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Influence of porosity and fibre diameter on the degradation of chitosan fibre-mesh scaffolds and cell adhesion

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Abstract The state of the art approaches for tailoring the degradation of chitosan scaffolds are based on altering the chemical structure of the polymer. Nevertheless, such alterations may lead to changes in other properties of scaffolds, such as the ability to promote cell adhesion. The aim of this study was to investigate the influence of physical parameters such as porosity and fibre diameter on the degradation of chitosan fibre-mesh scaffolds, as a possible way of tailoring the degradation of such scaffolds. Four sets of scaffolds with distinct fibre diameter and porosity were produced and their response to degradation and cell adhesion was studied. The degradation study was carried out at 37°C in a lysozyme solution for five weeks. The extent of degradation was expressed as percentage of weight loss of the dried scaffolds after lysozyme treatment. Cell adhesion was assessed by Confocal Microscopy. The results have shown that the scaffolds with higher porosity degrade faster and that, within the same range of porosity, the fibres with smaller diameter degrade slightly faster. Furthermore, the morphological differences between the scaffolds did not affect the degree of cell adhesion, and the cells were observed throughout the thickness of all four types of scaffolds.

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1 Introduction

One of the most widely used Tissue Engineering strategies is based on growing cells in a temporary 3-D scaffold made of a biodegradable polymer until maturation is achieved *in vitro* and then the engineered construct is implanted in the body [1]. The rate at which the polymer degrades and the scaffold erodes is of fundamental importance and must closely match the rate of new tissue formation [2]. Different applications require different degradation and erosion behaviours from the scaffolds [3, 4]. Therefore, the understanding of the mechanisms underlying these processes is of pivotal importance in defining how to tailor degradation/erosion towards specific applications. The breakdown of the scaffolds may be affected by several factors, which include the intrinsic properties and the morphology [5].

Amongst the wide range of available materials for Tissue Engineering applications, chitosan has shown to be a very promising option due to some of its properties like biocompatibility, biodegradability and antibacterial activity [6–11]. Chitosan is a partially de-acetylated derivative of chitin, found in arthropod exoskeletons. Structurally, chitosan is a linear polysaccharide consisting of $\beta(1\rightarrow4)$ linked D-glucosamine residues with a variable number of randomly located *N*-acetyl-glucosamine groups [12]. The presence of such chemical groups provides active sites for the grafting of relevant molecules for the improvement of cell-material interactions [13, 14]. This polymer is degraded by the action of several enzymes [15], but in an *in vivo* environment enzymatic degradation is mainly attributed to the action of lysozyme [16, 17]. As reported in several studies, the degradation rate of chitosan can be tailored by manipulating the molecular weight and the degree of de-acetylation [15, 18, 19]. Nevertheless, this kind of approach may be time

consuming [20] and sometimes requires the use of harsh conditions [21]. Furthermore, because it involves changes in the chemical structure of the material, it may lead to undesired changes in other properties of the polymer, such as cell-material interactions [22, 23].

The aim of this study was to investigate the influence of porosity and fibre diameter on the degradation rate of chitosan fibre-mesh scaffolds, as a possible way to tailor the degradation rate of such type of scaffolds. The use of these parameters as tools will provide the adjustment of the scaffolds degradation rate to specific applications circumventing the constraints associated with chemical modification.

2 Materials and methods

2.1 Scaffolds production

Chitosan powder (France Chitin) with a degree of deacetylation of 89% and molecular weight of 366 kDa was purified. The fibres were produced by spinning a 3% chitosan solution in 2% acetic acid (Sigma-Aldrich) into a sodium hydroxide/sodium sulphate (Sigma-Aldrich) solution, as previously described by Tuzlakoglu, Reis et al. [8]. In order to produce scaffolds with different porosities, 1 and 0.5 ml of chitosan solution were wet spun and the correspondent fibres were placed and dried in moulds with equal volume and shape. In order to achieve fibres with two different diameters, the fibres were wet spun using needles with 800 and 450 μm diameter. The produced scaffolds are cylindrical with approximately 5 mm diameter and 4 mm height.

2.2 *In vitro* degradation

The *in vitro* degradation of chitosan scaffolds was carried out at 37°C in 2 ml Phosphate-Buffered Solution (PBS, pH 7.4) (Sigma-Aldrich) containing lysozyme (hen egg-white, Sigma-Aldrich) and 0.02% of sodium azide (Sigma-Aldrich). The concentration of enzyme was adjusted to the mass of the different sets of scaffolds and it was 2 mg/ml for the samples produced out of 1 ml of chitosan solution and 1 mg/ml for the samples produced out of 0.5 ml. In order to guarantee that the eventual differences in the degradation rate of the different sets of scaffolds are not due to differences in the enzyme concentration, another set of scaffolds was prepared, using a lysozyme concentration of 1 mg/ml for all the scaffolds, regardless of their mass. The scaffolds with known dry weights ($n = 4$ samples of each set of scaffolds) were incubated in the lysozyme solution for the period of study. The lysozyme solution was refreshed regularly to guarantee continuous enzyme activity. After 7, 21, 35 and 49 days, samples were removed from the medium, rinsed with distilled water and dried to a constant weight. The extent of *in vitro* degradation was expressed as percentage of weight loss of the dried scaffolds after lysozyme treatment.

2.3 Morphological analysis

The different sets of scaffolds were scanned by micro computed tomography (micro-CT) (Scanco μCT40) using 55 kV energy and 144 μA intensity. The effective resolution of the reconstructions was 6 μm. A Gauss filter and threshold were applied to the reconstructions to segment the scaffolds structure, which was initially analysed for porosity, surface area and filament thickness. The morphological changes of the scaffolds during the degradation process were analysed by Scanning Electron Microscopy (SEM, HITACHI S-4500 field emission).

2.4 Cell adhesion

MG63 cells (human osteoblast-like cell line) were cultured in Dulbecco's Modified Eagle's Medium (DMEM - Biowest) supplemented with 10% Fetal Calf Serum (Biowest), 1% L-glutamine and 1% antibiotic and antimycotic solution (Sigma-Aldrich). When the cells reached 80% confluence they were trypsinized and labelled using the PKH26 Red Fluorescent Cell Linker Kit (maximum excitation wavelength at 551 nm, Sigma-Aldrich). The labelled cells were seeded into the different sets of scaffolds at a density of 1×10^6 cells per scaffold. The seeded scaffolds were cultured under the same conditions as for the cells. After 3 days of culture the scaffolds were observed using Confocal Scanning Microscopy (Olympus FLOUVIEW IX71) with laser lines at wavelengths of 488 nm and 543 nm. The autofluorescence of chitosan was detected on the same detection channel as the PKH26 fluorescent dye.

2.5 Statistical analysis

The differences in the weight loss of the various sets of scaffolds were analysed for statist ical significance by employing an ANOVA test. A p-level inferior to 0.05 was considered to be significant. The results are expressed as means $\pm$ standard deviation for $n = 4$.

3 Results

The morphological characterization of the scaffolds performed by Micro-CT analysis clearly showed that the method of processing used in this work enabled the production of scaffolds with distinct morphological features.

For simplification reasons, the different sets of scaffolds will be designated from A to D according to their morphological characteristics, as shown in Table 1. For the scaffolds

Table 1 The table shows some of the morphological parameters of the different sets of scaffolds determined by micro-CT analysis

Sample designation	Volume of chitosan (ml)	Average fibre diameter (μm)	Porosity (%)	Surface area (mm^2)
A	1	63	80	167.5
B	1	102	67	162.2
C	0.5	63	92	75.2
D	0.5	102	89	66.8

produced out of 1 ml of chitosan solution (A and B) two different porosities have been achieved, as a dependency of the fibre diameter. The porosity was calculated according to the following formula: 1 – (FV/TV), where FV is volume of the scaffolds occupied by the fibres and TV the total volume of the scaffold. For the same total volume, the partial volume occupied by the thicker fibres will be higher than for the thinner ones and the porosity will be lower. Conversely, the surface area of the scaffolds composed of thinner fibres is slightly higher. The same principle is also applied for the scaffolds produced from 0.5 ml of chitosan solution (C and D). The 3-dimensional reconstruction of the scaffolds (Fig. 1) reflects the differences described by the quantitative analysis.

The degradation study reveals significant differences in the degradation rate of the different scaffolds.

From the highest to the lowest values, the degradation rates after 35 days of study were as follows: $C \geq D > A \geq B$. The ANOVA test revealed two types of similar weight loss profiles: group A is similar to group B, and group C is similar to group D. For all the other pairwise comparisons the statistical difference was significant (p-level < 0.05) as shown in Table 2. Therefore, the scaffolds that showed a higher degradation rate were the ones that display higher porosity (C and D). Between these two structures, the one made of thinner fibres degraded slightly faster, but that difference was not statistically significant (p-level > 0.05). The scaffolds with lower porosity (A and B) degraded slower. Again, the degradation rate of the scaffolds made of thinner fibres

Table 2 Fisher's Least Significant Difference (LSD) of the difference between the scaffolds categories after 21 days of study, with a confidence interval of 95%

Contrast	Difference	Standardized difference	Critical value	Pr > Diff	Significant
C vs. B	3.601	2.719	2.179	0.019	Yes
C vs. A	3.539	2.671	2.179	0.020	Yes
C vs. D	0.514	0.388	2.179	0.705	No
D vs. B	3.087	2.331	2.179	0.038	Yes
D vs. A	3.025	2.283	2.179	0.041	Yes
A vs. B	0.063	0.047	2.179	0.963	No

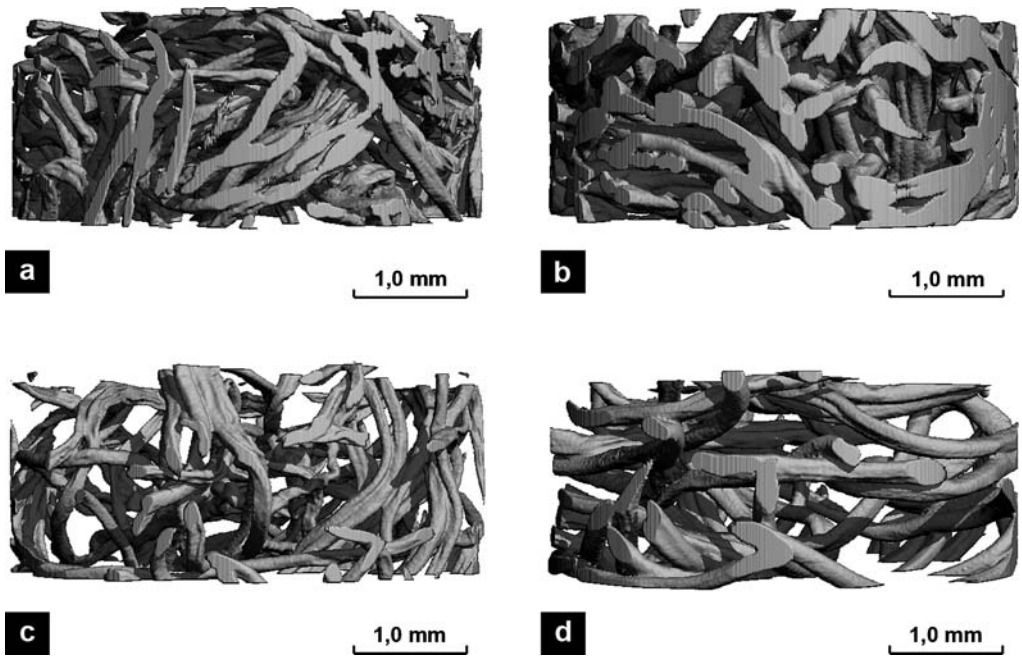

Fig. 1 Micro-CT 3-dimensional reconstruction of the scaffolds evidencing the different porosities and fibre diameters between the scaffolds. (a) Scaffold type A; (b) Scaffold type B; (c) Scaffold type C; (d) Scaffold type D

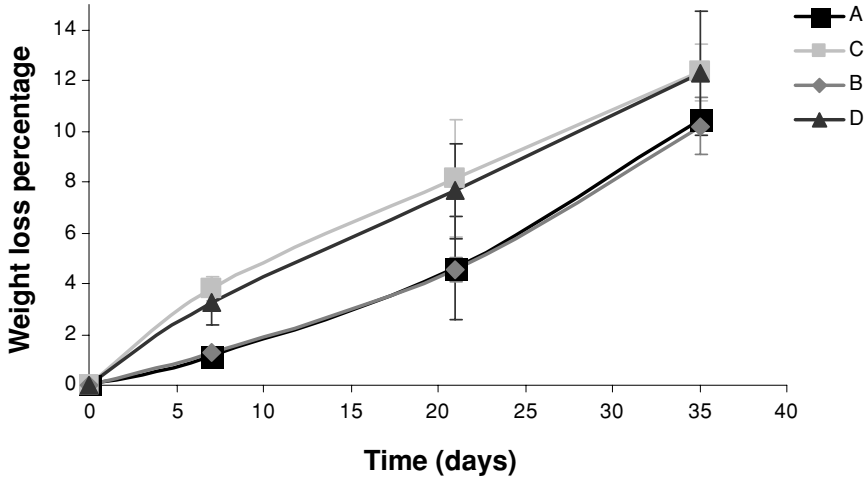

Fig. 2 Weight loss variation of the chitosan scaffolds during a five weeks degradation study in lysozyme solution. The lysozyme concentration was adjusted to the mass of each type of scaffold

was slightly higher. By the end of the third week of study, the degradation rate of the structures C and D was nearly the double of the structures A and B, as shown in Fig. 2. By the end of the fifth week of study, the weight losses of groups A and B was closer to groups C and D. At that stage, the differences found were not statistically significant (p-level > 0.05) for all pairwise comparisons.

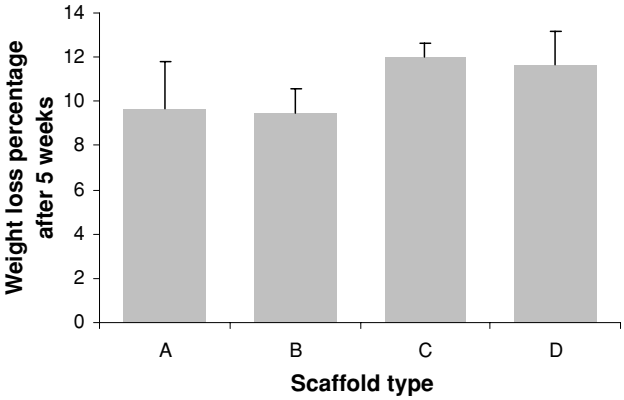

Fig. 3 Weight loss variation of the chitosan scaffolds after five weeks of degradation in lysozyme solution. The lysozyme concentration was 1 mg/ml regardless of the mass of each of the scaffolds

The changes in the surface topography were monitored by SEM. It was possible to observe an increase in the surface roughness of the samples that were subjected to lysozyme treatment. After 3 weeks of study, the increase in surface roughness was more pronounced on scaffold type C that on scaffold type B (Fig. 3).

The weight loss of another group of scaffolds A, B, C and D was studied during a 5 week period. In this group of scaffolds, the lysozyme concentration was 1 mg/ml for all the samples, regardless of their initial weight. Figure 4 shows that after the referred period, the weight loss of scaffold types A and B was around 10% and for scaffolds C and D 12%. These values are approximately the same as the ones depicted in Fig. 2.

The cell adhesion to the different sets of scaffolds was assessed in a qualitative way by Confocal Microscopy. After 3 days of culture the cells seemed to adhere evenly throughout the thickness of all four types of scaffolds. Figure 5 is representative of the cell distribution in the studied scaffolds.

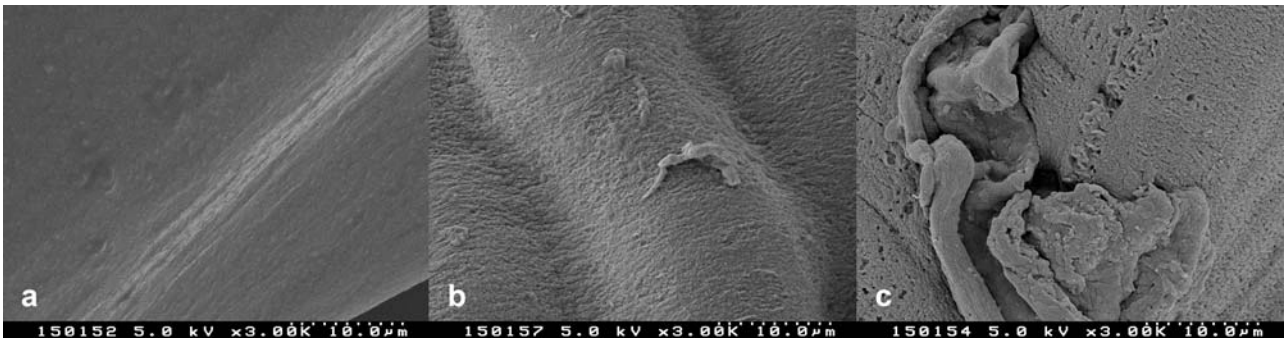

Fig. 4 SEM micrographs of the chitosan scaffolds. (a) Without lysozyme treatment; (b) Type B scaffold after 3 weeks of lysozyme treatment; (c) Type C scaffold after 3 weeks of lysozyme treatment (3000× magnification)

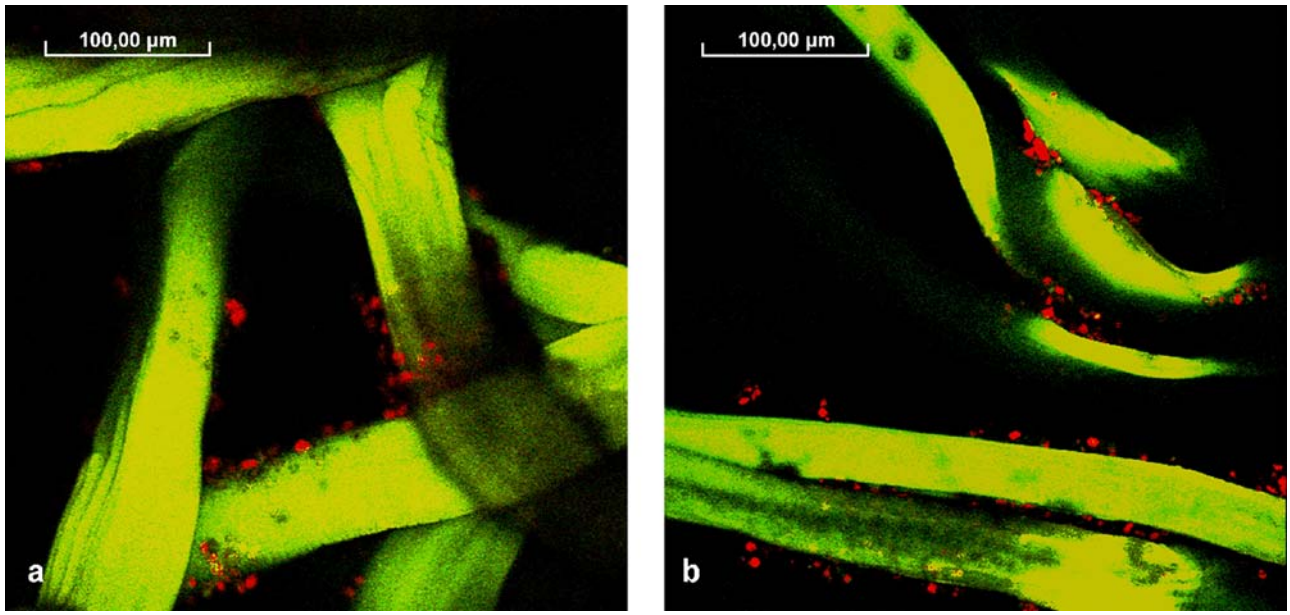

Fig. 5 Confocal microscopy of MG63 cells adhered to the fibres of the chitosan fibre-mesh scaffolds after 3 days of culture. (a) scaffold type A; (b) scaffold type C. The chitosan fibres appear in green due to the autofluorescence signal of chitosan (200× magnification)

4 Discussion

This degradation study revealed significant differences in the degradation profile of the different scaffolds. The scaffolds that showed a higher degradation rate were the ones that display higher porosity (C and D). For this type of scaffold, the degradation after the first week is very high and then continues to increase at a constant rate. Conversely, for scaffold type A and B, the rate is initially lower and keeps increasing. By the end of the study it is closer to the rate of scaffolds C and D. In fact, at that stage the differences in the weight loss profile of the four sets of scaffolds were not considered statistically significant (p-level > 0.05). This profile is probably due to the degradation phenomenon of the scaffolds itself. As scaffolds A and B undergo degradation, there will be an increase in their porosity, rendering them structurally similar to scaffolds C and D in earlier stages of degradation. The degradation rate of scaffolds A and B after 21 days of study is approximately the same of scaffolds C and D after 7 days of study. This clearly shows that the porosity has a major role in defining the degradation rate of these scaffolds, probably due to diffusional phenomena, as well as to the access of the degrading fluid to the bulk of the scaffold. As with any enzyme catalysed reaction, the degradation of chitosan will be controlled by the concentrations of the enzyme, substrate and products. A high concentration of degradation products or a low concentration of enzyme in the surroundings of the substrate will slow down the reaction. The scaffolds with a higher porosity enable a faster diffusion of the enzyme towards the surface of chitosan and of the degradation products in the opposite direction, thus accelerating the process of the degradation. Once both type of scaffolds reach the same porosity, the diffusional processes will no longer be the predominant factor in the degradation kinetics, and the degradation rate will be governed by other parameters, such as the specific surface area.

The values obtained for types C and D suggest that within the same range of porosity, another parameter influencing the degradation rate is the surface area (type C), which can be tailored by changing the fibre diameter. In this case, a higher surface area permits more contact points between the enzyme and the chitosan, favouring the degradation. This finding is supported by some of our previous studies (data not shown). In those studies, we found that when using porosities ranging from 86% to 92%, the degradation was not affected. In that case, only the surface area had an influence on the degradation rate. This can be explained by the differences in porosity which may not be high enough to alter the diffusion along the scaffold. The changes in the surface topography as a result of degradation were monitored by SEM. The micrographs support the results obtained from weight loss analysis. SEM analysis showed how the roughness of the scaffolds correlated with the scaffolds with a reduced weight.

In order to assess any potential effects of varying enzyme concentrations, a second batch of samples exposed to a constant enzyme concentration, regardless of the mass, was studied. After 5 weeks, the weight loss profile in these samples was the same as in the first experiment. This finding indicates that the lysozyme concentration is not a limiting step in this reaction, and that the kinetics of degradation is being governed by other parameters, such as diffusional processes, supporting our hypothesis.

Further confocal analysis of the cell adhesion to the scaffolds after 3 days of culture revealed no significant difference between the four constructs. The images indicate that regardless of the fibre diameter and porosity, the bone cells were able to adhere to the scaffolds. Due to the high porosity and high pore interconnectivity of these scaffolds, cell adhesion was not confined to the surface and cells were detected throughout the thickness of the scaffolds. Currently, a longer-term cell culture experiment is underway.

This study has shown that the degradation rate of chitosan fibre-mesh scaffolds can be tailored by changing their porosity. Because of that, special attention must be paid when planning cell seeding experiments. As the cells proliferate and spread along the scaffolds they will obstruct some of the pores. That phenomenon will be even more dramatic when the cells begin to produce extra cellular matrix and will lead to different degradation profiles. Therefore, it will be very important to understand in which way the presence of metabolically active cells changes the porosity of the scaffolds, in order to adjust the initial porosity.

Another rationale behind this study is to seek for feasible techniques which enable the monitoring of scaffolds' degradation in a non-destructive and more accurate manner. In this study we have used the mass loss as the main indicator of the scaffolds degradation and we have monitored the changes in the surface topography by SEM analysis. Nevertheless, neither of these techniques provides any information about the structural changes that come as a result of mass loss, such as changes in the dimensions, porosity, fibre diameter or surface area. Furthermore both techniques are destructive. The initial characterization of the scaffolds performed by micro-CT analysis provided a considerable amount of information regarding the morphology of the scaffolds and, it is important to highlight, both in a qualitative (3-dimensional reconstruction) and quantitative way. The diameter of chitosan fibres is different from the diameter of the needles used to produce them, due to their shrinkage during drying. The ratio between the diameter of the thicker and the thinner fibres, provided by micro-CT quantitative analysis (1.62) is approximately the same as the ratio between the needles diameter (1.78). This indicates that the technique can provide a high level of accuracy. Therefore, micro-CT analysis has shown to be a very promising accurate and non-destructive technique to monitor the scaffolds degradation.

5 Conclusions

This study has demonstrated that the manipulation of fibre diameter and porosity of chitosan fibre-mesh scaffolds is an effective tool for tailoring the degradation rate of such scaffolds without altering cell adhesion. Furthermore, the high pore interconnectivity of these scaffolds is shown to be ideal for promoting cell adhesion throughout the thickness of the scaffolds.

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References

1. R. LANGER and J. P. VACANTI, *Science* **260** (1993) 920.
2. X. MA, *Mater. Today*. **7** (2004) 30.
3. I. ADEKOGBE and A. GHANEM, *Biomaterials* **26** (2005) 7241.
4. D. W. HUTMACHER, *Biomaterials* **21** (2000) 2529.
5. A. GÖPFERICH, *Biomaterials* **17** (1996) 103.
6. P. J. VANDEVORD et al., *J. Biomed. Mater. Res.* **59** (2002) 585.
7. A. LAHIJI et al., *J. Biomed. Mater. Res*. **51** (2000) 586.
8. K. TUZLAKOGLU et al., *Macromol. Biosci.* **4** (2004) 811.
9. R. M. SILVA et al., *J. Mater. Sci. Mater. Med.* **15** (2004) 1105.
10. P. P. B. MALAFAYA et al., *J. Mater. Sci. Mater. Med.* **16** (2005) 1077.
11. S. S. SILVA et al., *J. Mater. Sci. Mater. Med.* **16** (2005) 575.
12. J.-K. F. SUH and H. W. T. MATTHEW, *Biomaterials* **21** (2000) 2589.
13. A. DI MARTINO, M. SITTINGERC and M. V. RISBUD, *Biomaterials* **26** (2005) 5983.
14. M. H. HO et al., *Biomaterials* **26** (2005) 3197.
15. H. ZHANG and S. H. NEAU, *Biomaterials* **22** (2001) 1653.
16. A. P. MARQUES, R. L. REIS and J. A. HUNT, *J. Mater. Sci. Mater. Med.* **14** (2003) 167.
17. K. M. VARUM et al., *Carbohydr. Res*. **299** (1997) 99.
18. H. ZHANG and S. H. NEAU, *Biomaterials* **23** (2002) 2761.
19. D. REN et al., *Carbohydr. Res.* **340** (2005) 2403.
20. J. LI et al., *Poly. Degrad. Stab.* **87** (2005) 441.
21. R. XING et al., *Carbohydr. Res.* **340** (2006) 2150.
22. T. FREIER et al., *Biomaterials* **26** (2005) 5872.
23. H. K. DHIMAN, A. R. RAYA and A. K. PANDA, *Biomaterials* **25** (2004) 5147.