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Original Citation

Mehrban, Nazia, Smith, Alan M. and Grover, Liam M. (2009) Evaluation of iota-carrageenan as a Potential Tissue Engineering Scaffold. In: 8th World Congress of Chemical Engineering (WCCE8), August 23 to 27, 2009, Montréal, Canada.

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EVALUATION OF *IOTA*-CARRAGEENAN AS A POTENTIAL TISSUE ENGINEERING SCAFFOLD

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Abstract: The present study evaluates the use of *iota*-carrageenan as a potential tissue engineering scaffold. Cell viability and cell attachment studies were performed on 3T3 fibroblasts cultured for a period of 12 days on the surface and when encapsulated within a 2% *iota*-carrageenan hydrogel. It was found that 3T3 fibroblasts seeded onto the surface of the gel did not show signs of attachment and the proportion of live cells decreased by 33% over a period of 12 days. The proportion of live cells encapsulated within the gel remained constant and there was evidence of cell proliferation throughout the 12 day study (4-fold increase in cell number). The extent of degradation of the gel for both encapsulated and non cell-seeded forms was also evaluated and showing no significant degradation for both types. These results indicate that *iota*-carrageenan may be a suitable scaffold material for use in tissue engineering and a potential tool for studying cell proliferation in three dimensions.

Keywords: *iota*-carrageenan, encapsulation, 3T3 fibroblasts, tissue engineering.

1. INTRODUCTION

Tissue engineering (TE) offers the potential for alleviation of problems associated with an ageing population, a shortage of donor organs and immune rejection of such tissue post-transplantation. Current methods used to enhance repair and regeneration of tissues include cell therapy, growth factors, gene delivery systems and biological scaffolds (Calve et. al., 2004). Seeding a patients' own cells onto a biodegradable scaffold which is inserted into the body, following culture *in vitro*, is of particular interest in regenerative medicine.

Recent research has focussed on the use of water soluble polymers for the encapsulation of cells (Konno and Ishihara, 2007), producing a temporary non-cytotoxic scaffold closely resembling the extracellular matrix of native soft tissue. Certain hydrogels, such as PHEMA (poly(hydroxyethylmethacrylate)) are able to retain water at a concentration similar to living tissue but do not degrade easily. Having a predictable degradation rate is an important factor to account for in tissue engineering as the gel acts as a temporary support to the cells, allowing the cells to grow and proliferate, providing mechanical support whilst the scaffold dissolves and is cleared from the body. The swelling of the gels allows nutrients to diffuse into the network whilst allowing cellular waste to diffuse out of the network (Jeon et. al., 2007). The mechanical properties exhibited by gels are also affected by the surfaces to which they adhere to (Jeon et. al., 2007).

In addition to the use of synthetic hydrogel scaffolds for tissue engineering there is a growing trend towards the use of natural hydrogel scaffolds (such as fibrin, alginate and chitosan). The benefit of using natural hydrogels is the mild and simple approach to 3D cell immobilization. However, some of the gels mentioned (such as alginate) have been known to contract after cells have been incorporated (Awad et. al., 1999). The investigation of proliferation and cell expansion abilities within the scaffolds is necessary as this is a prerequisite for tissue formation.

Carrageenan is a linear sulphated polysaccharide extracted from several species of red seaweeds (*Rhodophyceae*). There are three types of carrageenan commercially available *kappa*, *iota* and *lambda* of which *kappa* and *iota* can form self supporting hydrogels (*lambda* is non-gelling). The primary structure of all types of carrageenan is based on an alternating sequence of β(1-3) linked and α(1-4) linked D-galactose residues. The 4-linked residues in *kappa* and *iota* occur mainly as the 3,6 anhydride which are required for gel formation. The idealised structure of *iota*-carrageenan has one sulphate on every residue whereas idealised *kappa*-carrageenan has one sulphate group per disaccharide repeat unit. The central process of gelation in *iota* and *kappa*-carrageenan is the formation of intermolecular double helices on cooling (Rees et al 1982) and only chain

sequences with the 4-linked residues in the anhydride form are compatible for helix formation. Sol-gel transition of both *iota* and *kappa*-carrageenan can be displaced to higher temperatures by addition of salts, for *iota* this is a result of increasing ionic strength (Picullel et al 1993), however for *kappa* there is evidence of binding with specific cations in particular K^+ which results in suppression of the electrostatic repulsion between the negatively charged carrageenan helices which enables aggregation and ultimately increased gel strength. *Iota*-carrageenan is not believed to undergo cation mediated aggregation and requires substantially higher polymer concentrations for gel formation. An interesting feature of *iota*-carrageenan hydrogels is their ability to spontaneously reform below their melting point following mechanical disruption (Bixler et al 2001) unlike *kappa*-carrageenan and alginate, which may impact on degradation rates when used as cell scaffolds.

Whilst literature is widely available on the entrapment of bacterial cells within carrageenan gels (Lopez, A. et. al., 1997 and Woodward, J., 1998) little is known on the interaction of mammalian cells with carrageenan. We investigated the response of fibroblasts to *iota*-carrageenan. Previous, unpublished experiments, show a high proportion of living cells and good attachment to the *kappa*- form. Degradation, cell attachment to the surface of the gel, cell behaviour upon encapsulation and the proliferation of encapsulated cells were studied in depth.

2. MATERIALS AND METHODS

2.1Materials

All experiments requiring sterile conditions were conducted under laminar flow. Supplemented media consisted of 10% foetal calf serum, 2.4% L-glutamine, 2.4% HEPES buffer and 1% penicillin-streptomycin (P/S). Foetal calf serum was obtained from PAA (PAA Laboratories Ltd., Somerset, UK). All other chemicals were ordered from Sigma-Aldrich (Sigma Aldrich, Poole, UK).

2.2 Methods

Surface seeding. 2% sterile *iota*-carrageenan (w/v) was prepared to form 3ml gelled discs in 12-well cell culture plates and incubated at 37°C for 20 minutes. The gelled discs were seeded with 0.50×10^6 NIH 3T3's and 3ml supplemented media. Discs were incubated at 37°C and 5% CO_2 and the media changed every 3 days.

Encapsulation. 0.75×10^6 NIH 3T3's were encapsulated in 2% sterile *iota*-carrageenan (w/v) to form 3ml gel discs (created in 12-well culture plates). To ensure cells were evenly distributed within the gel, the cell suspension was repeatedly pipetted in liquid gel. Gelled discs were incubated at 37°C for 20 minutes to ensure gelation. All discs were topped with 3ml supplemented media and incubated at 37°C and 5% CO_2. The media was changed every 3 days to ensure a fresh supply of nutrients reached the cells.

Fluorescence- live/dead assay. 1ml of gelled samples (both encapsulated and surface-seeded samples) was stained with 2µl calcein-AM and 25µl propidium iodide (PI). The stained samples were each cut to an approximate 1mm thickness (with surface-seeded samples cut horizontally) and observed under ultraviolet light using a fluorescent microscope (Carl Zeiss Ltd, Hertfordshire, UK) at magnifications of x10 and x20. Live (green) and dead (red) cells were photographed using a Canon digital camera attached to the microscope (Powershot G5, Canon UK Ltd, Surrey, UK). Five representative samples were randomly chosen from both the surface seeded and encapsulated gels to determine the proportions of live and dead cells on a daily basis.

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The proliferation of encapsulated cells was evaluated by adding 200µl MTT (10% tetrazolium MTT in phosphate buffer solution) to a 2ml gelled disc. Calibration curves were initially conducted to determine the amount of MTT needed per gelled disc and the incubation time needed to obtain the maximum dissolution of MTT using HCL-isopropanaol (1ml HCL in 24ml isopropanol). After 18 hours incubation at 37°C, 1ml HCl-isopropanol was added to the well and incubated at 37°C and 5% CO_2 for a further 75 minutes. Absorbance was measured on a daily basis, in triplicate, at 620nm (Cecil C-7500, 7000 Series spectrophotometer, Cecil Instruments Limited, Cambridge, UK). Absorbance measurements obtained from the MTT assay were used to calculate the number of metabolising cells present in each daily sample.

Degradation. Degradation of gels with and without encapsulated cells was conducted by measuring daily wet and dry weights. Dry weights were obtained by slow vacuum drying in an Edwards model EF03 freeze dryer (High Vacuum Ltd., Crawley, UK). All measurements were taken in triplicate. Gels were suspended in 2ml of supplemented media and incubated at 37°C and 5% CO_2 with media being changed every 3 days.

Statistical Analysis. Comparisons among groups were made by the unpaired t-student's test or by analysis of variance (ANOVA) followed by post hoc Tukey test. Differences were determined statistically significant with $p < 0.05$. Data are expressed as mean ± SD.

3. RESULTS

3.1 Fluorescence- live/dead assay.

Figure 1 clearly illustrates that encapsulated cells remain alive and appear to proliferate with small, thin projections showing possible attachment to the hydrogel. However, cells seeded on the surface of the gel gradually reduce in number and do not aggregate. At day 12, very few cells (alive and dead) remained on the gel surface. The majority of cells photographed when both encapsulated and surface-seeded remain green, indicating that the cells remain alive.

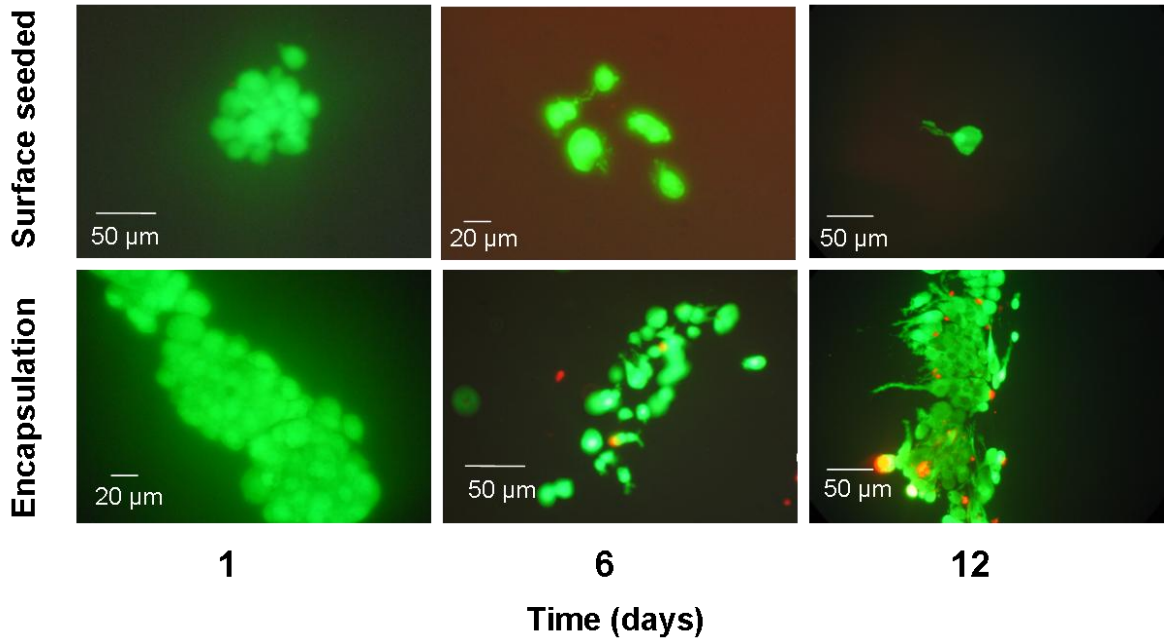

Figure 1. Fluorescence microscopy images of encapsulated and surface-seeded cells stained with calcein-AM and PI on days 1, 6, and 12 days. Live cells are stained green and dead cells red.

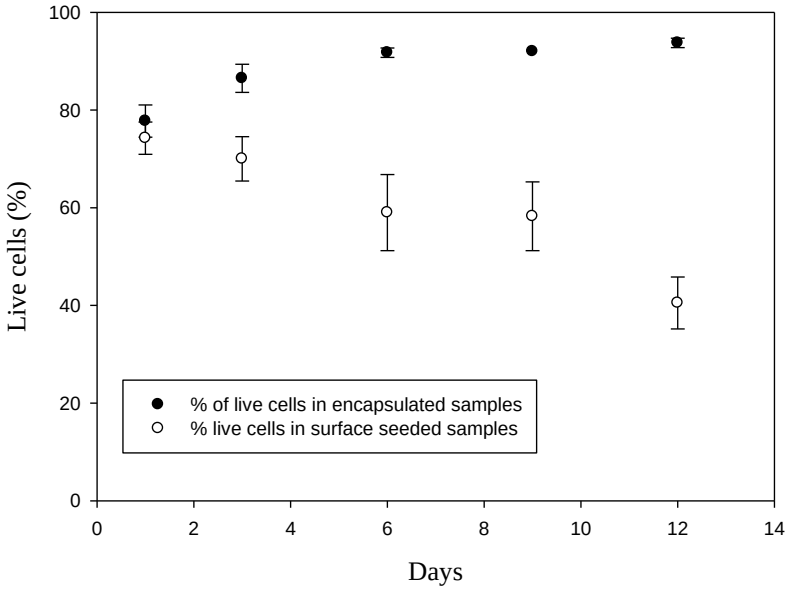

Figure 2. Percentage of live encapsulated cells as visualised using the live/ dead assay. Cell counts were conducted on days 1, 3, 6, 9 and 12.

Live cell counts shown in Figure 2 reveal that cell viability remains high (80 to 90%) when encapsulated. Indeed, a steady increase in the proportion of live encapsulated cells was witnessed (a total increase of 16%). In contrast, the proportion of live surface seeded cells showed a gradual reduction with a total loss of 33% over the 12 day study. It was also noticed that there was a dramatic reduction of the number of cells on the surface of the gel (live and dead) by day 12.

3.2 MTT cell proliferation assay.

Proliferation of cells was determined from daily absorbance readings of formazan. Figure 3 shows a four-fold increase in the daily number of encapsulated cells. The steady increase in cell number supports previous data obtained from the live/ dead assay and subsequent cell counts (Figures 1 and 2).

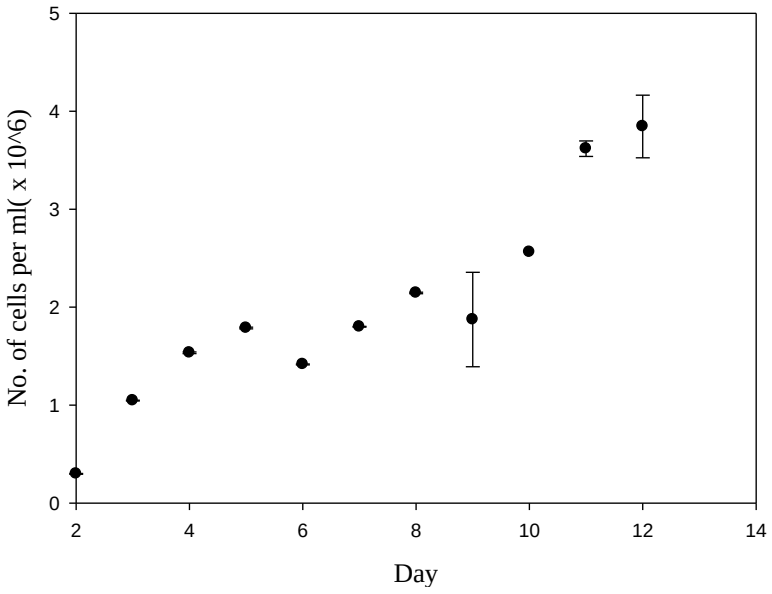

Figure. 3. Number of metabolising cells encapsulated within the carrageenan matrix following various periods of culture. As determined using the MTT assay.

3.3 Degradation

From the degradation comparison given in Figure 4 no significant difference is seen between the cell-seeded and non-seeded gels over the 12 day study and both sets of samples lost insignificant proportions of their original mass in this time.

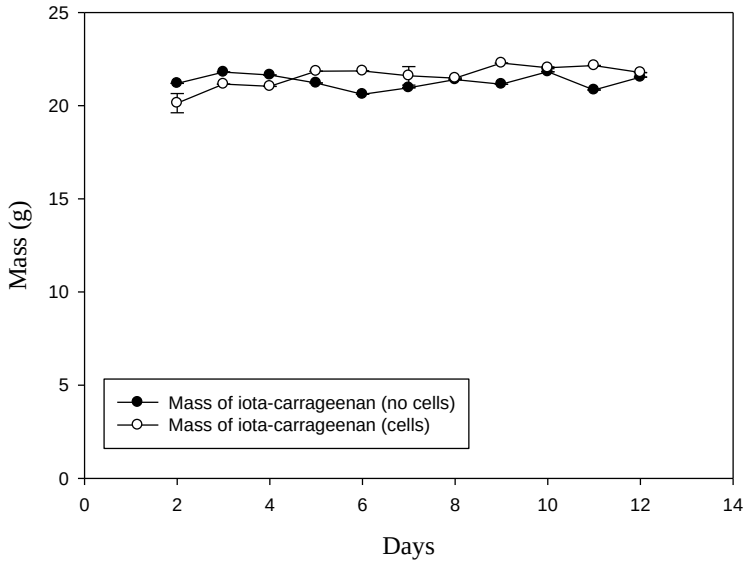

Figure 4. Mass lost from samples of *iota*-carrageenan in supplemented media over the duration of the study. Comparison of gels containing no cells to those containing cells.

4. DISCUSSION

Iota-carrageenan was chosen for investigation due to its well-known thermoreversible and thixotropic nature. We have investigated the attachment, viability and proliferation of the 3T3 fibroblasts in relation to *iota*-carrageenan gel. We have also looked at the degradation rate of *iota*-carrageenan, with and without cells.

Generally, to achieve gelation of carrageenan (*kappa* and *iota* forms), a source of cations (such as potassium) are required (as suggested by Picullel et. al., 1993), however in our system the salts present in the supplemented culture media were sufficient to cross-link the gel. Therefore, no additional cations were needed for gelation to occur offering extremely mild route to immobilising cells in 3D.

After encapsulation and surface seeding, live/dead assays (using calcein-AM and PI) were conducted on the gelled discs to investigate the viability and attachment of the cells to the gel. From the representative fluorescent images seen in Figure 1, it is immediately apparent that whilst both gels contain live cells on day 1, for the surface seeded samples these quickly diminish. At day 12, there are very few cells to be found on the surface of the gel. It is thought that weak attachment to the surface of the *iota*-carrageenan led to the cells being washed away when the media was changed. However, for the encapsulated cells, the cells appear to be proliferated. Cell counts based on the visual inspection of five fluorescent images (Figure 2), shows an increase in cell number when encapsulated and a reduction in cell counts when seeded on the surface of the gel. Although there is no significant difference between the two seeded gel types on day 1, by day 3 statistically significant ($P < 0.05$) differences are apparent.

These results suggest that after 12 days, *iota*-carrageenan allowed sufficient nutrients to diffuse to the centre of the gelled discs keeping cells alive throughout the construct whilst allowing waste products to diffuse out into the surrounding media. This may be explained by the fact that the structure of *iota*-carrageenan is not so orderly and spaces between the polymer chains caused by the swelling of the gel are sufficient in allowing the cells to grow and proliferate similar to the findings of Jeon et. al. (2007).

The increase in cell number seen in the encapsulated cells was further determined by MTT assay. Cell proliferation in encapsulated fibroblasts was evaluated through the reduction of yellow tetrazolium to purple formazan crystals. Changes in the metabolic activity of cells leads to changes in the amount of purple formazan produced (caused by mitochondrial dehydrogenases of viable cells cleaving the tetrazolium ring). The increasing optical density measured spectrophotometrically at 620nm was used in conjunction with the linear calibration curve obtained for MTT to calculate the number of viable cells in daily samples. The results, shown in Figure 3, exhibit the same general trend in cell viability and proliferation, indicated by the fluorescence images and cell counts. Cell number in encapsulated gel samples increases from 0.298×10^6 per ml to 4.297×10^6 per ml 12 days after encapsulation.

As well as a possible tissue engineering scaffold, this rapid proliferation of cells in *iota*-carrageenan makes the gel suitable for the expansion and proliferation of cells in three-dimensional (3D) conditions. This study shows that minimal work is required to obtain cells capable of producing their own extracellular matrix. Traditional two-dimensional (2D) cell culture techniques are time-consuming, requiring constant monitoring and the conditions required for their expansion are not mirrored by the conditions experienced upon implantation. In comparison to encapsulation in unmodified alginate, which is also an ionic binding gel (Constantinidis, I., 2007), cells have been shown to remain viable with little proliferation. The lack of proliferation is thought to be caused by minimal protein adsorption promoted by alginate hydrogels (Rowely, J. A., 1999).

Scaffold degradation studies shown in Figure 4 revealed no significant differences in degradation of samples containing cells in comparison to those containing no cells. This would suggest that although cells remain viable and proliferate in *iota*-carrageenan, they do not affect the degradation rate of the hydrogel and therefore it's overall structure. The reordering properties of *iota*-carrageenan gels are thought to be sufficient in allowing the cells to proliferate throughout the gel, over a 12 day of culture period. It is possible however that as the cells proliferate to a higher rate, the structure of *iota*-carrageenan will be affected. This initial degradation study further adds to the suitability of *iota*-carrageenan as a cell support, initially providing stability. Furthermore, chemical modifications may be made to *iota*-carrageenan to change its gelation behaviour and structure (Picullel et. al., 1993), which may be of use to engineer the scaffold further.

In conclusion, we have shown that although cells do not attach to *iota*-carrageenan, they remain viable and proliferate for up to 12 days when encapsulated, with no significant degradation. However, attachment of cells may be improved with the addition of cell adhesion factors. This preliminary study suggests that *iota*-carrageenan may be a suitable hydrogel for use as a scaffold for tissue engineering purposes and as a tool for examining cells as proliferation occurs.

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