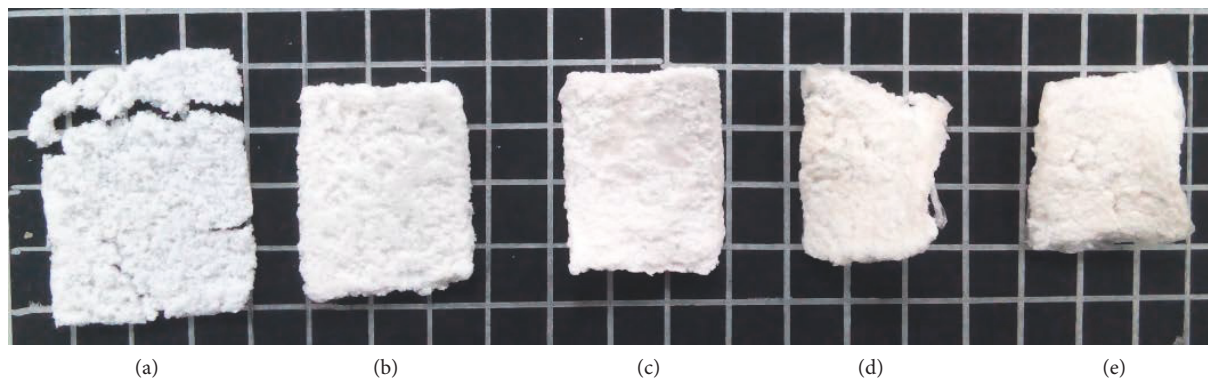


FIGURE 6: Aerial view of the infused carrageenan porous 3D scaffold 2 days after immersion: (a) 2% (w/v) carrageenan; (b) 4% (w/v) carrageenan; (c) 6% (w/v) carrageenan; (d) 8% (w/v) carrageenan; and (e) 10% (w/v) carrageenan.

water absorbed was inversely correlated with the prepared carrageenan solutions ( $p < 0.05$ ) in which the higher the concentration that is used, the lesser the amount of water that effectively gets into the porous 3D scaffold.

Meanwhile, based on the visual structural integrity assessment, the 3D scaffold infused with 2% (w/v) carrageenan (Figure 6(a)) has shown the least stable structural integrity as compared to other 3D scaffolds (Figures 6(b)–6(e)). Therefore, too much of carrageenan (filler) infused into the porous 3D scaffold would have eventually compromised the overall structural integrity which leads to breakages. With the help of water-like material (carrageenan), water was principally rushed into the porous structure via capillary action and the sudden gel expansion facilitates inner structure fractures.

### 3.6. Infused Carrageenan 3D Scaffold Surface Morphology.

Figure 7 shows the porous 3D scaffold surface morphologies containing dried carrageenan (filler) analysed by means of scanning electron microscope (SEM). Observably, it was found that the scaffold's pores wall (Figures 7(b), 7(c), and 7(d)) has been fully covered with carrageenan. On the contrary, scaffold which has been infused with 8% and 10% (w/v) carrageenan (Figures 7(e) and 7(f)) has the least covered carrageenan on the pores wall. Those highly concentrated carrageenan solutions were unable to diffuse efficiently into the porous structure due to the fast forming gel (Figures 4(e) and 4(f)). For that reason, only scaffolds with 2%, 4%, and 6% (w/v) carrageenan were selected for the degradability study in cell growth media (DMEM).

### 3.7. In Vitro Degradation Profiles of Modified 3D Scaffolds Containing Filler

**3.7.1. Scaffold Mass Reduction.** Prior to immersion in DMEM, moisture contents (MC) of all scaffolds were identified and removed using freeze drying process. All scaffolds contained acceptable level of MC (<12% (w/w)) and all remaining MC were removed prior to incubation in cell growth media. Figure 9 shows the degradation kinetic of all scaffolds (S0 [control], S1, S2, and S3) after DMEM incubation after 28 days. The mass reduction was observed from days 21 to 28

TABLE 2: Rate of degradation constant (mg/day) and moisture content (%) of the 3D scaffold infused with different carrageenan concentrations.

Concentration	*Degradation rate constant ( $K$ , mg/day)	*Moisture content traces (% w/w)
(S1) 2%	$0.011 \pm 0.0166$	$7.92 \pm 0.29$
(S2) 4%	$0.018 \pm 0.0155$	$10.49 \pm 0.28$
(S3) 6%	$0.032 \pm 0.0123$	$11.64 \pm 0.38$

\*  $p < 0.05$ : significantly different between samples ( $n = 3$ ).

for all scaffolds ranging from 5 to 20 mg ( $p < 0.05$ ), where the highest mass reduction was observed for carrageenan concentration of 6% (w/v) (S3) as compared to control (final mass:  $29.51 \pm 4.17$  mg). On the contrary, the mass reduction from day 7 to day 14 was observed to be an error value as all scaffolds (except control) contained excess amount of water which was due to the incomplete/improper drying process prior to weight measurement. The inverse logarithm profiles (Figure 8) were later converted to a 1st-order kinetic analysis to acquire the degradation rate constant value ( $K$ , mg/day) of all treated scaffolds.

Table 2 shows the degradation rate constant value ( $K$ , mg/day) and traces of moisture content (%) from the infused carrageenan of 2, 4, and 6% (w/v) 3D scaffolds. Scaffold infused with 2% (w/v) carrageenan produced the lowest degradation rate ( $0.011 \pm 1.66$  mg/day) as compared to control (S0: data not shown), 4% (S2) and 6% (w/v) (S3, the highest rate).

The degradation activity of the carrageenan infused scaffold was due to the breakage of glycosidic chain between molecular networks of carrageenan into smaller oligomers [11]. During the lyophilization process (removing water content from carrageenan gel solution), this thermally induced phase separation produced secondary pores (Figure 7(c)) inside the porous PHBV skeletal pores. In fact, scaffold that have too many pores have the potential to degrade easily due to its thin wall. Moreover, the carrageenan hydrophilicity is greater than the PHBV skeletal porous structures. Due to its water-like properties and high alkalinity, this material facilitates further breakages throughout its skeletal porous

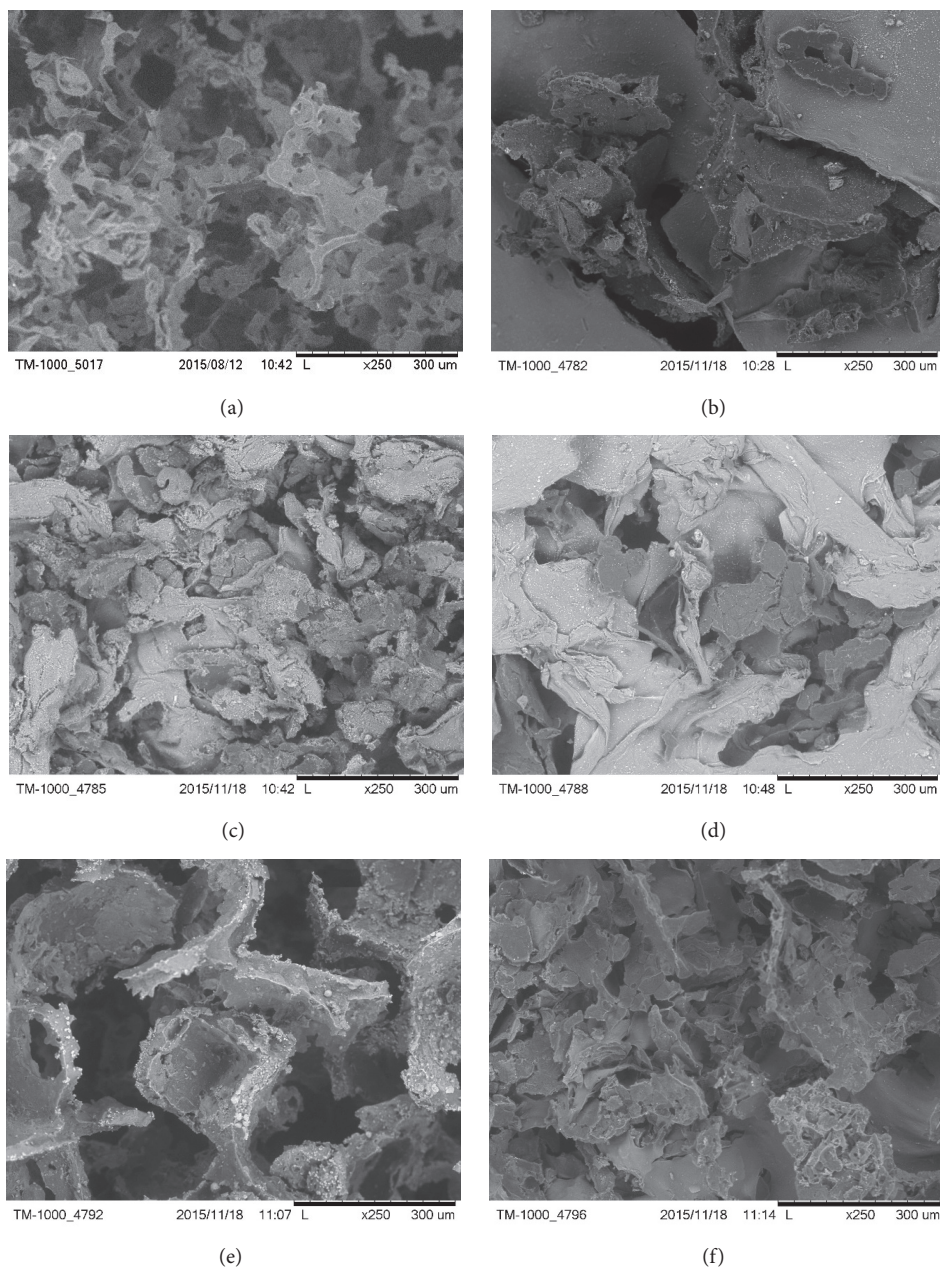


FIGURE 7: Surface morphology of porous 3D scaffolds infused with different carrageenan concentrations: (a) control (naked 3D scaffold); (b) 2% (w/v) carrageenan; (c) 4% (w/v) carrageenan; (d) 6% (w/v) carrageenan; (e) 8% (w/v) carrageenan; and (f) 10% (w/v) carrageenan.

structure as acidic by-products from both materials started to corrode the internal structure within [11, 15].

Moreover, solubility of carrageenan depends on the sulphate group concentration and accumulation of cation charges [15]. High sulphate content in the carrageenan infused scaffold (e.g., S3 – 6% (w/v)) and protein content in DMEM have affected the scaffolds physicochemical stability [18]. Interaction between those cation di-valences of the sulphate group causes the cross-bonding to change to mono-valence which produces a weak cross-bonding and less stable molecule [19]. Changes that occur in this chemical bonding will eventually compromise its structural integrity and lead to a faster degradation within the scaffold's core.

Meanwhile, based on the visual assessment (Figure 9), the scaffolds have begun to experience the process of degradation at day 14 and beyond. The finding was agreeably similar to Figure 9 that S1 has started to degrade into small pieces (Figure 9, S1 day 14) suggesting that the production of acidic by-products (hydrolytic chain reaction of carboxylic acid group as a catalyst) has resulted in speedy breakages. The media pH changes (from day 21) from low alkaline to moderate acidic condition up to day 28 in fact indicate that carboxylic acid has been generated during those incubation periods (Figure 11). Furthermore, the scaffolds degradation process which starts to seriously develop from day 14 was also influenced by the wall thickness and quantity of the pores

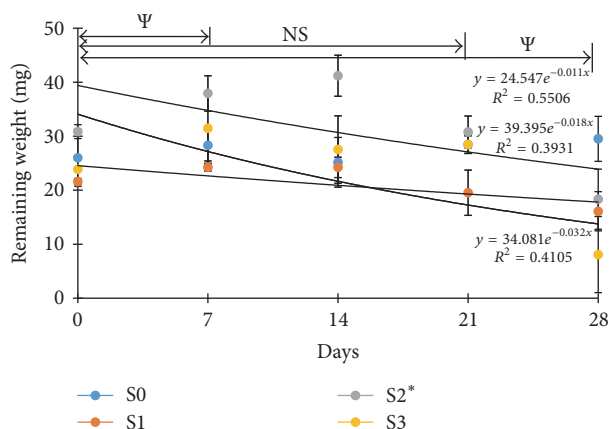


FIGURE 8: The inverse exponential of infused carrageenan 3D scaffolds remaining weight (mg) versus time of incubation (day). The experiments were carried out in triplicate ( $n = 3$ ). \* $\Psi$  ( $p < 0.05$ ): significantly different as compared to S0. NS: not significant.

available in the scaffold (e.g., secondary pores area created from the freeze-dried carrageenan). Scaffold with lower wall thickness will experience a high degradation rate due to large surface area which makes the fluid absorption (e.g., DMEM) become high [20, 21].

However, scanning electron micrograph images on day 7 revealed that the overall microstructures of the porous structures were very much intact with no sign of breakages for all treated scaffolds (Figure 10). This was consistent with all findings (especially based on the visually assessed images, Figure 9) that all scaffolds were pretty much in a good condition on the 1st week of incubation. The best scaffold for cell culture work should have the necessity to be tunable via physical-chemical approaches and moderately degrade accordingly to compensate the mechanical strength needed for good tissue generation [11, 22]. Therefore, scaffold that has been infused with 4% (w/v) carrageenan was considered the best selection for the composite 3D scaffold as it sustained moderate degradation for up to 14 days.

**3.7.2. Growth Media pH Changes.** The pH measurement on the cell growth media was carried out to determine any chemical changes that would possibly occur throughout 28 days of carrageenan infused 3D scaffolds incubation. pH of all scaffolds including control (naked 3D scaffold) did not produce drastic changes up to day 21 ranging from 7.80 to 8.40 (Figure 11). However, on day 21 onwards, the S1, S2, and S3 scaffolds (except control) experience a substantial decrease to a pH value of  $7.86 \pm 0.04$ ,  $7.21 \pm 0.01$ , and  $7.86 \pm 0.03$ , respectively ( $p < 0.05$ ). The optimum pH value for cell proliferation is between 6.9 and 7.8. Different cell phenotypes will have their own working microenvironment to thrive. Beyond those recommended array will result in slower cell growth which could possibly lead to cell necrosis (premature cell death) [23, 24]. For that reason, all carrageenan infused scaffolds were considered in a safe limit to be utilized for mammalian cell culture work. The only worrying aspect is in terms of its structural integrity in which when the 3D scaffold is loaded with high cell density, the acidic cell metabolites would possibly induce the *in vitro* degradation process even

faster [25]. The only possible way to reduce the effect is by blending this composite 3D scaffold with mineral like calcium apatite (HAP) as to counterbalance the acidic effects in the growth media.

## 4. Conclusions

3D porous scaffolds that have been infused with high carrageenan concentration (10%, w/v) have resulted in a lower percentage of the accumulated gel ( $5.49 \pm 1.22\%$ , w/w). Meanwhile, approximately  $13.79 \pm 1.52\%$  (w/w) has been effectively infused with the use of 2% (w/v) in which it was 2.5-fold higher than the highest concentration used. The highly concentrated carrageenan solutions (8% and 10%, w/v) were unable to diffuse efficiently into the porous structure due to its rapid gel solidification. Only scaffolds with carrageenan concentrations of 2%, 4%, and 6% (w/v) were chosen for 28 days of degradability study incubated in cell growth media (DMEM). The degradation rates of those selected scaffolds were in the range  $0.0111 \pm 1.66$  (mg/day) to  $0.032 \pm 3.23$  (mg/day). The infusions were heavily dependent on the concentration used. The higher the carrageenan concentration used for the infusion, the higher the degradation rate attained. The high hydrophilicity of the carrageenan has resulted in a rapid accumulation of acidic by-products inside the scaffold's core microstructure and eventually expedites breakages starting from the inner core to the outer section. On the other hand, based on the pH analysis, all carrageenan infused scaffolds were considered in a safe limit to be utilized for mammalian cell culture work. With all the presented data, the 4% (w/v) infused carrageenan porous 3D scaffold was considered the best selection for the composite 3D scaffold as it sustained moderate degradation for up to 14 days. However, its structural integrity needed some improvement and yet the scaffolds are not tested/loaded with high cell density. The problem will be even worrying, as the acidic cell metabolites would possibly induce the *in vitro* degradation process even at a greater rate. For that reason, further studies are needed to reduce those effects by blending this composite 3D scaffold with mineral like calcium apatite



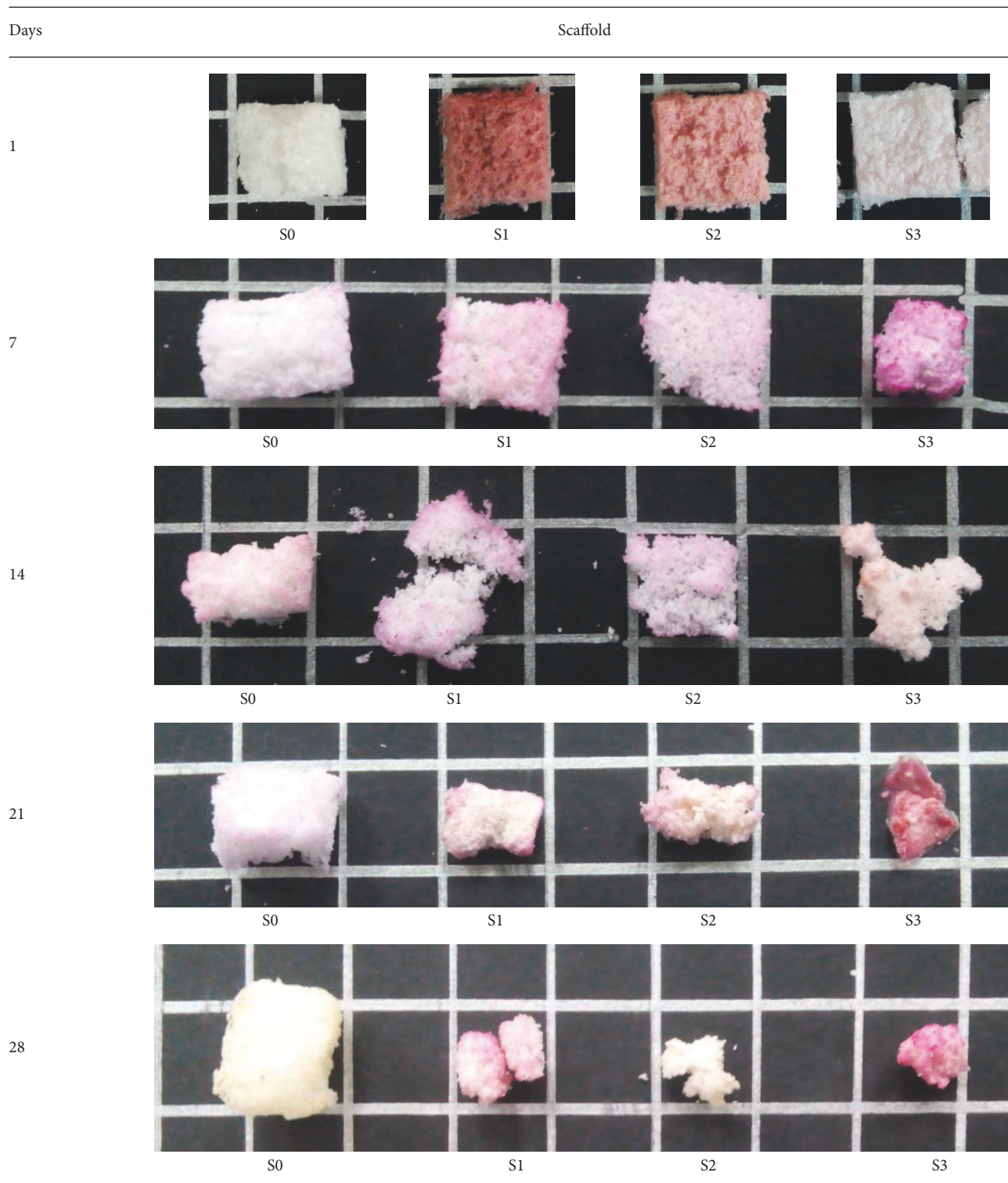


FIGURE 9: Visual assessment images: aerial view of the infused carrageenan 3D scaffolds incubated DMEM for 7, 14, 21, and 28 days. Scaffold were coded as S0 (naked 3D scaffold), S1 (2%, w/v) carrageenan, S2 (4%, w/v) carrageenan, and S3 (6%, w/v) carrageenan.

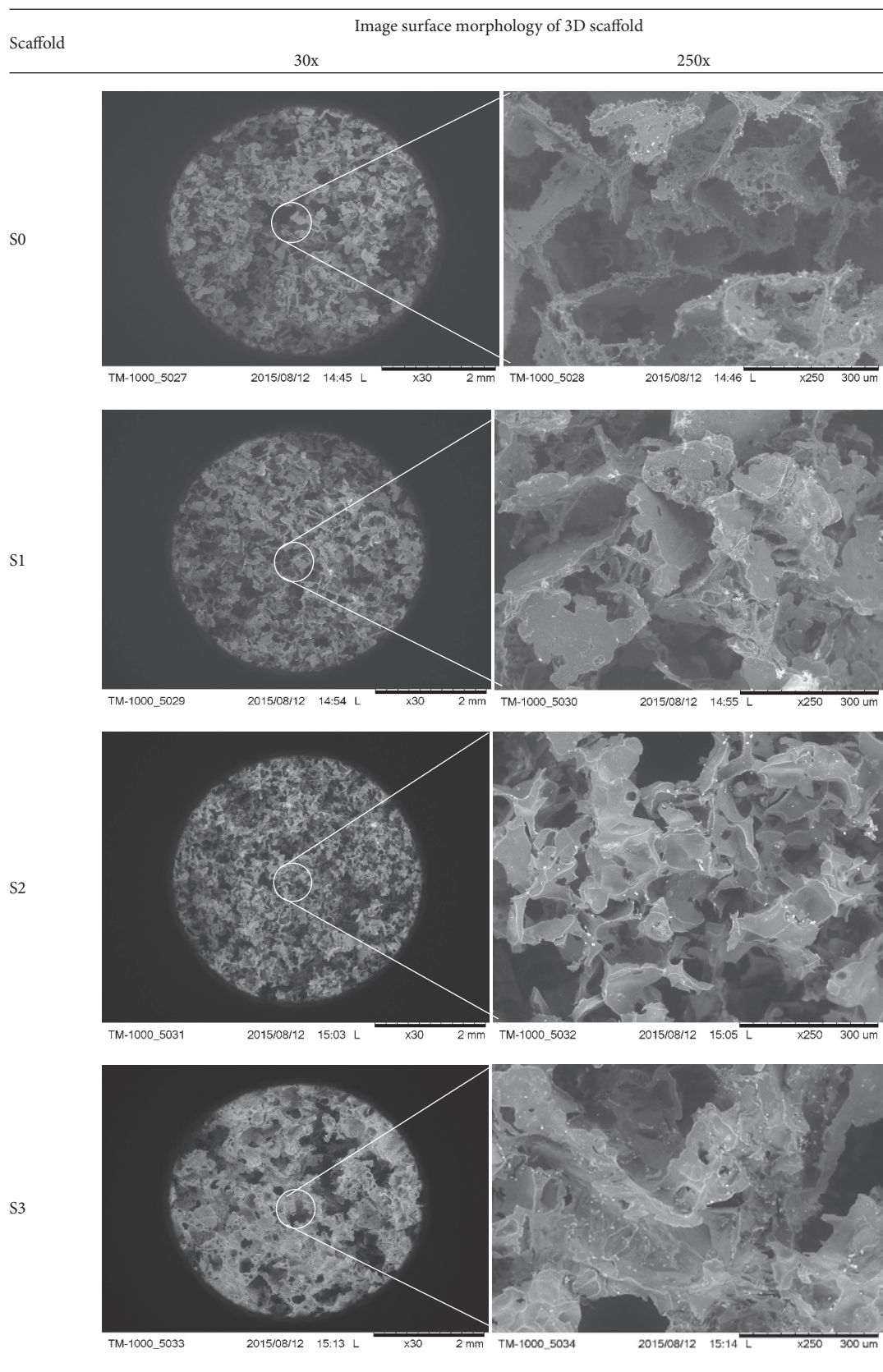


FIGURE 10: Scanning electron micrograph images of the infused carrageenan 3D scaffolds (S0, S1, S2, and S3) 7 days after incubation. Images were enlarged from 30x (white circle) to 250x.

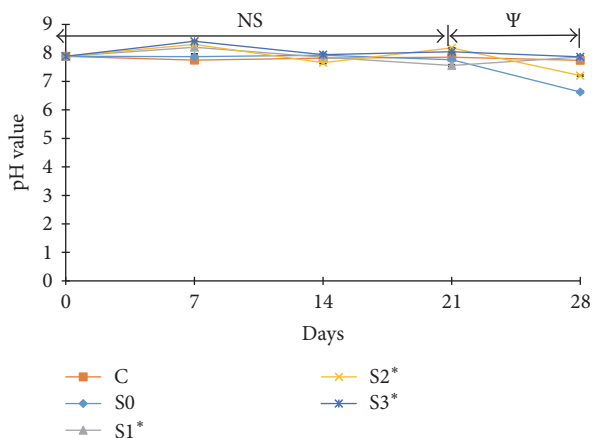


FIGURE 11: The DMEM pH profiles incubated with infused carrageenan 3D scaffold (S0, S1, S2, and S3 and control media without scaffold (C)) for 7, 14, 21, and 28 days. The experiments were carried out in triplicate ( $n = 3$ ). \* $\Psi$  ( $p < 0.05$ ): significantly different as compared to S0. NS: not significant.

(HAP) as to counterbalance the acidic effects in the growth media.

## Conflicts of Interest

The authors declare that they have no conflicts of interest.

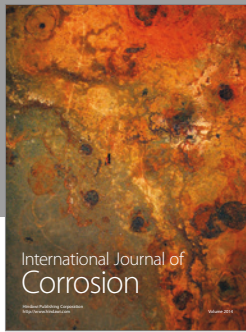
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