Glucose diffusivity in cell-seeded tissue engineering scaffolds

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Abstract

Objective A series of well-defined diffusion experiments was carried out to determine the effective glucose diffusion coefficient in cell-seeded porous scaffolds to understand the importance of nutrient diffusion in tissue engineering bioreactor.

Results Cell growth changed the morphological structure of the scaffolds reducing the effective pore space and inevitably decreased the effective glucose diffusivity in the chosen scaffolds, namely, collagen, poly(L-lactide) and poly(caprolactone) scaffolds from 3.71 x 10^{-9} m^2/s to 3.23 x 10^{-9} m^2/s, 1.39 x 10^{-10} m^2/s to 9.09 x 10^{-11} m^2/s and 1.78 x 10^{-10} m^2/s to 1.32 x 10^{-10} m^2/s, respectively.

Conclusions The presence of cells over time during cell culture reduces the mobility of glucose. The results of this study should be possible to use to predict the glucose concentration profiles in thick engineered tissues.

Keywords: Diffusion coefficient; Glucose; Osteoblast cell; Scaffold; Tissue engineering

Introduction

The main goal of tissue engineering field is to design, construct, regenerate and repair damaged tissues in the human body (Abdullah et al. 2009; Ahn et al. 2014). Many tissue engineering scaffolds have emerged to mimic extracellular matrices (ECM) which exhibit a biologically induced stable environment

and therefore should promote cell and tissue growth as well as providing mechanical support (Daniele et al. 2014). They play a crucial role in the successful reconstruction of diseased tissues due to the porous, biocompatible and non-toxic characteristics. They also must have interconnected porous network for easy access of nutrients into the cells and removal of metabolic wastes such as lactate from the cells (Ahn et al. 2014). As the amount of interconnected pores varies in different tissue engineering scaffolds, the nutrient diffusivity in these scaffolds may also vary as discussed recently by Suhaimi et al. (2015).

In general, the solutes for cell growth (e.g., nutrients and O_2) are transported in the scaffold and into the cells by diffusion process. The morphological structure of the scaffolds (e.g., pore size and shape distribution, average porosity, tortuosity, and any other) has a significant effect on the solute transport processes (Wu et al. 2010; Park et al. 2014; Chao and Das 2015). For example, increasing the cell mass grown in scaffolds has been shown to affect the diffusivity of O_2 by Kang et al. (2011) who reported a decrease in O_2 diffusivity with increasing tissue formation within a tissue engineering scaffold.

In principle, the presence of cells should have affected the solute diffusivity in scaffolds due to the possibility of the cells exerting significant contractile forces on the scaffolds which is also dependent on the cell and scaffold type. For example, fibroblasts and mesenchymal stem cells were shown to exert contractile forces on collagenous scaffolds, as reported by Brown et al. (2002) and Awad et al. (2000), while Leddy et al. (2004) reported the effects of changes in scaffold material leading to decreased diffusivity as a result of contractile forces exerted on the scaffold by cells.

In contrast to O_2 diffusivity which has been reported in a number of studies (Bettinger et al. 2006; Kang et al. 2011; Cheema et al. 2012; Fiedler et al. 2014), there is limited study on the nutrient diffusivity especially glucose within cell-seeded tissue engineering scaffolds. In addressing this issue, we report the glucose diffusivity of tissue engineering scaffolds seeded with human osteoblast cells in cell culture media (CCM) at 37°C. The scaffolds employed in this study have been used in our previous work (Suhaimi et al. 2015). However, these materials, at the time, were not seeded with any biological cells as the previous work aimed only at quantifying passive diffusion of glucose through the materials, i.e., the

relationship between glucose diffusion with different amount of connected pores and pore morphology in different tissue engineering scaffolds. In contrast to this study (Suhaimi et al. 2015), the goal of the present study is to quantify the diffusive properties of cell-seeded scaffolds and compare with our previous results of non-seeded tissue engineering scaffolds. It is shown how the glucose diffusivity would change with morphological changes of the scaffolds and cell culture time. Specifically, the glucose diffusion coefficient is shown decrease with increasing cell mass grown on the surface and inside the scaffolds.

Materials and methods

Materials

Human osteoblast HOSTE85 cell line (European Collection of Cell Culture (ECACC), UK) was donated by the Centre for Biological Engineering, Loughborough University. The CCM used was Dulbecco's modified eagle medium (DMEM), supplemented with 10% (v/v) foetal bovine serum (FBS) and 2% (v/v) non-essential amino acids (NEAA). Poly(caprolactone) (PCL), poly(L-lactide) (PLLA) and collagen scaffolds were employed in this study. Initial porosity of the materials (i.e., before cells were seeded) was evaluated by a pycnometric method, as described in Suhaimi et al. (2015). The glucose was of analytical grade powder (D-glucose-anhydrous).

Pore size distribution determination and morphological structure of scaffolds

Pore structures and distribution as well as the morphological structure of the scaffold materials were observed by a scanning electron microscopy (SEM). The cell culture medium was removed and the scaffold specimens were washed with phosphate buffered saline (PBS) to discard any remaining DMEM. The scaffolds were then left to dry inside a class II biological safety cabinet before the SEM analysis. The dry specimens were coated with carbon for 120s by a sputter coater. The images were taken at a

voltage of 5 keV. The minimum, mean and maximum pore sizes of the scaffolds were determined using the ImageJ version 1.48 software (Wayne Rasband, National Institute of Mental Health, USA) where the SEM images were uploaded. Briefly, lines were drawn between the pores and measurements were tracked and recorded by the software (Suhaimi et al. 2015).

Cell culture and seeding

Prior to cell seeding, both PCL and PLLA scaffolds were pre-wetted in 20% (v/v) ethanol for 30 min followed by washing with DMEM twice in another 30 min. Collagen scaffolds were highly purified and could therefore be seeded in the dry state without previous washing with DMEM. This procedure was carried out after sterilizing the class II biological safety cabinet by exposure to ultraviolet (UV) light for 30 min.

The HOSTE85 human osteoblast cells were cultured in DMEM supplemented with 10% (v/v) FBS and 2% (v/v) NEAA at 37°C and 5% CO_2 – 95% air (v/v) in a humidified incubator. The medium was changed every 2 days and the HOSTE85 cells were detached using 0.25% trypsin – 0.1% ethylenediaminetetraacetic acid (EDTA) (w/v) followed by re-suspending in the supplemented DMEM. After pre-wetting with 20% (v/v) ethanol and washing both the PCL and PLLA scaffolds twice with DMEM, 1 ml of the HOSTE85 cell suspension containing 1.2 x 10^5 cells and 1.5 x 10^5 cells was seeded onto both the PCL and PLLA scaffolds in a 16-well plate, respectively. As for collagen scaffolds, 1 ml of the HOSTE85 cell suspension containing 6 x 10^5 cells was seeded onto collagen in the dry state, also in a 16-well plate. The cell suspension was also added into an empty well (no scaffold) of the 16-well plate as control. After seeding, the collagen scaffolds were incubated at 37°C for 2 h before adding additional medium. All seeding plates were maintained at 37°C in the humidified incubator and the medium was changed every 2 days.

After 1, 2 and 3 weeks post seeding, the culture medium was removed and both the control and wells containing scaffold specimens were washed out with PBS solution to discard any remaining medium.

The scaffolds were then removed for SEM analysis. Trypsin-EDTA solution was used to detach cells followed by re-suspending in the supplemented DMEM. The mixture solution was centrifuged for 5 min followed by re-suspending in DMEM for cell count using a haemocytometer.

Comment [H1]: Approximate position of Figure 1

Glucose diffusivity measurement

A diffusion cell was constructed to measure the glucose diffusivity within the seeded scaffolds in CCM. The design and the operation principle of the diffusion cell were described in detail in another paper (Suhaimi et al. 2015). Briefly, the cell consisted of two acrylic chambers, namely, donor and receptor chamber. Both chambers held equal volumes of 41 ml per chamber with an internal geometry of length 20 mm x height 45 mm x width 45 mm. The seeded scaffold was fixed in between the chambers (Figure 2). The donor chamber was filled with 8 mg/ml of glucose solution dissolved in CCM while the receptor chamber contained pure CCM. The glucose powder was dissolved without further purification in a beaker containing pure CCM before the start of the diffusion experiment. Both solutions of pure CCM and glucose solution containing CCM were placed inside a heated water bath at 37°C for 1 h for the purpose of equilibrating to the experimental temperature. The whole apparatus was placed in the water bath at 37°C.

The change in the glucose concentration was measured using the same method described by Suhaimi et al. (2015). Briefly, the diffusion of glucose was monitored using a glucose analyser (YSI 2300 STAT PLUS, YSI UK Ltd, Hampshire, UK). The samples were taken simultaneously from both chambers using a plastic syringe. The samples were then placed inside a glass cuvette where 25 µl were aspirated by the sipper of the glucose analyser. After the measurements were recorded, the samples were poured back into the diffusion cell to keep the volume constant. The measurements were taken at an hourly interval until equilibrium was achieved.

The diffusion study was conducted at the time points of 1, 2 and 3 weeks post seeding for both PLLA and collagen scaffolds while 1 and 2 weeks post seeding for PCL scaffold. After 1, 2 and 3 weeks post

seeding, scaffold specimens were removed from the well plate and placed in between the chambers of the diffusion cell for diffusion experiments.

The corresponding diffusivities were calculated according to Fick's first law which was modified to include the effective diffusivity by Gutenwik et al. (2004) defined by:

$$J = -D_e \frac{\partial C}{\partial z} \quad (1)$$

Assuming that there was no change in volume in the diffusion cell, Eq. (1) was translated into Eq. (2) as given below:

$$V_d \frac{\partial C_d}{\partial t} = -D_e A \frac{C_d - C_r}{l} \quad (2)$$

where l was the scaffold thickness, A was the area of the scaffold, D_e was the effective diffusivity of glucose in the seeded scaffold and V_d was the donor volume. The effective diffusivity was determined by fitting the experimental data into Eq. (2) as described in Suhaimi et al. (2015).

In this work, all errors in results have been calculated in terms of standard deviation which is taken to imply a deviation of the results from a mean value of the results. No other statistical calculation is made. All experiments have been repeated twice unless there is significant dispersion in the results in which case the experiments have repeated at least three times.

Comment [H2]: Approximate position of Figure 2

Results and discussions

As mentioned earlier, we have carried out a number of experiments for characterising the materials in our diffusion studies. The thickness, pore size, porosity, minimum, mean and maximum pore size of PLLA are 50 µm, 12-18 µm, 80%, 4.04 µm, 13.67±4.25 µm and 25.87 µm, respectively while that of PCL are found to be 50 µm, 20-30 µm, 80%, 5.8 µm, 21.69±6.85 µm and 44.84 µm, respectively. Collagen's thickness, pore size, porosity, minimum, mean and maximum pore size are found to be 1500 µm, 80 µm, 72%, 12.55 µm, 75.15±5.21 µm and 175.18 µm, respectively.

146

147 Cell proliferation on scaffolds

148 To confirm the morphological changes of the cell-seeded scaffolds at various cell culture time intervals
149 the materials were viewed for surface morphology and cross-sections using SEM. Figure 3 illustrates
150 some typical micrographs of osteoblasts seeded on the surface of collagen, PLLA and PCL scaffolds after
151 1, 2 and 3 weeks of culture as well as the blank scaffolds (no cells). A clear comparison is depicted on
152 the morphological change between blank scaffolds and seeded scaffolds. The cells have gradually
153 covered the surface and have almost filled all of the pores by week 3. Figure 4 illustrates the cross-
154 sectional view of the fibres of the scaffold where cells have migrated. It is observed that more cells are
155 attached on the surface rather than in between the fibres of the scaffold. Figure 5 presents the number
156 of seeded cells on all scaffolds at 1, 2 and 3 weeks of culture time which shows similar pattern as the
157 cell growth kinetics curve (Figure 1).

158

Comment [H3]: Approximate position of Figure 3, 4 and 5

159 Glucose diffusion analysis

160 Typical curves for the temporal change in glucose concentration for both donor and receptor chambers
161 are shown in Figure 6 which depict the changes in the two chambers during the diffusion experiment for
162 collagen, PCL and PLLA scaffolds. The diffusion coefficients of the cultured scaffolds are calculated by
163 fitting the experimental data into Eq. (2) and the diffusivity values are listed in Table 1. Table 1 also
164 shows the percentage difference between scaffolds with no cells and cultured scaffolds. Tissue
165 engineering scaffolds should be biodegradable for successful tissue formation *in vivo*; however, PCL,
166 PLLA and collagen scaffolds were stable for culturing periods of up to three weeks. This shows that they
167 have a long time of degradation in comparison to the culturing periods and diffusion experiments and
168 hence, we define that any biodegradation has no effect on the diffusivity.

169 There is an obvious decrease in the values of effective diffusivity between scaffolds with no cells

Comment [H4]: Approximate position of Figure 6

170 attached (blank) and cultured scaffolds on week 1. However, we did not observe significant difference in

the effective diffusion coefficients of the cultured scaffolds between weeks 1, 2 and 3 which is likely due to a slower proliferation rate of osteoblast cells. To further quantify the relationship between the effective diffusion coefficient and number of cells grown on and inside the scaffold, a graph of the effective diffusivity against cell number was plotted as shown in Figure 7 for PLLA scaffold. This figure shows a decrease in the effective diffusion coefficient values as cell number increases. Both collagen and PCL scaffolds follow similar trends and are not shown in the figure. Diffusion is generally defined by a random motion of molecules from a higher concentration to a lower concentration. In this case, glucose molecules have diffused from the donor chamber into the receptor chamber through the connected pores of the scaffold. As the cells gradually cover the surface and almost all of the pores of the scaffold starting from week 1 to week 3, the effective diffusion coefficient of glucose seems to decrease monotonically with the cell number which is attributed to the change in the pore volume available for diffusion.

Comment [H5]: Approximate position of Table 1

Conclusion

A diffusion cell has been constructed to carry out glucose diffusion experiments through cell-seeded scaffolds saturated in CCM at 37°C. The results show that cell growth changes the morphological structure of the scaffold which affects the effective diffusion coefficient of glucose. The pore volume for glucose diffusion has been reduced and it is concluded that increasing cell mass grown on and inside the scaffold decreases the mobility of glucose. Further investigation on cell proliferation rate by deoxyribonucleic (DNA) quantification is necessary to further understand the relationship between effective diffusion coefficient and culture time. Observing the cell specific glucose consumption rates from one tissue construct to another may also be useful for future work as the consumption rates may vary.

Comment [H6]: Approximate position of Figure 7

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241 Table 1. Effective diffusion coefficients with standard deviations for glucose across blank and cultured
242 scaffolds saturated in cell culture medium. The table also shows the errors in terms of standard
243 deviation of the data.

Scaffold	Pore size (μm)	Effective diffusion coefficient (m^2/s)				Difference between values calculated from scaffolds with no cells and scaffolds with cells grown on week 1 (%)
		Effective diffusion coefficient of scaffolds with no cells (Suhaimi et al. (2015))	Effective diffusion coefficient of scaffolds with cells grown on week 1	Effective diffusion coefficient of scaffolds with cells grown on week 2	Effective diffusion coefficient of scaffolds with cells grown on week 3	
Collagen	80	$3.71 \pm 2.78 \times 10^{-9}$	$3.23 \pm 0.16 \times 10^{-9}$	$3.22 \pm 0.16 \times 10^{-9}$	$3.07 \pm 0.11 \times 10^{-9}$	12.9
Poly (L-lactide)(PLLA)	12-18	$1.39 \pm 0.28 \times 10^{-10}$	$9.09 \pm 0.67 \times 10^{-11}$	$8.44 \pm 0.17 \times 10^{-11}$	$7.56 \pm 0.46 \times 10^{-11}$	34.6
Poly(caprolactone)(PCL)	20-30	$1.78 \pm 0.50 \times 10^{-10}$	$1.32 \pm 0.10 \times 10^{-10}$	$1.17 \pm 0.04 \times 10^{-10}$	NA	25.8

Figure 1. The cell growth curve for HOSTE85 as a function of time where the doubling time of HOSTE85 cells is 1.49 days. The cells were cultured for 5 days and cell number was calculated on each day using a haemocytometer where trypan blue was added into the cell suspension to differentiate between live and dead cells. The cell growth study was replicated twice with an error of <20% in most cases. The figure also shows the errors in terms of standard deviation of the results

Figure 2. Schematic drawing of a diffusion cell to measure the glucose diffusivity across the seeded scaffolds saturated in cell culture medium at 37°C (dimensions of the cell are shown in the figure)

Figure 3. SEM micrographs showing morphological changes on the surface of collagen, PLLA and PCL scaffolds from no cells attached (blank scaffold) to cells cultured on week 1, week 2 and week 3

Figure 4. SEM images showing the cross-sectional cell distribution in collagen, PLLA and PCL scaffolds where cells have migrated into on the time points of week 1, week 2 and week 3 of culture period

Figure 5. The approximate number of cells grown on collagen and PLLA scaffolds in week 1, 2 and 3 and that of PCL scaffold in week 1 and 2 of culture periods. The difference between cell number contained inside the control well and cell number contained inside the wells that initially were present with scaffold specimens, represents the number of cells that have grown on the scaffolds. All data were replicated twice with an error of <15% in most cases

Figure 6. Diffusion cell experiments with 8 mg glucose ml^{-1} for cultured collagen, PLLA and PCL scaffolds saturated in cell culture medium at 37°C. All diffusion experiments were replicated twice with an error of <20% in most cases

Figure 7. The relationship between effective diffusivity and seeded cell number for PLLA scaffold. The percentage difference for both cell number and effective diffusivity at different culture time were calculated at the time points of 1, 2 and 3 weeks of culture time with reference to initial time (at 0 week)

Figure 1

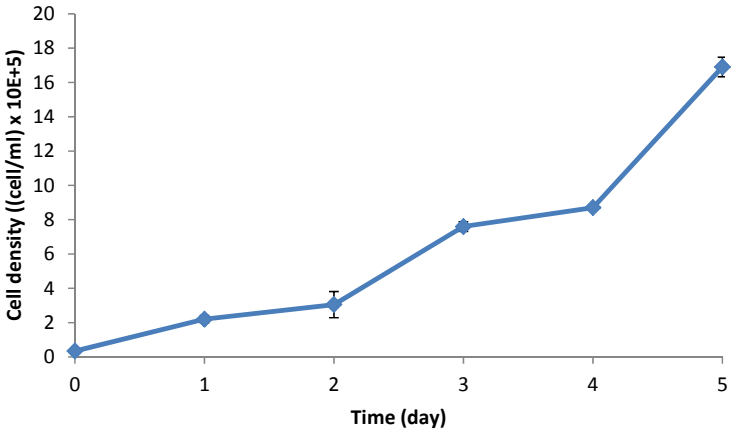

Cell density ((cell/ml) x 10E+5)
20
18
16
14
12
10
8
6
4
2
0
0
1
2
3
4
5
Time (day)

Figure 2

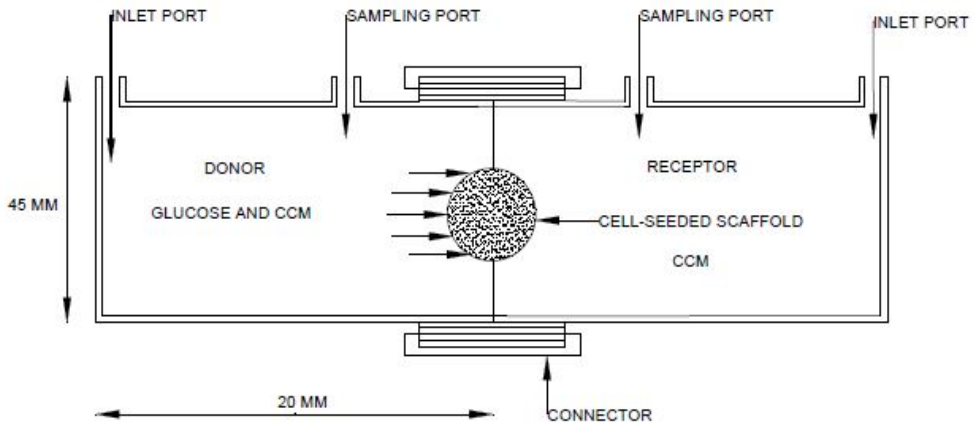

INLET PORT
SAMPLING PORT
SAMPLING PORT
INLET PORT
45 MM
DONOR
GLUCOSE AND CCM
RECEPTOR
CELL-SEEDED SCAFFOLD
CCM
20 MM
CONNECTOR

Figure 3

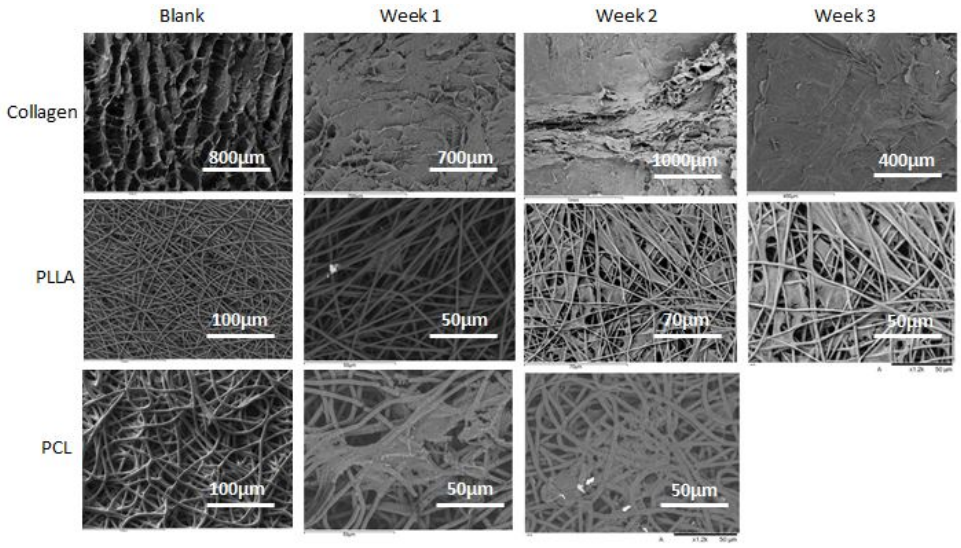

Blank
Week 1
Week 2
Week 3
Collagen
PLLA
PCL
800μm
700μm
1000μm
400μm
100μm
50μm
70μm
50μm
100μm
50μm
50μm

Figure 4

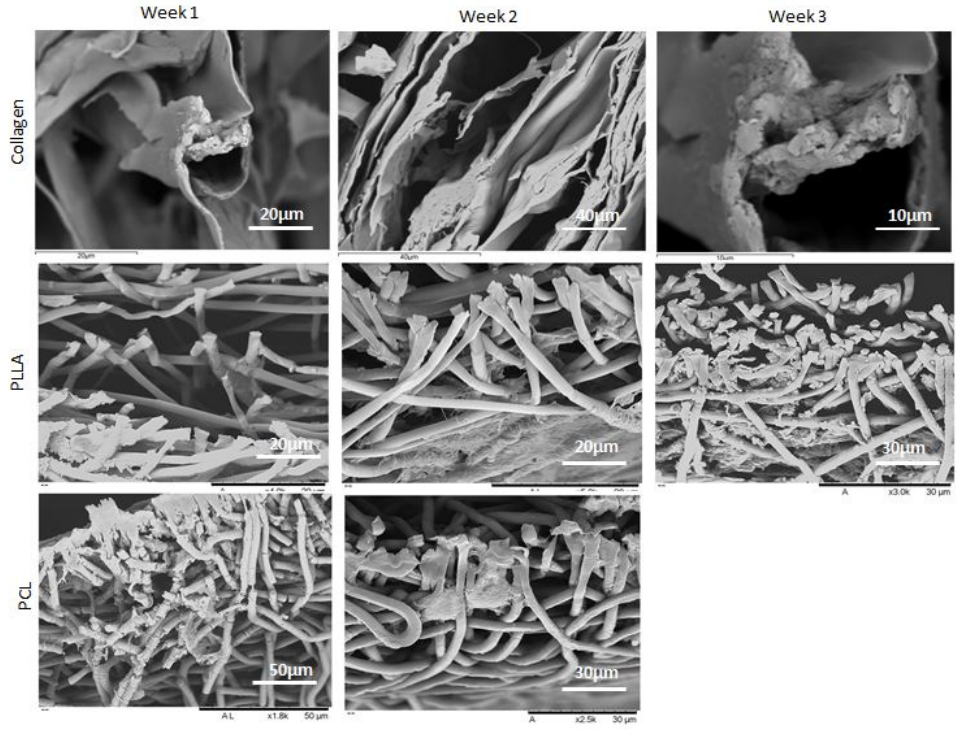
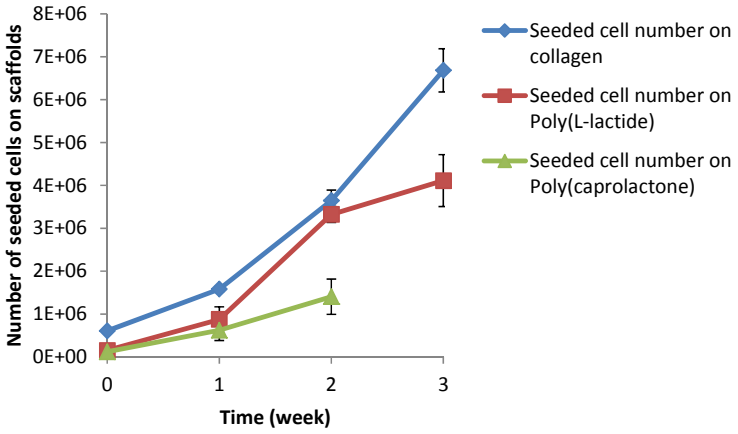

Week 1
Week 2
Week 3
Collagen
PLLA
PCL
20µm
40µm
10µm
20µm
20µm
30µm
50µm
30µm

Figure 5



[Collagen] Data from 21-day cell growth were used for calculation of number of cells grown in collagen on week 1, 2 and 3

[Poly(L-lactide)] Data from 21-day cell growth were used for calculation of number of cells grown in poly(L-lactide) on week 1, 2 and 3

[Poly(caprolactone)] Data from 14-day cell growth were used for calculation of number of cells grown in poly(caprolactone) on week 1 and 2

Figure 6

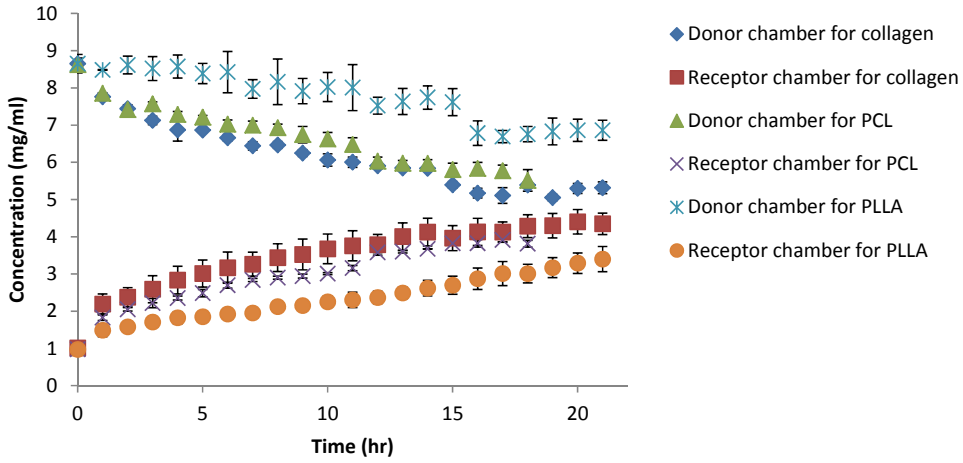
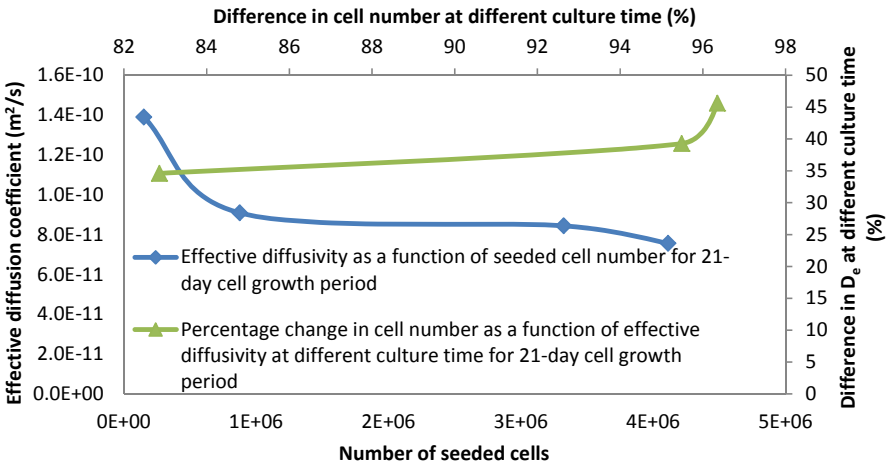

Concentration (mg/ml)
Time (hr)
0
1
2
3
4
5
6
7
8
9
10
0
5
10
15
20
Donor chamber for collagen
Receptor chamber for collagen
Donor chamber for PCL
Receptor chamber for PCL
Donor chamber for PLLA
Receptor chamber for PLLA

Figure 7


Difference in cell number at different culture time (%)
82
84
86
88
90
92
94
96
98
1.6E-10
1.4E-10
1.2E-10
1.0E-10
8.0E-11
6.0E-11
4.0E-11
2.0E-11
0.0E+00
Effective diffusion coefficient (m²/s)
50
45
40
35
30
25
20
15
10
5
0
Difference in De at different culture time (%)
Effective diffusivity as a function of seeded cell number for 21-day cell growth period
Percentage change in cell number as a function of effective diffusivity at different culture time for 21-day cell growth period
0E+00
1E+06
2E+06
3E+06
4E+06
5E+06
Number of seeded cells