A COMPARATIVE STUDY OF IOTA CARRAGEENAN, KAPPA CARRAGEENAN AND ALGINATE HYDROGELS AS TISSUE ENGINEERING SCAFFOLDS

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1 INTRODUCTION

The increasingly aging population and lack of donor tissue has pushed tissue regeneration to the forefront of modern biomedical research and biopolymers traditionally found in the food and pharmaceutical industry are increasingly being used in tissue engineering. The development of tissue engineering provides the opportunity to take a sample of cells from a patient which are then cultured *in vitro* to organise into functional tissue that can then be implanted back into the patient, ultimately, overcoming problems of tissue rejection and the need for donor tissue. To grow replacement tissue requires a suitable substrate or scaffold for cells to attach, proliferate and organise into viable tissue that can be safely implanted into the body. Biopolymers and hydrogel forming biopolymers in particular, have been shown to be a promising scaffold material due having properties that resemble the environment of the mammalian extracellular matrix (ECM). In addition properties such as mild gelation conditions, potential for good mass transport of nutrients and waste molecules, nontoxic nature and biological compatibility provide an advantage over synthetic polymers and ceramic and metallic materials which have also been used as tissue engineering scaffolds.

As well as supporting cell growth and proliferation, the scaffold must have the mechanical characteristics needed to retain its structure for as long as it is needed [1]. Ideally gels used as scaffolds for tissue engineering would have a mild sol-gel reaction that can be controlled at 37°C and at physiological pH, have rapid gelation kinetics and be robust enough for 3D culture and implantation. This explains why much of the research on biopolymers for tissue engineering has been performed on materials such as alginate[2] and chitosan[3] collagen[4] agarose[5] and fibrin[6]. The relative success of the cell culture of the mentioned biopolymers has generated interest in other gel forming biopolymers such as gellan gum[7] and chemically modified gels such as RGD alginate[8]. Performance of the cells when encapsulated in these biopolymers varies due to the cellular interaction with the polymer chemistry and gel network structure.

In this study we have investigated the use of carrageenan as a 3D tissue engineering scaffold for mammalian cells and looked at the impact of structural differences between kappa and iota carrageenan has on the attachment, survival and proliferation of fibroblasts and in comparison with alginate which, is widely used in tissue regeneration. Carrageenan has been used in bacterial culture[9] and has been evaluated as a delivery vehicle for growth factors for mammalian tissue engineering applications[10] however there have been no reports of carrageenan being used exclusively for the 3D culture of mammalian cells.

2 METHODS AND RESULTS

Unless otherwise specified all reagents were obtained form Sigma Aldrich (Poole, UK).

2.1. Formation of hydrogel scaffolds for encapsulation of fibroblasts

NIH 3T3 murine fibroblasts were added to 2% (w/v) Na-alginate at a density of 0.75 x 10^6 cells/ml. The cell-gel suspension was then added drop wise into a bath of 100mM $CaCl_2$ and left to incubate at 37ºC for 2h to form cross-linked spheres of 3.0±0.2 mm diameter.

1% *kappa* and 2% *Iota* carrageenan discs were formed using 3ml autoclaved gel which was cooled to 40°C before a 0.75 x 10^6 NIH 3T3 murine fibroblast cell suspension was added and pippetted repeatedly to ensure homogenisation. No extra cations were added to the gel solution as there were sufficient salts present within the supplemented media added to cell suspensions to cause the gel to cross-link.

All samples were cultured in high glucose Dulbecco's Modified Eagle's Media (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 and maintained at 37ºC with 5% CO_2 and 100% relative humidity and media was changed three times weekly. Control samples without cell seeding were also prepared for degradation studies.

2.2 Formation of hydrogel scaffolds for surface seeding

Alginate discs were prepared using sterile 2% low viscosity Na-alginate. The discs were formed by covering 3ml Na-alginate with 100 mM $CaCl_2$ for 2h in 6-well tissue culture plates. Following crosslinking, NIH 3T3 murine fibroblasts were seeded on the surface of the alginate at a density of 0.44×10^6 cells/ml

Iota and *Kappa* carrageenan gel cylinders were formed using sterile 2% (w/v) *iota*-carrageenan and 1% (w/v) *kappa* carrageenan in 12-well culture plates containing 3ml of the gel solution per well before addition of 1 ml 200 mM KCl. The cross-linked *kappa* and *iota*-carrageenan discs were then seeded at a cell density of 0.75×10^6. The gel discs were topped with supplemented DMEM and maintained at 37ºC with 5% CO_2 and 100% relative humidity.

2.3 Analysis of cell morphology and survival

Following 10 days in culture, samples of the gels were removed and the cells were analysed using a Live/Dead® Viability/Cytotoxicity Kit. This procedure, based on the uptake of calcein AM and conversion to fluorescent calcein (green) by intracellular esterases in live cells and the uptake of propidium iodide (red) by cells with damaged plasma membranes, was used in conjunction with fluorescence microscopy to assess cellular responses to the alginate and carrageenan scaffolds. The results for the encapsulated samples showed that >95% of cells remained viable in alginate. The rounded morphology, however, also indicate that they were not well attached to the gel (Fig. 1A). Similarly cells encapsulated in *kappa*-carrageenan are rounded in appearance on day 10 (Fig 1B) with > 95% of the cells remaining live. Samples of *iota*-carrageenan (Fig. 1C) also had a >95% survival, however, the appearance of thin projections and overall elongation of the cells suggests attachment of the cells to the hydrogel. It is hypothesised that the lack of ionically bound junction zones within the structure of iota-carrageenan gels

provides spaces between the polymer chains that are sufficient in allowing the cells to grow and proliferate similar to the findings of Jeon et. al. (2007)[11]. For surface seeded samples only a few cells were seen to be attached to alginate and *iota*-carrageenan after 10 days (Fig. 2A and 2B). These cells were shown to be alive, but with a rounded morphology without cytoplasmic projections indicating that the cells were poorly attached to the surface of the gel. Although the cells in alginate exhibit similar behaviour to kappa carrageenan when they are encapsulated, there is a marked reduction in cell number (<50%) and overall lack of attachment to the surface of the alginate samples. This poor attachment was similar to reported found by Harris et al (2007) who used gellan gum for surface seeding. The authors hypothesised that poor attachment was due to poor cell traction which prevented the cells from adhering to the surface of the material and exerting forces sufficient to allow migration, which may be the case for alginate and kappa carrageenan. Indeed, it has been shown that modifying alginate with the tri-peptide RGD dramatically improves cell attachment[8]. For *iota*-carrageenan, however, the cells appear to be attached to the surface of the gel although this cannot be truly confirmed without performing assaying for the presence of focal contacts or adhesion proteins.

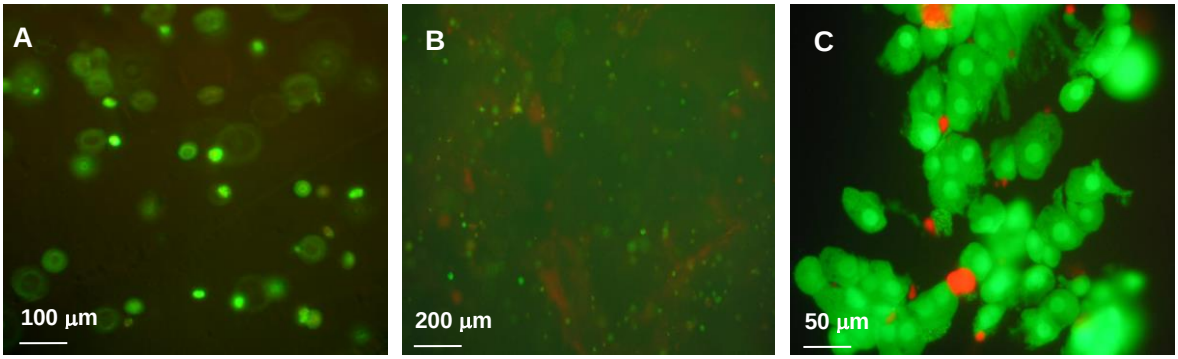

Fig. 1. 3T3 Fibroblasts stained with calcein-AM and PI 10 days post encapsulation in alginate (A), *kappa*-carrageenan (B) and *iota*-carrageenan (C).

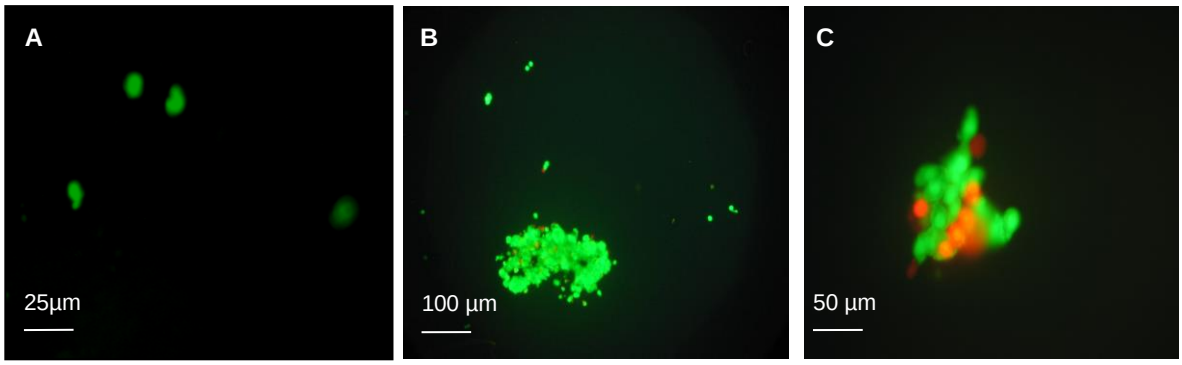

Fig. 2. 3T3 Fibroblasts stained with calcein-AM and PI 10 days after application to the surface of alginate (A), *kappa* -carrageenan (B) and *iota*-carrageenan (C).

2.2 MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay

To identify and quantify any cell proliferation within the gels an MTT assay was performed, whereby the tetrazolium ring of the MTT is cleaved by the mitochondrial dehydrogenases in living cells, producing purple formazan crystals. Dissolving these crystals and measuring the absorbance is an indication of the cell population's metabolic activity. This assay was applied to the cultures by adding 200 µl MTT to a 2 ml gelled sample. The absorbance readings (at 620 nm) obtained from the MTT assay over 12 days were used to determine the number of viable cells present in each daily sample. Figure 3 shows a comparison of the calculated cell number for all three gels. It can be seen that whilst there was a significant difference between the cell counts of all three gels ($P < 0.05$) there appeared to be no proliferation in the number of cells encapsulated in alginate. Cells encapsulated in *iota*-carrageenan show a gradual increase in the cell number over 5 days. This may be explained by the fact that iota-carrageenen gels have the ability to spontaneously reform below their melting point following mechanical disruption[12] allowing cells to migrate throughout the gel without disrupting the network, unlike in kappa-carrageenan and alginate where the cells are confined within a more tightly bound gel network. After 5 days in culture, this number reduced (from 4.9×10^6 to 1.1×10^6 by day 12). Proliferation was also seen in *kappa*-carrageenan, however, not at the same rate as in *iota*-carrageenan and it was not until day 10 that the cells in kappa carrageenan were of comparable number to those in the iota-carrageenan.

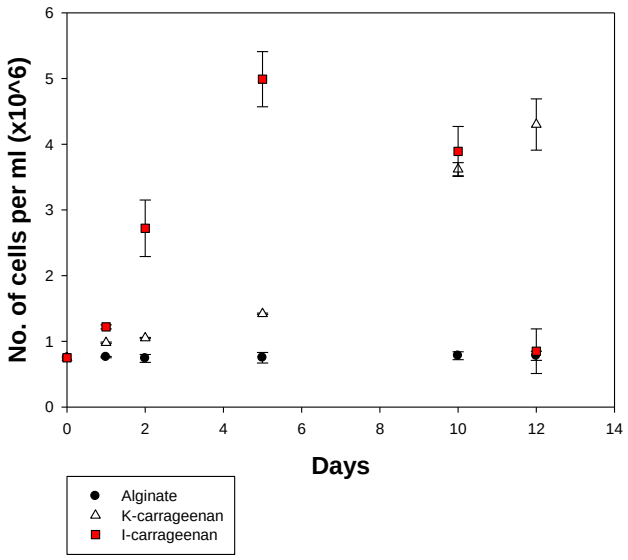

Fig. 3: Number of viable cells calculated from the absorbance of formazan of the encapsulated cells. Vertical error bars represent the standard deviation of the reported mean values (n=3).

2.4 Degradation

The changes in mass of gels with and without cells was determined by measuring the dry mass of the samples (by slow vacuum drying) on a daily basis in a vacuum freeze-drier (Edwards, EF03, Sussex, UK) for 16h. This was to quantify any fluctuations in mass

within the samples which may occur as a consequence of polymer degradation, protein absorption from the culture media or formation of extracellular matrix.

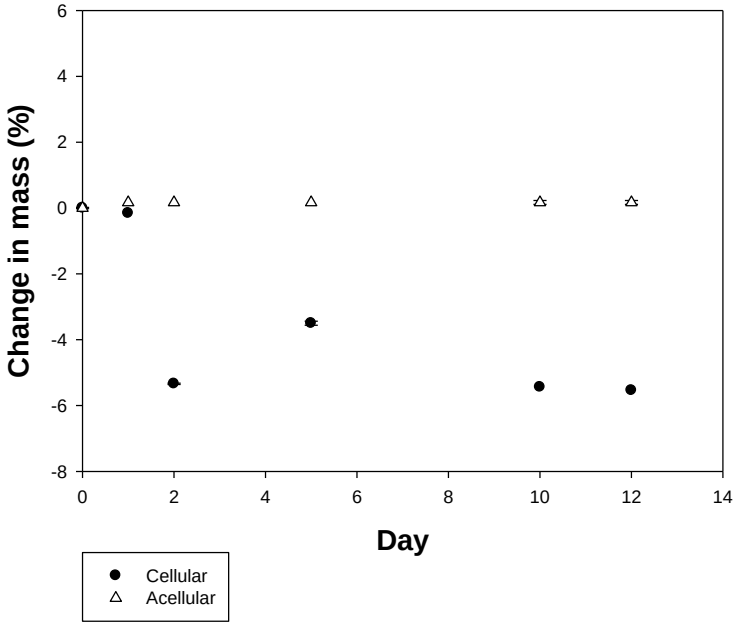

Fig. 4: Changes in the mass for *Kappa*-carrageenan with and without cells. Vertical error bars represent the standard deviation of the reported mean values (n=3).

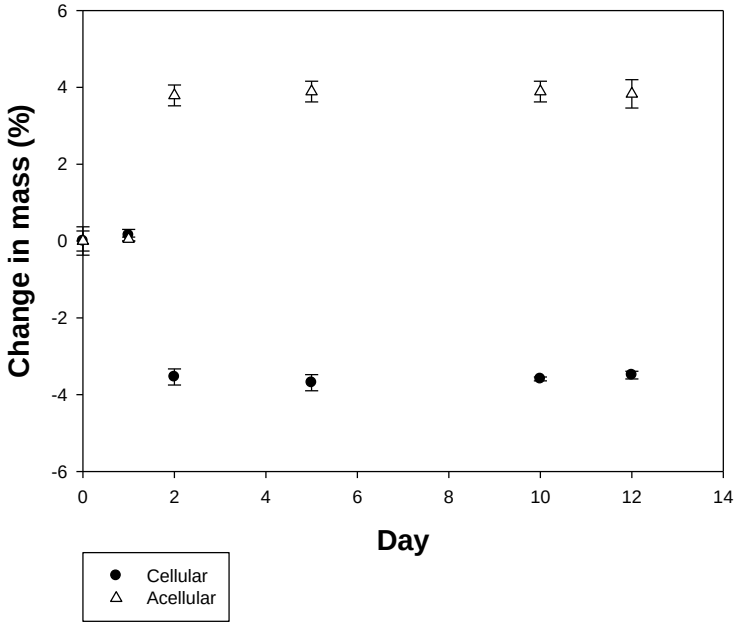

Fig. 5: Changes in the mass for *Iota*-carrageenan with and without cells. Vertical error bars represent the standard deviation of the reported mean values (n=3).

The degradation rates of the three gels were found to vary considerably, with no statistical difference seen between the cellular and acellular samples of alginate ($P > 0.05$) (results not shown). The acellular samples of *kappa*-carrageenan remained unchanged for the duration of the study, however, the addition of cells within the gel caused a gradual loss in total mass suggesting degradation of the polymer. The gel mass of acellular samples of *iota*-carrageenan increased by a total of 3.9% 2 days post encapsulation indicating the possibility of protein absorption from the culture media to the carrageenan, enabling cell attachment. This supports the findings from the proliferation assay and the observations of cell attachment. The *iota*-carrageenan containing cells showed a 3.5% reduction in mass which, is thought to be due to degradation of the gel which was visually apparent when samples were being transferred to the freeze dryer for measurements. The kappa carrageenan samples where observed to be more robust than the iota-carrageenan however the gel integrity of both types did reduce through the duration of the study. Kappa-carrageenan was initially observed to be a stronger gel in comparison with iota-carrageenan and interestingly, these physical properties may be the cause of initial cell proliferation observed in iota-carrageenan which were not seen in the kappa form. Eventual dissolution of iota-carrageenan lead to difficulty in quantifying the proliferation of encapsulated cells within the polymer at day 12 which accounts for the reduction in cellular proliferation measured using the MTT assay. The gel network of the kappa-carrageenan samples also began to lose its integrity, however, this was not apparent until day 10 of the study which correlates with a dramatic increase in the proliferation of encapsulated cells. This is in contrast to the alginate samples which appeared unchanged throughout. This demonstrates the necessity of investigating the mechanical properties of hydrogel scaffolds in relation to cellular behaviour.

3.0 CONCLUSION

In conclusion we have shown that cells can be encapsulated and remain viable in both kappa and iota carrageenan for up to 12 days which is comparable with alginate which is a widely used tissue engineering substrate. There was no evidence of attachment of cells to the alginate or the kappa carrageenan however some attachment was evident when using iota carrageenan as the scaffold for both encapsulated cells and surface seeded cells. It was also shown that proliferation of the cells within the scaffolds occurs at different rates possibly due to the mechanical properties and network architecture of the gels. Fluctuations in mass were also measured in the scaffolds which correlated with changes in cellular proliferation.

Subsequently, this study has highlighted the need for investigations into the mechanical properties of hydrogels when applied as cell scaffolds. The potential for utilising biopolymers and mixtures of biopolymers traditionally used in the food and pharmaceutical industry for this purpose is apparent as the subtle differences in gel architecture can have a dramatic effect on cell culture. This preliminary study suggests that both kappa and iota-carrageenan may be suitable hydrogels for use as a scaffold for tissue engineering purposes and as a tool for examining cells as proliferation occurs. Iota-carrageenan in particular, may have applications as a cell delivery vehicle due to the initial proliferation and subsequent dissolution. Furthermore, chemical modifications, and the development of polymer blends to change the structure and gelation behaviour may be of use to tailor the scaffolds further.

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