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# Encapsulation of fibroblasts causes accelerated alginate hydrogel degradation

N.C. Hunt [a], A.M. Smith [a], U. Gbureck [b], R.M. Shelton [c], L.M. Grover [a,*]

[a] School of Chemical Engineering, University of Birmingham, Birmingham B15 2TT, UK
[b] Department of Functional Materials in Medicine and Dentistry, University of Wurzburg, Wurzburg D97070, Germany
[c] School of Dentistry, University of Birmingham, Birmingham B4 6NN, UK

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ABSTRACT

Calcium-alginate hydrogel has been widely studied as a material for cell encapsulation for tissue engineering. At present, the effect that cells have on the degradation of alginate hydrogel is largely unknown. We have shown that fibroblasts encapsulated at a density of $7.5 \times 10^5$ cells $ml^{-1}$ in both 2% and 5% w/v alginate remain viable for at least 60 days. Rheological analysis was used to study how the mechanical properties exhibited by alginate hydrogel changed during 28 days in vitro culture. Alginate degradation was shown to occur throughout the study but was greatest within the first 7 days of culture for all samples, which correlated with a sharp release of calcium ions from the construct. Fibroblasts were shown to increase the rate of degradation during the first 7 days when compared with acellular samples in both 2% and 5% w/v gels, but after 28 days both acellular and cell-encapsulating samples retained disc-shaped morphologies and gel-like spectra. The results demonstrate that although at an early stage cells influence the mechanical properties of encapsulating alginate, over a longer period of culture, the hydrogels retain sufficient mechanical integrity to exhibit gel-like properties. This allows sustained immobilization of the cells at the desired location in vivo where they can produce extracellular matrix and growth factors to expedite the healing process.



## 1. Introduction

As the mean age of the population in the developed world continues to increase, the demand for tissue grafts continues to outgrow donor tissue availability. There is therefore, an increasing need for tissue engineering (TE) to replace diseased or damaged tissue. TE often involves the culture of cells on a scaffold which plays the role of an artificial extracellular matrix (ECM). Hydrogels are scaffold materials that are commonly used for tissue engineering applications since the hydrated macromolecular structures expose the cells to a 3D environment which is similar to the natural ECM of soft tissues [1]. This is important since the 3D environment has a marked influence on cell phenotype [2] and so it is preferable that a niche is created that resembles that in vivo.

Alginate is a polysaccharide isolated from seaweed and is composed of two uronic acid salts β-D-mannuronate (M) and α-L-guluronate (G). These residues occur as homopolymeric blocks of both polymannuronate and polyguluronate and heteropolymeric sequences of both monomers. Solutions of alginate can be cross-linked by a mild gelling reaction with calcium ions to form a hydrogel. Since the mild gelling reaction is compatible with cell survival and alginate is abundant, cheap and non-toxic [3], cell encapsulation in alginate hydrogel has been investigated for a variety of tissue engineering applications, as have been recently summarized [4]. The mechanical properties of alginate gels can be tailored by controlling the molecular weight of the polymer [5], the G:M ratio [6,7], cross-linking species [8] and concentration of cross-linking cations [5]. This allows scope for the design of scaffolds with mechanical properties tailored for a specific application.

Calcium cross-links are known to dissipate from the scaffold over time as a result of exposure of the scaffold to ions such as sodium, potassium, magnesium and phosphate, which cause the scaffold to degrade both in vitro and in vivo [9,10,7]. In order that a tissue engineering approach is successful the rate of tissue growth should ideally occur at the rate of scaffold degradation [11]. It is therefore important that the scaffold remains in situ for sufficient time to support new tissue formation. Scaffold degradation results in changes in mechanical properties of hydrogel scaffolds [12]. Moreover, changes in mechanical properties have been shown to impact on the cell proliferation, cell phenotype, growth factor production, ECM deposition and tissue development [13–15]. Assessing the change in mechanical properties of scaffolds during culture is therefore important in determining the efficacy of a hydrogel as a cell-encapsulating substrate.

The mechanical properties of hydrogels can be measured in a number of ways, including compression and tensile testing [12,16]. When testing in compression, the sample's cross-sectional

* Corresponding author. Tel.: +44 121 414 3887; fax: +44 121 414 5324.
*E-mail address:* L.M.Grover@bham.ac.uk (L.M. Grover).

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area increases during the test and so it is difficult to obtain meaningful value for modulus and failure. While testing in tension gives less disputable results and gross mechanical deformation has been used previously to measure the tensile strength and modulus of alginate gels in tissue engineering [17], this method is subject to high variability which can be attributed to difficulties associated with clamping the sample.

In this study small deformation oscillatory rheology was used to measure changes in alginate hydrogel mechanical properties over time. Data collected using this technique can be analysed to precisely identify and discriminate between samples that are true gels (viscoelastic solids) or are concentrated polymer solutions which may appear gel-like, but are actually viscoelastic liquids [18]. The mechanical spectra of alginate (acellular and cell-encapsulating) samples were characterized by measurement of the storage modulus ($G'$), loss modulus ($G''$) and complex viscosity ($\eta^*$) as a function of angular frequency, to quantify the effect of culture media and cell presence on gel degradation over a period of 28 days in vitro. The changes in mechanical properties were also compared with changes in gel morphology and the release of calcium ions from the structure. Furthermore, live/dead staining was used in order to ensure that the encapsulated cells remained viable within both 2% and 5% w/v hydrogels for extended periods of time, so that they can facilitate the repair of the target tissue at the site of implantation.

## 2. Materials and methods

Unless otherwise specified, all materials were obtained from Sigma–Aldrich, Poole, UK.

### 2.1. Gel preparation

Two per cent and 5% w/v low viscosity sodium-alginate (20–40 centipoise (cps) for 2% w/v at 25 °C, Cat. No. 180947, Lot. 08620BJ, $M_W$ 102,000–209,000, M:G ratio 1.56) was autoclaved prior to dispersion of NIH 3T3 cells (LGC, Middlesex, UK) at a density of $7.5 \times 10^5$ cells ml$^{-1}$ to create cell-encapsulating samples, or used directly after autoclaving for preparation of acellular samples. The alginate/3T3 dispersion or acellular alginate hydrocolloid was pipetted into disc-shaped moulds placed on and covered with filter paper impregnated with 100 mM $CaCl_2$. The moulds were then immersed in 100 mM $CaCl_2$ and left to incubate at 37 °C for 3 h to form cross-linked hydrogel discs 20 mm in diameter and 5 mm high. In order to prepare alginate hydrogel beads of 3 ± 0.2 mm diameter the hydrocolloid with or without cells dispersed within it was added dropwise to a bath of 100 mM $CaCl_2$ and the resulting spheres were incubated in the solution for 2 h at 37 °C. Bead and disc samples were washed three times in non-supplemented Dulbecco's modified Eagle's medium or water to remove excess $CaCl_2$ (DMEM) before cultivation in either culture media or water, respectively. Disc-shaped samples were utilized for rheological evaluation and beads for ICP–MS analysis.

### 2.2. Cell culture conditions

NIH 3T3s and alginate samples were maintained in fibroblast culture media (FCM) prepared from high glucose DMEM supplemented with 10% fetal bovine serum (PAA, Somerset, UK), 1% penicillin–streptomycin, 1% Fungizone (Gibco, UK), 2.25% HEPES and 2% L-glutamine. All cultures were maintained at 37 °C with 5% $CO_2$ and 100% relative humidity and media was changed three times weekly. For encapsulation purposes NIH 3T3 fibroblasts stored in 10% DMSO in FCM under nitrogen were revived and passaged twice before trypsinization, centrifugation and resuspension in either 2% or 5% w/v alginate hydrocolloid.

### 2.3. Live/dead staining

Sections of 1 mm thickness were taken from the centre of alginate discs containing 3T3 cells after 60 days encapsulation using a razor blade. The sections were immersed in 0.2 μg ml$^{-1}$ calcein acetoxymethylester (calcein-AM) for 15 min and 2.5 μg ml$^{-1}$ propidium iodide (PI) (Invitrogen, Paisley, UK) for 5 min in supplemented DMEM at 37 °C to stain live cells green and dead cells red, respectively, when visualized using fluorescence microscopy.

### 2.4. Degradation analysis

The degradation of the alginate hydrogels was monitored by capturing images of the discs after 28 days in vitro culture (Canon Powershot GS, Surrey, UK) and by performing small deformation oscillatory rheology on the alginate hydrogel disc-shaped samples. For both 2% and 5% w/v alginate the effect of cell culture media on alginate degradation was assessed by comparing acellular samples incubated in media with acellular samples incubated in water. The effect of encapsulated cells on degradation was assessed by comparing acellular samples and cell-encapsulating samples incubated in culture media. Samples were trimmed to size where necessary using a razor blade to obtain samples 20 mm in diameter with planar surfaces. Measurements were taken in the linear viscoelastic region using parallel plate geometry 20 mm mounted on a Bohlin CVO Rheometer (Malvern Instruments, UK) fitted with Peltier plate thermal control. The plate gap corresponded to sample thickness. Measurements of storage modulus ($G'$), loss modulus ($G''$) and dynamic viscosity ($\eta^*$) were taken at frequencies from 1 to 100 rad s$^{-1}$ to ascertain mechanical spectra of the gels at an isotherm of 37 °C, at a fixed strain of 0.5%. Measurements were performed in triplicate and mean values shown at 1, 7, 14, 21 and 28 days. Comparisons of $G'$, $G''$ and $\eta^*$ were made at a frequency of 6 rad s$^{-1}$.

### 2.5. Inductively coupled plasma–mass spectroscopy (ICP–MS)

One milliliter samples of 5% w/v alginate beads were prepared and immersed in 4 ml supplemented DMEM. The samples were prepared and maintained according to the method described for encapsulated fibroblasts. Before assaying the ion concentrations the media were unchanged for 4 days. The calcium, phosphate, potassium and sodium ion concentrations in the culture medium were determined using ICP–MS (Varian, Darmstadt, Germany) against standard solutions of 50 and 100 ppm (Merck, Darmstadt, Germany). Measurements were performed in triplicate and the mean ± SD is shown at each time point.

### 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, USA).

## 3. Results

Live/dead staining of fibroblasts encapsulated in 2% and 5% w/v alginate 60 days post-encapsulation stained the viable cells green and dead cells red and indicated that >90% of the cells were viable in both 2% (Fig. 1a) and 5% w/v (Fig. 1b) alginate hydrogels. The cells were evenly distributed throughout the alginate hydrogels. After 60 days there was no obvious change in viability or cell number from day 1 (results not shown).

Rheological measurements of $G'$, $G''$ and $\eta^*$ were performed to evaluate the impact of cell encapsulation and the effect of culture