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Incorporation of proteins within alginate fibre-based scaffolds using a post-fabrication entrapment method

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Abstract

In this study, a physical entrapment process was explored for the incorporation of proteins within preformed fibrous alginates and the release profile was tuned by varying the processing parameters. The entrapment process was carried out in a series of aqueous solutions at room temperature and involved pre-swelling of the fibrous alginate within a Na^+-rich solution, followed by exposure to the protein of choice and entrapping it by re-establishing cross-links of alginate with $BaCl_2$. Entrapment and release of fluorescein isothiocyanate-labelled bovine serum albumin (FITC-BSA), a model protein, was studied. It was found that a sustained release of the incorporated protein in cell culture medium for about 6 days was achieved. The main factors determining the release profile included the $NaCl/CaCl_2$ ratio in the pre-swelling solution, protein concentration, and the exposure time. To retard protein release, alginate fibres with entrapped FITC-BSA were processed together with poly(D, L-lactide) (PDLLA) into porous alginate fibre/PDLLA composites using supercritical CO_2. In this manner, release of the protein for up to 3 months was achieved.

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Introduction

A potential application of tissue engineering scaffolds, including alginate fibre meshes, is the stimulation of tissue regeneration via the release of growth factors or other protein-based drugs (Sheridan et al 2000; Whitaker et al 2001). Many growth factors have short half-lives, and concentration-dependent effects (Babensee et al 2000). Therefore, controlled release of growth factors from scaffolds to the surrounding environment may be beneficial. The production of scaffolds with embedded growth factors is challenging because the instability of the protein component limits the processing environments that can be employed. To address this problem, several approaches have been developed, including the supercritical fluid processing method (Watson et al 2002), the double-emulsion-solvent-extraction technique (Cleek et al 1997; Franssen & Hennink 1998), and use of hydrogel systems (Tabata et al 1999; Holland et al 2005). In this study, we demonstrate a simple and rapid method of adding growth factors to pre-fabricated fibrous alginate scaffolds.

Alginate is a naturally occurring linear polysaccharide that forms a hydrogel in the presence of multivalent cations. It is composed of two sterically different repeating units, (1→4)-α-L-guluronate (G unit) and (1→4)-β-D-mannuronate (M unit) in varying proportions and sequential arrangements. The gelation process is caused by ionic cross-linking involving the formation of egg-box junction zones (Bryce et al 1975), where regular packing and co-ordination of the cations in the voids of associated segments takes place. Moreover, the sol–gel transition is reversible through ion exchange. Because of its low toxicity, biocompatibility, abundant availability, and controllable gelation and mechanical properties, alginate has been investigated as scaffold material for angiogenesis (Ko et al 1997; Peters et al 1998; Richardson et al 2001; Tanihara et al 2001), and tissue regeneration (Yang et al 2001; Alsberg et al 2002; Dar et al 2002; Mierisch et al 2002; Hashimoto et al 2004). However, the lack of specific cellular interactions limits its potential wider application, as the initial cell adhesion and growth on the substrate is critical to the regenerative process. To

address this issue, for initial cell attachment and recruitment, adhesion peptide sequences, such as Arg-Gly-Asp (RGD), have been introduced into alginate (Rowley et al 1999). However, for long-term cellular function and promotion of tissue-specific gene expression, sustained release of growth factors is required for some regenerative medicine applications.

Growth factors have been incorporated into various forms of alginate such as beads (Downs et al 1992; Ko et al 1997; Liu et al 1997; Peters et al 1998; Mierisch et al 2002; Arnold et al 2003), membrane (Milella et al 2001), sponges (Caterson et al 2001; Tanihara et al 2001), and microspheres (Elcin et al 2001; Chinen et al 2003). However, no attempt has been made to incorporate proteins within preformed alginate fibres. In fact, alginate fibres are of significant potential interest in tissue engineering because they can be readily processed into macroporous three-dimensional scaffolds. These scaffolds differ from the conventional formats of alginate scaffolds used in cell culture, such as beads or hydrogels, because the interconnected macroporosity allows rapid diffusion of culture medium components through millimetre-scale structures. Hence, these types of scaffold are promising for macroscopic tissue growth. However, incorporation of protein into alginate fibres can be problematic. The processing conditions during fibre production, which generally involve the use of organic solvents and high temperatures, may damage the activity of the protein. The storage and shelf life of these fibres incorporated with proteins is another major concern. Alternatively, incorporation of protein into preformed alginate fibres can be advantageous as the incorporation process is separated from the production process and the protein of choice can be introduced into the fibres just before use.

Recently, we have developed a physical entrapment process for the incorporation of bioactive molecules within pre-formed alginate fibres (Hou et al 2005). The aim of the present work was to process proteins into fibrous alginates by this technique and to study the release behaviour using fluorescein isothiocyanate-labelled bovine serum albumin (FITC-BSA) as a model protein. The retention of biological activity of the protein after entrapment and release processes, and the effect of the entrapment process on cell viability during cell culture were also investigated. To the best of our knowledge, this is the first report on the controlled release of proteins from alginate fibres after entrapment engineering. Our previous work focused on surface exposed ligands to promote cell adhesion. In this study, we adjusted the entrapment process to control the release of the protein component.

Materials and Methods

Materials

Alginate fibres and felts were obtained from Advanced Medical Solutions plc (Cheshire, UK). The pore size of the felts was over the range of 200–500 μm as measured by scanning electron microscope. The porosity of the felts was $95 \pm 3\%$ as calculated from the densities of the felts and the alginate. Calcium chloride dihydrate, sodium chloride, barium chloride, hydrochloric acid, tris[hydroxymethyl]aminomethane, fluorescein isothiocyanate-labelled bovine serum albumin (FITC-BSA) and ribonuclease A were purchased from Sigma-Aldrich, UK.

Incorporation of FITC-BSA within alginate felts and the release behaviour

Thin strips of alginate felt (approx. 1 mm in thickness) were cut as discs to fit 12-well non-tissue culture treated plates (Falcon). The alginate felt discs were first pre-swollen in a sodium-rich $NaCl/CaCl_2$ solution for 10 min before exposure to FITC-BSA solution of certain concentration for a given period of time. The felts were then immersed in barium chloride solution (10% w/v) for 30 min, followed by washing with distilled water for 30 min and drying in air at ambient temperature to a constant weight. The processing conditions were varied during the entrapment process as detailed in Table 1. The presentation of FITC-BSA within the alginate felts was visualized using a confocal laser-scanning fluorescence microscope (Leica DMLFS).

For the protein release study, the alginate felts incorporated with FITC-BSA were placed in 12-well non-tissue culture treated plates. Then, 6 mL of Dulbecco's modified Eagle's medium (Sigma) was pipetted into each well. The plates were wrapped in aluminum foil and left at room temperature. The release behaviour of FITC-BSA from the alginate felts was investigated using a Hitachi F-4500 fluorescence spectrophotometer. The excitation and emission wavelength settings were 492 and 521 nm, respec-

Table 1 Processing parameters for the entrapment of fluorescein isothiocyanate-labelled bovine serum albumin (FITC-BSA) within alginate felts

Experiment	Variable conditions	Constant conditions
Varying the $NaCl/CaCl_2$ ratio in the pre-swelling solution	X (% w/v) 0.5:0.1 1.0:0.1 2.0:0.1 10.0:1.0	FITC-BSA concentration: 0.5 mg mL^{-1}; exposure time: 10 min
Varying the FITC-BSA concentration	X (mg mL^{-1}) 0.25 0.50 0.75 1.00	Pre-swelling in $NaCl/CaCl_2$ 1%:0.1% w/v for 10 min; FITC-BSA exposure time: 10 min
Varying the exposure time in FITC-BSA solution	X (min) 2 5 10 20	Pre-swelling in $NaCl/CaCl_2$ 1%:0.1% w/v for 10 min; FITC-BSA concentration: 0.5 mg mL^{-1}

tively. The photomultiplier was set at 700 V, with 2.5/10.0 nm slits. The experiments were carried out in triplicate. At each set time point, 2 mL of medium was removed from each well and replaced with 2 mL of fresh medium.

Activity study of the released protein from alginate fibres

Ribonuclease A was used as a model protein to study the activity of the released protein from alginate fibres. The enzyme was incorporated into alginate fibres as described above, and then released into a Tris buffer (pH 7.13) containing 25 mM tris[hydroxymethyl]aminomethane, 25 mM sodium chloride and 25 mM hydrochloric acid at 37°C for 15 min. The activity of the released enzyme was monitored by measuring the rate of conversion of a ribonuclease-specific substrate, cytidine-2′,3′-monophospate to cytidine 3′-phosphate that could be detected spectrophotometrically, as described previously (Watson et al 2002). Ribonuclease activity was normalized to the total amount of protein present, determined by Coomassie protein assay.

Fabrication of porous poly(D,L-lactide) (PDLLA) structures embedded with protein entrapped alginate fibres and the protein release behaviour

PDLLA structures embedded with FITC-BSA incorporated alginate fibres were prepared by a two-step process. First, alginate fibres were pre-swollen in a sodium-rich $NaCl/CaCl_2$ solution ($NaCl/CaCl_2$ ratio (w/w) 20:1; NaCl concentration 10% w/v) for 5 min before exposure to 5 mg mL^{-1} of FITC-BSA solution for 30 min. The fibres were then immersed in barium chloride solution (5% w/v) for 15 min, followed by washing with distilled water and drying in air at ambient temperature to a constant weight. The alginate fibres loaded with FITC-BSA were then cut into fibrils of 1–2 mm in length. Then, porous composites were fabricated using supercritical fluid CO_2. Briefly, the protein entrapped fibres (0.01 g) were mixed with PDLLA (Purac, The Netherlands) particles (0.10 g) in a Teflon mould. The mould was then sealed inside a 60-mL autoclave, which was heated to 35°C before filling with CO_2 for 30 min to a pressure of 207 Bar. The plasticized CO_2 polymer mixture was equilibrated for 20 min before venting to atmospheric pressure for 8 min. The pressure was controlled throughout the preparation process while the temperature remained below 38°C throughout the filling step. The flow rate of CO_2 during the equilibrium step was 12 mL min^{-1}. After CO_2 processing, the mould containing foamed polymer was removed from the autoclave and the residual gas was allowed to escape for 2 h to obtain porous alginate/PDLLA composites. The morphology of porous alginate fibre/PDLLA composites was examined using a Jeol JSM-6060LV scanning electron microscope. Cross-sections of the composites were sputter-coated with gold before analysis.

FITC-BSA release from the porous alginate fibre/PDLLA composites was carried out at 37°C in a phosphate buffer solution (pH 7.4; PBS). The FITC-BSA release behaviour from alginate fibres under the same entrapment and release conditions was also studied as a control.

Cell viability within alginate felts after the protein entrapment process

Alginate felts were cut to fit in 24-well non-treated plates (Costar). Alamar Blue assay on Mouse 3T3 fibroblast cells was used to test the cell viability within alginate felts after the FITC-BSA entrapment process. A control experiment was carried out with alginate felts without protein incorporation. Cell seeding density was 750 000 cells/felt.

Statistical analysis

All data were expressed as mean ± s.d. The effect of processing parameters (ion ratio, protein concentration and immersion time) and time on protein release was statistically examined using Friedman's test. A post-hoc test was then used to discern between differences in individual parameters and time. The effect of each system on fluorescence at each time point was compared using the Kruskal–Wallis test (non-parametric). Individual differences between the various systems were then examined using an appropriate post-hoc test. Differences between groups were considered significant when $P < 0.05$.

Results and Discussion

Physical entrapment of FITC-BSA within alginate felts

The entrapment process exploited the reversible sol–gel transition of alginates by using a series of aqueous solutions at room temperature. The pre-swelling of the alginate felts in a Na^+-rich $NaCl/CaCl_2$ mixture solution led to the formation of a moderate dissociation layer, into which the protein could diffuse. Subsequent addition of a large excess of Ba^{2+} ions resulted in re-establishing cross-links of alginate and entrapment of the protein within the felts. Ba^{2+} was used to restore the cross-links between the polymer strands because of its high affinity with alginates (Gombotz & Wee 1998). A typical confocal image of an alginate felt entrapped with FITC-BSA is shown in Figure 1. It was noted that the presentation of fluorescent FITC-tagged protein at the surface of alginate felts was clearly visible after the entrapment process.

Release of FITC-BSA from alginate felts

The release of the entrapped protein from alginate felts was achieved in cell culture medium as a result of Ba^{2+} diffusion out of the felts into the surrounding medium. The gradual loss of cross-links led to a

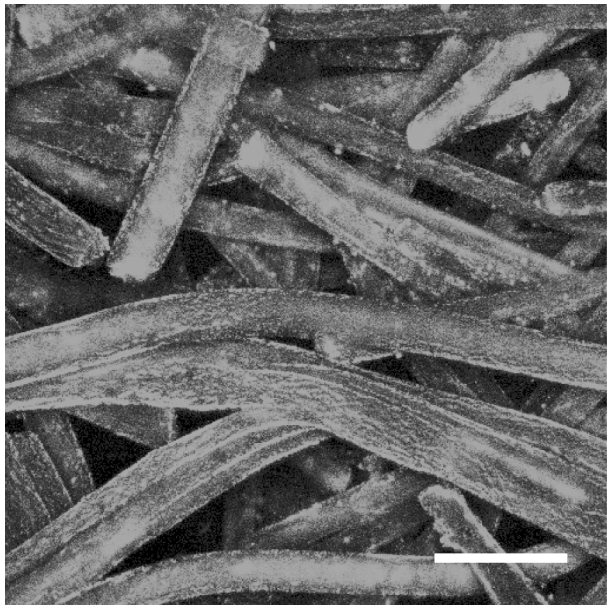

Figure 1 Confocal microscopy of alginate felts entrapped with fluorescein isothiocyanate-labelled bovine serum albumin ($0.25\,mg\,mL^{-1}$) (bar = $50\,\mu m$).

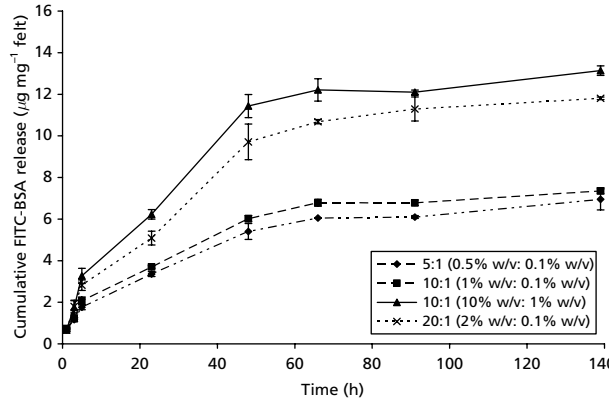

Figure 2 Effect of $NaCl/CaCl_2$ ratio in the pre-swelling solution on the release of fluorescein isothiocyanate-labelled bovine serum albumin (FITC-BSA) from alginate felts.

diffusion-controlled release of the entrapped protein. The effects of three main processing parameters, including Na^+/Ca^{2+} ratio in the pre-swelling solution, protein concentration and exposure time during the entrapment process, on the protein release profile were studied in detail.

Effect of Na^+/Ca^{2+} ratio in the pre-swelling solution

$NaCl/CaCl_2$ solutions with different Na^+/Ca^{2+} ratios were used to dissociate the alginates at the surface by removal of guluronic acid associated Ca^{2+} ions, responsible for the cross-links between the polymer strands (Gombotz & Wee 1998). It was hypothesized that by increasing the Na^+ ions relative to the Ca^{2+} ions in the $NaCl/CaCl_2$ pre-swelling solution, a greater number of cross-links would be dissociated, allowing greater swelling of the felt and consequently more FITC-BSA to be entrapped.

Figure 2 shows the effect of Na^+/Ca^{2+} ratio in the pre-swelling solution on the protein release profile from alginate felts. It was noted that all four sets of alginate felts shared similar profiles, with the cumulative FITC-BSA released increasing over 140 h. The gradient of the trend line for each set of felts gradually became less steep over the course of the experiment, with a plateau forming from t = 66 to t = 91 h. The felts treated with lower NaCl concentrations had consistently lower FITC-BSA cumulative release at time-points between 5 and 91 h ($P < 0.05$). Increasing the Na^+ ions from 0.5% to 2.0% w/v while keeping the Ca^{2+} concentration constant at 0.1% w/v resulted in the final cumulative FITC-BSA released increasing from 7.0 to $11.8\,\mu g\,mg^{-1}$ felt. Interestingly, the greatest cumulative release of FITC-BSA ($13.2\,\mu g\,mg^{-1}$ felt) was achieved by increasing the concentrations of both the Na^+ and Ca^{2+} ions (to 10% and 1% w/v, respectively), while maintaining the ratio of NaCl to $CaCl_2$ (w/w) at 10:1. It was thus shown that while increasing the Na^+ ions relative to the Ca^{2+} ions increased the swelling of the alginate felts, the number of Na^+ ions available in the lower concentration $NaCl/CaCl_2$ solutions prevented a greater number of cross-links from being removed. The extent to which the Na^+ ion concentration was increased relative to the Ca^{2+} ion concentration was also known to be important as the immersion of the alginate felts in a NaCl (7.5% w/v)/$CaCl_2$ (0.1% w/v) solution resulted in total fragmentation of the felts within a few minutes. Therefore, the concentration of Na^+ cannot be increased indefinitely without adjusting the concentration of Ca^{2+}, otherwise excessive cross-links will be removed resulting ultimately in total dissolution of the felts.

Effect of FITC-BSA concentration during the entrapment process

Following the pre-swelling process, the hydrated felts were immersed in a FITC-BSA solution of certain concentration. The effect of the concentration of the FITC-BSA solution on the release profile was examined. During the entrapment process, alginate felts were subject to FITC-BSA solutions with concentrations ranging from 0.25 to $1.00\,mg\,mL^{-1}$. It can be hypothesized that increasing the concentration of FITC-BSA resulted in a steeper FITC-BSA concentration gradient between the immersion solution and the hydrated felts, allowing a greater proportion of FITC-BSA to diffuse into the felts and thus to be entrapped.

Figure 3 shows the effect of protein concentration during the entrapment process on the cumulative release profile of FITC-BSA. All four FITC-BSA entrapment concentrations shared similar profiles, with cumulative FITC-BSA release increasing until tending towards a plateau between t = 66 and t = 91 h. As the FITC-BSA entrapment concentration increased, it was observed that there was an increase in the cumulative release of FITC-BSA from the felts at time points between 3 and 139 h ($P < 0.05$). For instance, when the concentration of FITC-BSA was increased from $0.25\,mg\,mL^{-1}$ to

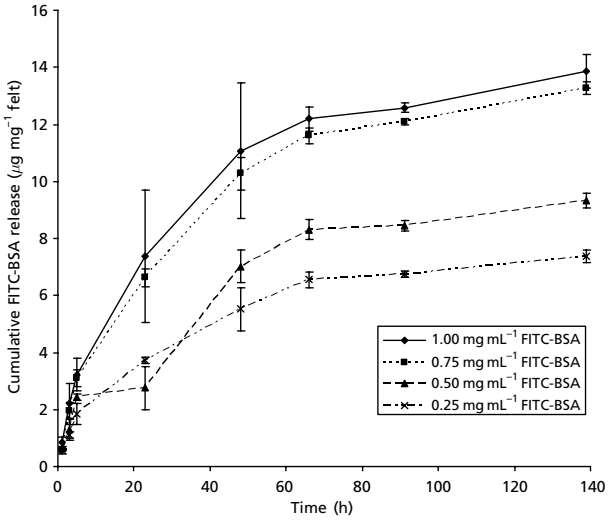

Figure 3 Effect of protein concentration during the entrapment process on fluorescein isothiocyanate-labelled bovine serum albumin (FITC-BSA) release from alginate felts.

1.00 mg mL^{-1}, the final cumulative FITC-BSA released was almost doubled from 7.4 to 14.0 μg mg^{-1} felt.

Effect of exposure time in FITC-BSA solution during the entrapment process

The effect of exposure time in the protein solution during the entrapment process on the protein release profile is shown in Figure 4. The exposure time of the alginate felts in 0.5 mg mL^{-1} FITC-BSA solution varied from 2 to 20 min. The cumulative release of FITC-BSA increased over the course of the experiment, tending towards a plateau at t = 66 h. The felts treated with FITC-BSA solution for 2 and 5 min produced almost identical profiles, with the final cumulative FITC-BSA released at about 5 μg mg^{-1} felt. The felts immersed for 10 min and 20 min gave higher final cumulative FITC-BSA release of approximately 7.2 μg mg^{-1} felt. This result indicated that the release of the protein from the alginate felts increased with exposure time during the entrapment process within the range of 2–10 min at time points between 48 and 91 h ($P < 0.05$). Thereafter, further increasing the exposure time did not yield greater cumulative release, probably because a diffusion balance was reached within 10 min. We also noticed that the felts started to slightly break down at 20 min exposure time. This may be the reason why there was a decrease in the amount of released protein from the felts with an immersion time of 20 min as compared with those with an immersion time of 10 min.

The controlled release profiles observed here indicate that the amount of protein entrapped within alginate felts can be regulated by altering a range of processing parameters without changing the general trends in the release process. The initial burst effect of protein release followed by a slower release has also been noted in the case of alginate microspheres (Liu et al 1997; Gombotz & Wee 1998).

Biological activity of the released protein from alginate fibres

A major concern regarding the incorporation of bioactive molecules is their activity upon release. In this study, ribonuclease A was used as a model protein to investigate the activity of the released protein incorporated within alginate fibres. As shown in Figure 5, it was found that the maximum conversion rate of the ribonuclease-specific substrate cytidine-2′,3′-monophospate to cytidine 3′-phosphate and normalized protein present in the medium after release correlated well with the standard curve, indicating that the released protein maintained its full biological activity.

Protein release behaviour from porous alginate fibre/PDLLA composites

The fast release of the entrapped protein within the fibrous alginates may limit their potential use for long-term tissue regeneration. Furthermore, fibrous hydrogel

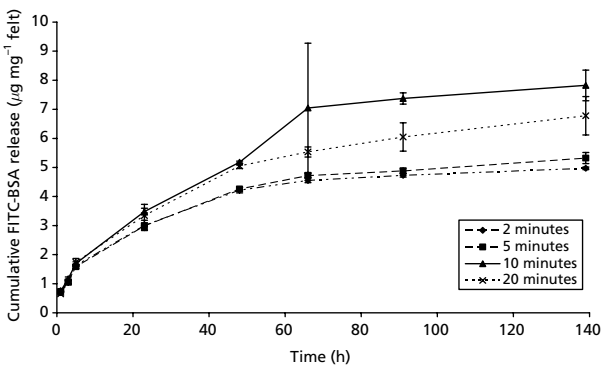

Figure 4 Effect of exposure time in fluorescein isothiocyanate-labelled bovine serum albumin (FITC-BSA) during the entrapment process on the release of FITC-BSA from alginate felts.

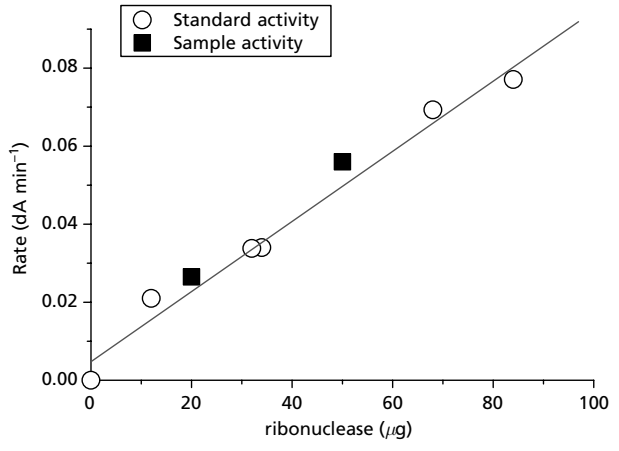

Figure 5 Evidence of retention of ribonuclease A activity. Sample activities of the released protein from alginate fibres correlate well with the calibration data.

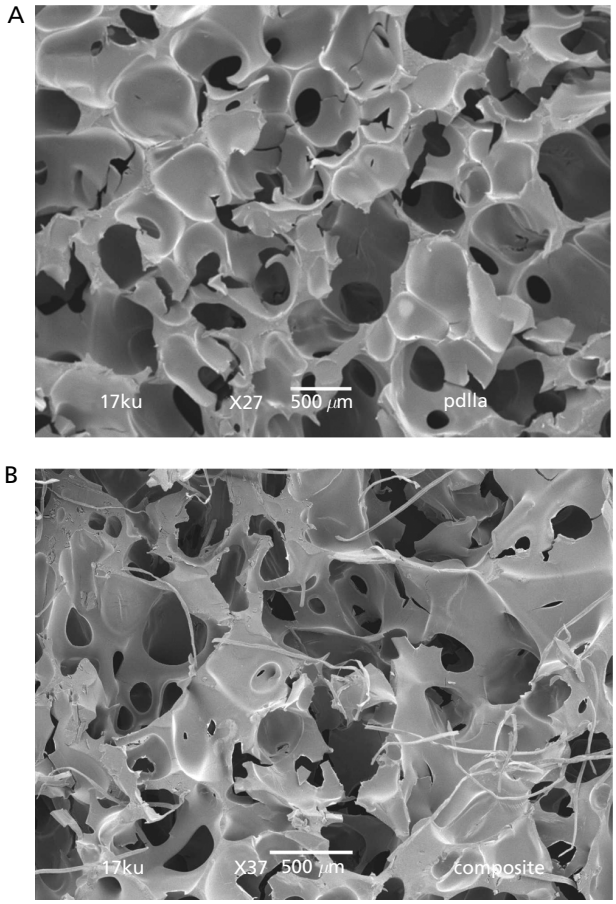

Figure 6 Scanning electron microscope images of porous poly(D,L-lactide) (A) and a poly(D,L-lactide) composite embedded with fluorescein isothiocyanate-labelled bovine serum albumin loaded alginate fibres (B).

scaffolds generally possess low mechanical strength. To further retard the release of the entrapped protein, and to improve the strength of the scaffolds, porous alginate fibre/PDLLA composites were fabricated using a supercritical fluid mixing process. Figure 6 shows scanning electron microscope images of a porous PDLLA and a porous alginate fibre/PDLLA composite prepared in this manner. It can be seen that in the porous composite, the alginate fibres were embedded within the matrix of the porous PDLLA structure. In this way, FITC-BSA was incorporated into the porous composites but still restricted within the alginate fibres. During the supercritical fluid-processing step, only the PDLLA was plasticized, while the alginate fibres maintained their intact fibrous morphology. This allows for a good control over the spatial distribution of the protein in the porous composites and thus a localized release of the incorporated proteins.

The release of the protein from the porous alginate fibre/PDLLA composites was studied in PBS at 37°C, as shown in Figure 7. After the initial burst effect, the FITC-BSA was released in a sustained manner for over 90 days. For comparison, the release profile of FITC-BSA from alginate fibres in PBS was also shown in Figure 7. The

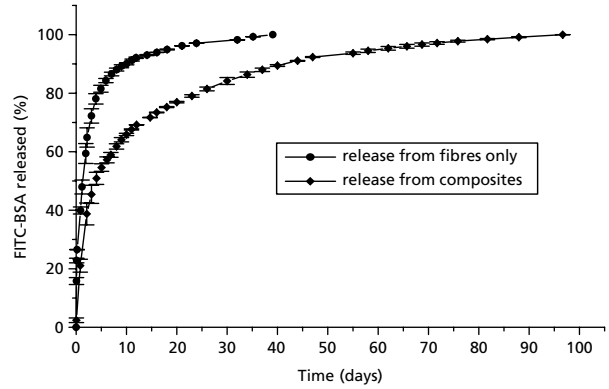

Figure 7 Comparison of fluorescein isothiocyanate-labelled bovine serum albumin (FITC-BSA) release behaviour from alginate fibres and from porous alginate fibre/poly(D,L-lactide) composites. Data are expressed as mean ± s.d., n = 2.

protein was incorporated within the fibres under the same entrapment conditions. It can be seen that the protein release from the fibres was much faster as compared with that from the alginate fibre/PDLLA composites. In the former case, about 83% of the incorporated protein was released after 5 days.

The hydrophobic nature of PDLLA contributed to the prolonged release of the entrapped protein. The release profile can be further tuned by adjusting the processing parameters of the entrapment and the supercritical fluid fabrication processes. For example, the protein concentration during entrapment, the amount and the distribution of the protein-loaded fibres within the composites, and the porosity and interconnectivity of the resulting composites may affect the protein release behaviour. This method provides a new approach to the localized and sustained release of one or more types of protein drugs from a porous polymeric composite. To our knowledge, this is the first report on the preparation of porous composites made of a synthetic polymer matrix and natural polymer fibres loaded with proteins. The mild entrapment and fabrication conditions make this approach promising for drug delivery and tissue regeneration applications.

Cell viability within alginate felts after the protein entrapment process

Alamar blue assay was performed to test cell viability of mouse 3T3 fibroblasts within alginate felts. The results during 2-day cell seeding are shown in Figure 8. It was noted that cell viability of the fibroblasts seeded within alginate felts entrapped with FITC-BSA was slightly higher than the control, indicating that the entrapment process did not affect cell viability in a negative way. There was no significant difference in cell viability between the treated felts and the controls ($P > 0.05$). These results were in line with the mild processing conditions during the whole entrapment process. Hence, the approach demonstrated here can potentially be used for

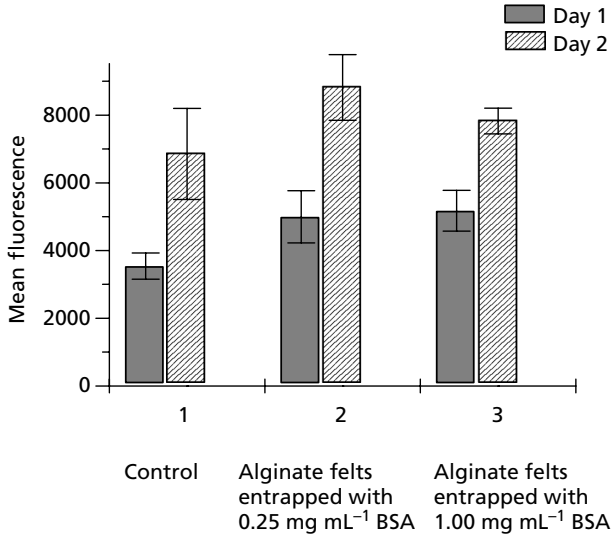

Figure 8 Cell viability of mouse 3T3 fibroblasts within alginate felts.

the incorporation and release of biologically active proteins, such as growth factors, for tissue regeneration.

Overall, the ability of the alginate fibres and felts to entrap proteins through a simple yet effective process while maintaining their fibrous morphology potentially allows for mass production of fibrous alginates with proteins incorporated. The controlled release achieved in these fibrous matrices has the potential to be used in angiogenesis and other rapid tissue formation processes that require a high loading of growth factor at the site of tissue repair. Alternatively, porous PDLLA structures embedded with protein-entrapped alginate fibres can be used when a long-term sustained protein release is required in a scaffold with higher mechanical strength. Finally, the entrapment strategy demonstrated here can also be employed for the incorporation and sequential release of multiple proteins.

Conclusions

A physical entrapment method was used for the incorporation of proteins within preformed fibrous alginates. The whole process was carried out in aqueous solutions at room temperature and no organic solvents or toxic chemicals were involved. A sustained release of the entrapped protein from the pre-formed alginate fibre-based scaffolds over a period of 6 days was achieved. By varying the processing parameters of protein entrapment, the quantity and the timing of the protein release were regulated. Protein-loaded alginate fibres were alternatively further processed together with PDLLA into porous polymer/fibre composites using supercritical fluid technology and an even more sustained release of protein for a period of up to 3 months was achieved. Moreover, the released protein maintained its full biological activity, and the entrapment process did not affect cell viability within alginate felts due to the mild processing conditions used.

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