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Research Article

An alginate hydrogel matrix for the localised delivery of a fibroblast/keratinocyte co-culture

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Abbreviations: A/L, air-liquid interface; **ECM**, extracellular matrix; **H&E**, haematoxylin and eosin; **HVG**, haematoxylin Van Gieson; **PI**, propidium iodide

There is significant interest in the development of tissue-engineered skin analogues, which replace both the dermal and the epidermal layer, without the use of animal or human derived products such as collagen or de-epidermalised dermis. In this study, we proposed that alginate hydrogel could be used to encapsulate fibroblasts and that keratinocytes could be cultured on the surface to form a bilayered structure, which

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could be used to deliver the co-culture to a wound bed, initially providing wound closure and eventually expediting the healing process. Encapsulation of fibroblasts in 2 and 5% w/v alginate hydrogel effectively inhibited their proliferation, whilst maintaining cell viability allowing keratinocytes to grow uninhibited by fibroblast overgrowth to produce a stratified epidermal layer. It was shown that the alginate degradation process was not influenced by the presence of fibroblasts within the hydrogel and that lowering the alginate concentration from 5 to 2% w/v increased the rate of degradation. Fibroblasts released from the scaffold were able to secrete extracellular matrix (ECM) and thus should replace the degrading scaffold with normal ECM following application to the wound site. These findings demonstrate that alginate hydrogel may be an effective delivery vehicle and scaffold for the healing of full-thickness skin wounds.

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1 Introduction

There is a considerable demand for the development of novel methods to replace diseased or damaged tissues. Synthetic materials have been used as tissue replacements for many centuries, but issues remain surrounding the integration of these materials into the body. Tissue engineers seek to produce and condition tissues *in vitro* with the intention of implanting the resulting construct into the body. The use of the patients' own cells in this process eliminates the chances of immunological

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rejection and enhances the prospects of complete integration into the patient's body [1].

Skin is composed of two main layers known as the epidermis and the dermis. The epidermis is a keratinocyte-rich layer that comprises four sub-layers, which contain keratinocytes at different stages of differentiation, upon the surface of a basement membrane [2]. The epidermis is mainly responsible for providing a barrier function to prevent water loss and infection, which is achieved by the most ventral layer of the epidermis known as the stratum corneum [3]. The dermis is rich in ECM, of which the main component is type I collagen and the most prevalent cell type in this layer is the fibroblast [4]. In severe wounds where both these tissue layers are lost it is important to replace both layers to achieve effective healing [5]. The first tissue-engineered skin analogue was developed in 1975, which simply aimed to replace the epidermal layer using stratified keratinocyte cultures [6]. Since then, a number of other substitutes have been developed which aim to replace either the dermal layer alone or both the epidermal and dermal layers [7–9]. However, the problem with many of these is the presence of human- or animal-derived components such as de-epidermalised dermis (DED) or collagen, which carry the risk of disease transmission and promoting immune rejection [10, 11]. It has also been suggested that the inclusion of collagen in the initial construct may be detrimental to the healing process in terms of tissue cell migration as collagen appears at the later stages of wound healing [9, 12]. There is, therefore, a significant need of development of a bilayered structure capable of assisting in wound healing in which the dermal layer is derived from a non-animal source [5, 11].

In addition, it is beneficial to have both the keratinocytes and fibroblasts present, as the paracrine interactions between these two cell types are also very important to

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effective healing [13, 14]. When co-cultures of these cells are used, however, it is important to mitotically inhibit the fibroblasts to prevent over-growth of the culture. This is typically accomplished by the use of gamma-irradiation or Mitomycin C [15, 16]. These mitotically inhibited fibroblasts facilitate formation of the epidermis *in vitro*, but are not actually delivered to the wound site and thus play no role in the replacement of the lost dermis at the wound site. It is therefore desirable to develop a method in which the mitotic inhibition is reversible, so that autologous fibroblasts can be delivered to the wound along with the keratinocytes to help maintain the keratinocyte culture at the wound bed and facilitate replacement of the dermis. The fibroblasts also secrete angiogenic mediators that encourage the neo-vascularisation of the site [17], which is essential for survival of the tissue-engineered construct.

In order to mimic the 3-D organisation of cells in a range of tissues, typically cells are seeded onto a scaffold composed of a macro-porous sponge-like material. Although such structures provide a certain amount of 3-D organisation, the cells still adhere to a 2-D surface. It has been demonstrated that cells cultured on 2-D substrates exhibit distinct phenotypes when compared with cells in the ECM [18]. In order to produce a tissue representative of that found *in vivo,* it is therefore desirable to culture the cells in an environment that mimics both the structure and spatial resolution of the ECM. Consequently, there is currently a focus on the encapsulation of cells in hydrogels [19, 20]. The highly hydrated structure of hydrogels allows for good mass transport properties [21] so that nutrients, waste products, and signalling molecules can effectively perfuse the scaffold to maintain cell viability and thereby facilitate paracrine interactions.

Alginate is a polysaccharide that is derived from brown algae, which can be cross-linked by calcium in a mild gelling reaction to form a hydrogel, and is suitable for cell

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encapsulation [21]. Alginate hydrogel has been successfully used for the encapsulation of a variety of cell types including hepatocytes, chondrocytes, osteoblasts and bone marrow stromal cells [22–25]. Alginate is approved by the Food and Drug Administration (FDA) for medical applications and has been used in wound dressings for around 20 years with no apparent toxicity. A number of wound dressings, including SeaSorb and Kaltostat, are currently commercially available [26].

In this study, the use of an alginate hydrogel matrix to support the growth of fibroblasts and keratinocytes to facilitate healing of severe skin wounds has been examined. Primary rat keratinocytes and 3T3 fibroblasts were used as models for human autologous keratinocytes and fibroblasts, respectively. This alginate hydrogel seeded with both autologous keratinocytes and fibroblasts could be applied to a wound bed and should be capable of fully replacing both the epidermal and dermal component of the lost skin. The proliferation of the encapsulated fibroblasts should be inhibited to allow for the keratinocytes growing uninhibited upon the alginate surface to produce a stratified epidermal layer, whilst still receiving growth factors and cytokines that are supplied by fibroblasts to promote growth [8, 9, 27]. This should be achieved by using a high-concentration alginate, where the encapsulated cells are mono-dispersed throughout the hydrogel, to mechanically confine the fibroblasts. This alginate scaffold should then degrade to allow for the proliferation of the encapsulated fibroblasts and secretion of ECM to replace the alginate hydrogel matrix and the migration of other cell types into the scaffold to restore functional and structural properties of the skin [27].

2 Materials and methods

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Unless otherwise specified all chemicals were supplied by Sigma Aldrich, Poole, UK.

2.1 Encapsulation of NIH 3T3 murine fibroblasts

Low viscosity alginate (20–40 centipoise (cps) for 2% w/v at 25°C) was sterilised by autoclave then used to encapsulate NIH 3T3 cells (LGC, Middlesex, UK) at density of 7.5×10^5 cells/mL. The alginate/3T3 dispersion was added drop-wise into a bath of 100 mM $CaCl_2$ and left to incubate at 37ºC for 2 h to form cross-linked spheres of 3.0 ± 0.2-mm diameter and then washed three times in non-supplemented DMEM. To form an alginate encapsulating cell sheet, the suspension was used to cover the surface of a petri-dish and immersed in 100 mM $CaCl_2$ for 2h to allow for complete cross-linking of the alginate. The displacement of alginate by $CaCl_2$ was prevented by covering the alginate hydrocolloid with a layer of filter paper impregnated with 100 mM $CaCl_2$. Cells were grown and maintained in high glucose DMEM supplemented with 10% v/v foetal bovine serum (FBS) (PAA, Somerset, UK), 1% v/v penicillin-streptomycin (P/S), 2.25% v/v HEPES and 2% v/v L-glutamine. All cultures were maintained at 37ºC with 5% CO_2 and 100% relative humidity and media was changed three times weekly.

2.2 Keratinocyte cultures

Keratinocytes were obtained from neonatal rat sacrificed by cervical dislocation. The keratinocytes were isolated using 0.25% trypsin–0.02% EDTA and mechanical dissociation and cultured in 3:1 DMEM:Ham's F12 supplemented with 50 mM hydrocortisone, 10 µg/mLl insulin, 25 ng/mL EGF, 10% v/v FBS (PAA), 1% v/v P/S,

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2.25% v/v HEPES, 2% v/v L-glutamine as monolayers, with the support of a fibroblast feeder layer prepared by treatment with 4 mM Mitomycin C for 2 h at 37ºC, until a sufficient number of keratinocytes were obtained, as determined by counting using a haemocytometer. The keratinocytes were seeded at a density of 0.2 × 10^6cells/cm^2 onto the surface of alginate containing fibroblasts. The keratinocytes were cultured for a further 7 days on the alginate scaffold before being raised to the air-liquid (A/L) interface to allow for stratification of the keratinocytes. All keratinocyte cultures were maintained at 37ºC with 5% CO_2 and 100% relative humidity.

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2.3 Live/dead staining

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Calcein acetoxymethylester (calcein AM) and propidium iodide (PI) (Invitrogen, Paisley, UK) were used to stain live cells green and dead cells red, respectively, when visualised using fluorescence microscopy (Carl Zeiss, Axiolab, UK). The 1-mm thick sections were taken from the centre of alginate beads containing 3T3 cells using a razor blade and immersed in 0.2 µg/mL calcein-AM for 15 min and 2.5 µg/mLl PI for 5 min in DMEM at 37ºC before examination with a fluorescence microscope (Carl Zeiss) and capture of images (Canon Powershot GS, Surrey, UK).

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2.4 MTT assay for fibroblast growth

A stock solution of 5 mg/mL MTT in PBS was added to supplemented DMEM at a concentration of 10% v/v. Samples of alginate/3T3 beads were removed from the media, washed in DMEM and immersed in the resulting 10% solution of MTT for

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18 h. The MTT solution was then removed and replaced with HCl-isopropanol and left for 2.5 h to allow the formazan produced to dissolve and diffuse out of the alginate. The optical absorbance of the solution at 620 nm was measured using a spectrophotometer (Cecil, Cambridge, UK). This amount of formazan produced by cellular reduction of MTT was determined from the A at 620 nm according to a calibration of different cell concentrations in alginate, as determined by counting on a haemocytometer, and the A at 620 nm after application of the MTT assay.

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2.4 Histology

An adult rat was humanely sacrificed by cervical dislocation before skin removal. Alginate encapsulated cells and skin samples were fixed in formalin buffer for 24 h before progressive dehydration in cassettes (Thermo Shandon, Citadel 1000, UK). The dehydrated samples were then embedded in paraffin wax (Sakura, Tissue Tek, TEC) and stored at room temperature. Samples were then cooled to 5°C and 5-µm sections were taken using a microtome (Leica RM 2035), which were attached to glass slides and dried at 60°C in an oven for 1 h before removal of the wax in xylene and staining with Haematoxylin Van Gieson (HVG) (Merck, Nottingham, UK) according to a standard protocol to demonstrate presence of collagen [28] or Haematoxylin and Eosin (H&E) (Surgipath Europe, Peterborough, UK) to demonstrate tissue architecture [29]. Coverslips were then applied to the slides using XAM (BDH Laboratory Supplies, Poole, UK) and images of the slides obtained with a light microscope (Carl Zeiss) and captured (Canon Powershot GS, Surrey, UK). The images were then corrected using Image J software (NIH, MD) to remove any uneven illumination from the image.

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2.5 Determining alginate degradation

Degradation profiles were obtained over 33 days for acellular and fibroblast encapsulating samples of both 2 and 5% w/w alginate beads of 3 ± 0.2-mm diameter under the normal culture conditions described above. The degradation was assessed by measuring the alginate mass after dehydration in a vacuum freeze-drier (Edwards, EF03, Sussex, UK) for 16 h. Samples were measured in triplicate and the mean and standard deviation for each data point were shown. The release of fibroblasts from the alginate samples was determined by visual examination of the culture dish using a light microscope 33 days after introduction of the alginate samples.

2.6 Statistical analysis

Statistical significance (p <0.05) between test groups was determined by one-way analysis of variance (ANOVA) and Tukey post-hoc test (SPSS v.17, Chicago, IL).

3 Results

To evaluate the effect of long-term encapsulation of fibroblasts in 5% w/v alginate hydrogel, the encapsulated cells were stained *in situ* with a Calcein-AM/PI live/dead stain. The encapsulated fibroblasts were showed to remain viable for the duration of the study (28 days post encapsulation) in 5% w/v alginate (Fig. 1). The cells were evenly dispersed throughout the alginate matrix and after 28 days there had been no evident change in either cell viability or number. The formation of cell spheroids was

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not detectable in any of the samples. Cell number, as quantified using the MTT assay (Table 1), did not change significantly over the duration of the study ($p > 0.05$), with very little fluctuation from the initial cell seeding density (7.5×10^5mL).

((Figure 1)) ((Table 1))

To determine whether the encapsulated cells had deposited ECM during the encapsulation period histological sections of alginate-encapsulated fibroblasts were stained with HVG and compared with the normal dermis of rat skin. In Fig. 2a the distribution of cells and collagen in the dermis of normal rat skin can be seen. The nuclei of cells stained blue-black due to the haematoxylin, demonstrating the cell distribution, whilst the Van Gieson solution stains the collagen pink. It can be seen that the dermis has a low cell content and that the cells are present in small clusters or individual units and the main component of the dermis is collagen, which is present in small bundles of around 10–35-µm diameter. In Fig. 2b the cells encapsulated within the alginate matrix remained as dispersed units within the hydrogel after 21 days of encapsulation and had not proliferated or migrated within the gel to form cell clusters as seen in the dermis of normal skin so the cell concentration was lower in the hydrogel than in normal rat skin. The haematoxylin also stained the alginate fibres in the hydrogel blue-black due to their negative charge and the positive charge of the haematoxylin [28, 29]. After 21 days, there was no evidence of collagen, which indicated that the encapsulated fibroblasts fail to secrete ECM during this time.

((Figure 2))

The dry mass of the tissue engineered dermis consisting of 5% w/v alginate and fibroblasts was shown to decrease over 33 days, so that by day 33 around 80% of the original mass remained (Fig. 3). It is evident that by reducing the alginate concentration to 2% w/v the rate of degradation was increased and that by day 33 only

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around 50% of the original alginate remained. The cells appeared to play very little part in this degradation, as with both 2 and 5% w/v alginate, the degradation profiles are extremely similar for both acellular and cell-seeded samples. All four samples showed a significant decrease in dry mass between days 0 and 33 (p <0.05). Degradation of the alginate hydrogel has been shown to facilitate the release of entrapped material [30]. In the present study, it was observed that as the alginate degraded the cells were released from the alginate matrix and attached to suitable substrates such as glass or tissue culture plastic in which the alginate-encapsulated cells were being cultured, as has been seen for fibroblasts released form a different alginate hydrogel after 14 days cultivation [24]. HVG staining illustrated that cells released from alginate hydrogel were able to secrete collagen on the surface of the glass slide to which the cells had attached, as before they were encapsulated (Fig. 4).

((Figure 3)) ((Figure 4))

The alginate-fibroblast dermal scaffold was seeded with primary neonatal keratinocytes and after 7 days the keratinocyte culture was raised to the A/L interface. After 14 days at the A/L interface the resulting epidermal layer was examined. Staining with H&E (Fig. 5) illustrated that the keratinocytes had stratified to form a 3–4 cell-thick layer, but no stratum corneum was observed.

((Figure 5))

4 Discussion

Encapsulated fibroblasts were shown to remain viable within the alginate hydrogel for up to 28 days post encapsulation. The long-term viability of other encapsulated cells has been demonstrated, for example with chondrocytes [31] and human embryonic

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stem cells [32] and two other fibroblast cell lines [33]. In the present study, the viability of cells in the centre of both 2 and 5% w/v alginate hydrogel beads of 3-mm diameter was observed (Fig. 1) and illustrated that there was no critical necrosis and that oxygen tension was high enough to support cell survival, in contrast with what has been found with other alginates and cell types [34]. The non-aggregated fibroblasts remained viable within un-modified alginate throughout the duration of the study, in contrast with previous reports [22, 31, 32]. The encapsulated fibroblasts, however, did not proliferate throughout the 28 days (Table 1) and the cells remained as dispersed units and failed to form cell aggregates (Figs. 1 and 3), which have been seen to form when embryonic stem cells were encapsulated in alginate [31].

It is proposed that the lack of mitotic activity is due in part to the use of a relatively low cell-seeding density [35], the encapsulation of dispersed cells rather than spheroids [36], the use of a non-liquefied core [37] and the use of high concentrations of alginate, which resulted in the fibroblasts being mechanically confined from each other by cross-linked chains of alginate. These results demonstrate that encapsulation in alginate effectively mitotically inhibits the fibroblasts without the need for treatment with Mitomycin C or gamma-irradiation, to allow for co-culturing of keratinocytes with the fibroblasts.

Evaluation by HVG after 21 days encapsulation of fibroblasts in 5% w/v alginate revealed that there was no collagen present, indicating that the encapsulation inhibited the fibroblasts production of ECM. It is proposed that this is because the fibroblasts sensed the confinement of the 3-D environment through cytoskeletal arrangement [38], which altered cell behaviour in comparison with that exhibited in the 2-D environment [18]. In the body, fibroblasts only begin to secrete large quantities of

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ECM after injury [12], when the cells are released from the 3-D confinement of the damaged ECM.

The present study has shown that calcium cross-linked hydrogel degrades over time by acellular mechanisms. This is due in part to the fact that mammalian cells do not possess alginases to hydrolyse the alginate molecules [39] and the degradation is possibly due to the calcium cross-links being replaced by monovalent cations in the media, such as sodium or potassium, which cannot cross-link the structure or chelation by phosphate anions [40]. As the matrix degraded, the encapsulated cells were released [24], other cells present at the wound site should be able to migrate into the alginate hydrogel scaffold to assist the healing process and integration of the engineered tissue with the surrounding host tissue [41]. It was shown that degradation could be tailored simply by varying the alginate concentration (Fig. 3).

When the fibroblasts were released from alginate hydrogel they were able to adhere to glass and resume the normal activity that was observed upon 2-D culture, whereby the fibroblasts proliferate to cover the glass substrate and secrete ECM (Fig. 4), similar to when the dermis of normal skin is wounded [9, 12]. When the construct is implanted into the body the fibroblasts should eventually replace the degrading alginate with natural ECM. As the alginate scaffold degrades, other cells at the wound site should also be capable of migrating into the scaffold and this should facilitate reinnervation and angiogenesis of the site, which is important for the success of the treatment.

It was possible to culture keratinocytes on the surface of the alginate/fibroblast dermal layer, suggesting that this was an effective way of providing the keratinocytes with a fibroblast feeder-layer compatible with application to the wound site. Unlike the feeder-layer produced by irradiation of the cells or treatment with Mitomycin C, which ultimately causes cell death, the fibroblasts in the present study did not need to

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be continuously replaced, as encapsulated fibroblasts remained viable throughout the duration of the experiment (Table 1). Histological evaluation with H&E staining illustrated that the keratinocytes formed stratified layers in areas where the keratinocytes had attached, but the stratum corneum was not evident (Fig. 5). Calcium is a key regulator in keratinocyte maturation [42], but when keratinocytes were grown on the surface of the alginate hydrogel-based dermal analogue no terminally differentiated keratinocytes were observed, suggesting that the amount of calcium released by the degrading alginate scaffold was not significant to induce terminal differentiation of all the keratinocytes (Fig. 5). Improvement of cell attachment to the alginate could be achieved by modifying the alginate with adhesion-promoting ligands [21, 43], or by using an alginate with a higher content of guluronic acid residues [44], which should facilitate formation of a full-thickness epithelium over the entire surface of the alginate and upon implantation into the body. The additional growth factors and cytokines present at a wound should also facilitate formation of a more complete epidermal layer [45].

In conclusion, alginate hydrogel may prove to be a desirable scaffold for tissue-engineering skin. This tissue-engineered skin may have applications in the clinical setting for treatment of chronic skin wounds and in the pharmaceutical industry for high-throughput testing of drugs and pharmaceuticals. Further work should seek to evaluate how the construct behaves *in vivo* when seeded with autologous fibroblasts and keratinocytes.

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Figure 1. Fibroblasts stained *in situ* with calcein AM/PI live/dead stain in 2% w/v and 5% w/v alginate at 3, 14 and 28 days post encapsulation. The evidence of spheroid formation or cell proliferation is visible. The viability of cells throughout the experiment was illustrated by the presence of mainly green cells and very few red cells. This indicates that the diffusion of nutrients and waste products through the matrix was sufficient to maintain the cells' viability and that the alginate scaffold was not necrotic.

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Figure 2. Haematoxylin Van Gieson staining (a) of normal dermis of skin shows that there is a large presence of collagen in bundles of 10–35-μm diameter, the main component of the ECM. (b) The 5% w/v alginate hydrogel 21 days post encapsulation of fibroblasts demonstrated no collagen.

Figure 3. Degradation profiles of acellular and cell-seeded alginate. Samples were either of 2or 5% w/v alginate concentration and inclusion of cells in the scaffolds had no appreciable effect on the degradation profile. It is also apparent that the 2% w/v alginate degrades faster than the 5% w/v alginate.

Figure 4. HVG staining of fibroblasts cells grown on glass coverslips. (a) Before encapsulation this reveals that fibroblasts cells secrete collagen. (b) When encapsulated fibroblasts are released from 5% w/v alginate after 21 days encapsulation the fibroblasts retain the ability to secrete collagen.

Figure 5. Keratinocytes cultured on the surface of the alginate/fibroblast dermal analogue produce a stratified layer around 3–4 cells thick (a), as is seen in normal epidermis of skin (b). The presence of cell nuclei indicates that these are basal and/or suprasal cells which can undergo further differentiation to form the stratum corneum, which is not seen on the surface of the stratified layer (a).

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Table 1. Mean number of viable fibroblasts per ml alginate and SD at each time point as determined by the MTT assay. The viable cell number remains relatively constant throughout the duration of the experiment.

Day	Cell number ± SD ($\times 10^5$)	
	2% w/v alginate	5% w/v alginate
3	7.5±0.64	7.2±0.78.
14	7.6±0.68	7.8±0.78
28	7.8±0.80	7.6±0.70

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Figure 2 – Haematoxylin Van Gieson staining a) of normal dermis of skin shows that there is a large presence of collagen in bundles of 10-35µm diameter, the main component of the ECM. b) of 5% w/v alginate hydrogel 21 days post encapsulation of fibroblasts demonstrated no collagen. ¶

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Figure 3 - Degradation profiles of acellular and cell-seeded alginate. Samples were either of 2% or 5% w/v alginate concentration and inclusion of cells in the scaffolds had no appreciable effect on the degradation profile. It is also apparent that the 2% w/v alginate degrades faster than the 5% w/v alginate.¶

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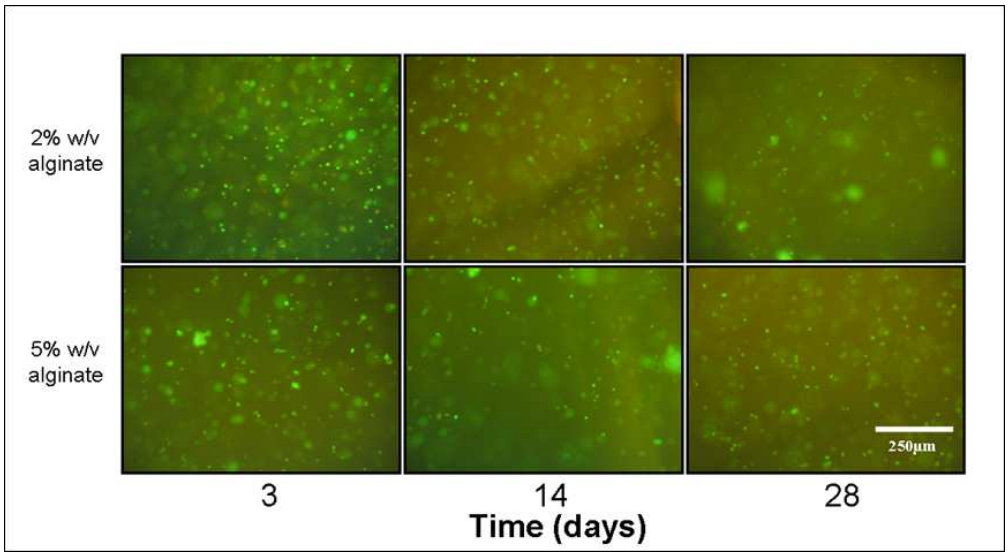

Figure 1 Â□ cells stained in situ with Calcein AM/ PI live/ dead stain in 2% w/v and 5% w/v alginate at 3 days, 14 days and 28 days post encapsulation. The evidence of spheroid formation or cell proliferation is visible. The viability of cells throughout the experiment was illustrated by the presence of mainly green cells and very few red cells. This indicates that the diffusion of nutrients and waste products through the matrix was sufficient to maintain the cells' viability and that the alginate scaffold was not necrotic.
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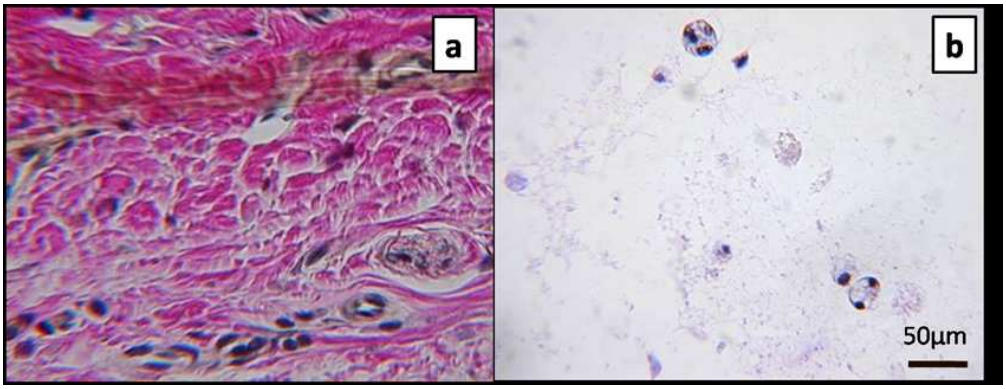

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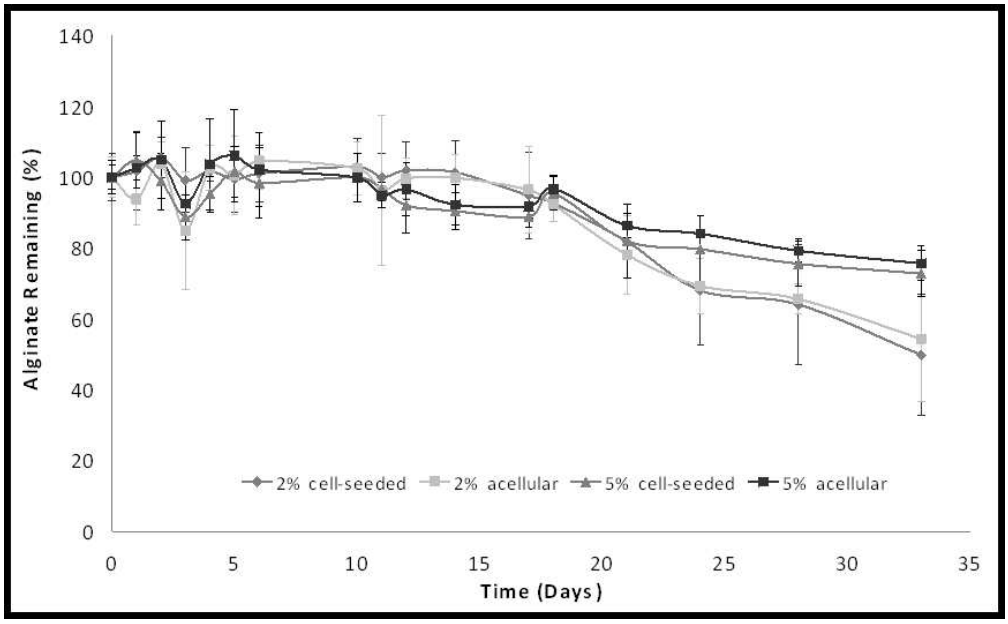

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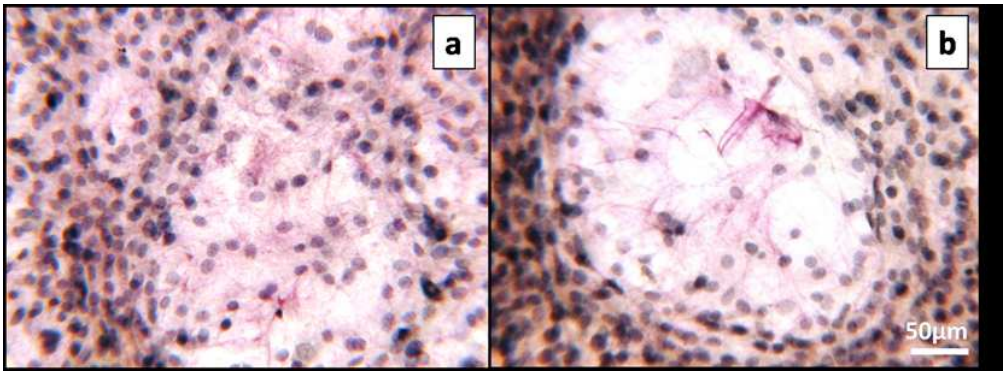

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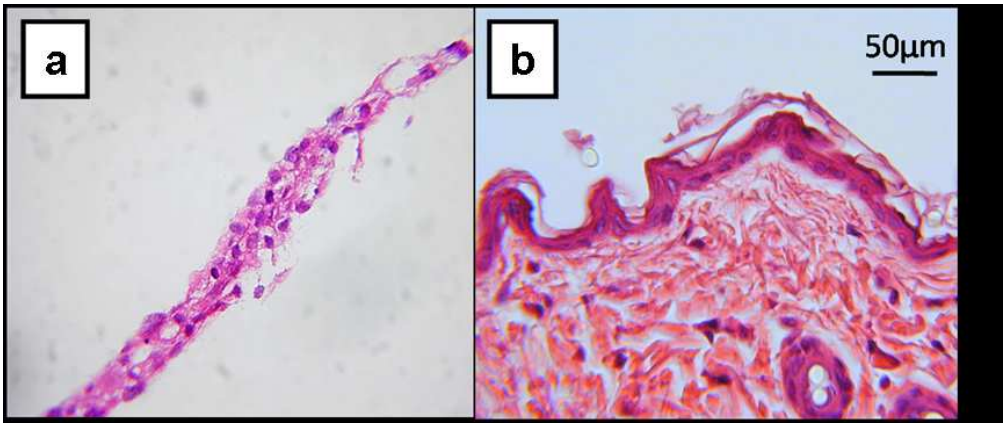

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