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EFFECT OF ULTRASOUND ON PRODUCTION OF EXTRACELLULAR MATRIX BY CELLS IN CULTURE

by

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ABSTRACT

The thesis starts with characterization of alginate that involved the viscosity, ageing, and degradation of different concentrations of alginate. Live/Dead, MTT, and Hoechst 33258 assays were used to investigate cell viability and proliferation in alginate discs. Pulsed-low intensity ultrasound (PLIUS) treatment was applied to cells encapsulated in alginate to see if it has an effect on viability/proliferation of encapsulated cells (Hoechst 33258 assay), extracellular matrix (ECM) deposition (Alcian blue staining, hydroxyproline and glycosaminoglycan (GaG) assay) and cell morphology (scanning electron microscopy (SEM)). Study of cell behaviour in alginate discs showed that by altering both alginate concentration and cell seeding density, cell proliferation in alginate can be controlled. PLIUS in conjunction with growth factors (ascorbic acid and TGF-β1) leads to a higher amount of collagen production for 3T3 cells when compared to PLIUS alone. However, PLIUS exposure alone leads to a greater increase in HDF cell proliferation and collagen production than when supplemented with growth factors. No effect was seen on chondrocytes when PLIUS was applied. SEM images showed no damage to cells as a result of PLIUS exposure. These results indicate that PLIUS treatment of cells encapsulated in alginate showed a potential effect for enhancing ECM production by cells in culture.

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Glossary

2D	Two dimension
3D	Three dimension
Ca^{2+}	Calcium ion
$CaCl_2$	Calcium chloride
CO_2	Carbon dioxide
cP	Centipoise (1 cP = 0.001 Pa.s)
DMB	1,9 dimetylmetylene blue dye
DMEM	Dulbecco's modified Eagle's medium
ECM	Extracellular matrix
g/l	gram/litre
GaG	Glycosaminoglycan
HCl	Hydrochloric acid
HDF	Human dermal fibroblast
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl] ethanesulfonic acid
$MgCl_2$	Magnesium chloride
mM	Milimolar

MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
M_w	Molecular mass (mass ratio of one molecule of a substances to 1/12 of the atom carbon-12)
Na^+	Sodium ion
Pa.s	SI unit of viscosity
PBS	Phosphate-buffered saline
P-DMBA	p-dimethylaminobenzaldehyde
PEG	Polyethylene glycol
PLIUS	Pulsed-low intensity ultrasound
RGD	Sequence of the three amino acids: arginine, glycine, aspartic acid
SEM	Scanning electron microscopy
TGF-β	Transforming growth factor
v/v%	Percentage volume of solute divided by volume of solvent
wt/vol	Mass of solute divided by the volume of solvent.

TABLE OF CONTENTS

1. INTRODUCTION

In this study, focus will be on therapeutic ultrasound or specifically pulsed-low intensity ultrasound (PLIUS) as a potential physical method for stimulation of enhanced extracellular matrix (ECM) production by encapsulated cells in culture. In tissue engineering, many studies have used biochemical stimulation (growth factors) for promoting ECM production by cells in culture [Bittencourt *et al.,* 2009, Chojkier *et al.,* 1989, Clark *et al.,* 1995, Kim *et al.,* 2009, Neidert *et al.,* 2002, Park *et al.,* 2009, Ross and Tranquillo 2003]. However, physical stimulation offers several advantages such as providing a non-invasive treatment and lack of toxicity to living cells [Doan *et al.,* 1999, Iwashina *et al.,* 2006]; also it can be used alone or in conjunction with biochemical stimulation [Miyamoto *et al.,* 2005].

This thesis will investigate the suitability of alginate as a medium for cell viability/proliferation, and of PLIUS as a method for physical stimulation of ECM production by the encapsulated cells. Chapter 2 will introduce alginate as a scaffold, therapeutic ultrasound, and at the same time provide some other basic background information that is necessary for understanding this thesis. Further detailed information will be given at the beginning of each chapter. The experimental work will be divided into five chapters, as described below.

Investigating alginate properties: this chapter will focus on the rheology of alginate solutions subsequently used for cell encapsulation. It will cover the viscosity, ageing and degradation of different concentrations of alginate. This study aimed to determine whether the

concentration, ageing and the degradation of alginate could have an influence on the encapsulated cells.

Viability and proliferation of 3T3 cells: this chapter presents a detailed study of the influence of different alginate concentrations with different seeding densities on cell viability/proliferation, and time of spheroid formation occuring in culture. These variables are important for understanding cell behaviour in alginate discs. 3T3 cells are mouse fibroblasts and fibroblasts are responsible for making ECM. In this preliminary study, 3T3 cells were used to see if alginate is a good material for cell encapsulation. This is important before further study can be carried out to investigate ECM production by cells in culture.

PLIUS effect on 3T3 cells: PLIUS use intensities low enough to avoid promoting thermal effects on the cells. This is essential as high temperatures could kill the treated cells. Many previous studies reported that PLIUS showed a positive effect on cell proliferation [Doan *et al.,* 1999, Iwashina *et al.,* 2006] and increasing ECM production of treated cells [Iwashina *et al.,* 2006, Miyamoto *et al.,* 2005, Schumann *et al.,* 2006, Zhang *et al.,* 2003] . However, these previous studies used different frequencies, intensities and duty cycles for PLIUS than in the present study. Furthermore, there are no previous studies on PLIUS treatment of 3T3 cells cultured in alginate. For this reason, this study was carried on to investigate effect of PLIUS on 3T3 mouse fibroblasts

PLIUS effect on human dermal fibroblast (HDF) cells: this chapter investigates the effect of PLIUS on human dermal fibroblasts (HDF), as a continuation of the study of 3T3 mouse fibroblasts. This is essential to see if there is any difference to the observations/findings when

using different fibroblasts from a different species. If this study with HDF cells shows a promising effect, it would demonstrate potential for the application of tissue engineering to treat some human health problems, such as sun-damaged-skin and diabetes mellitus.

PLIUS effect on chondrocytes: the chondrocytes were originally from calf knee cartilage and are reported to require a 3D culture system in order to maintain their morphology and increased their ECM production [Bittencourt *et al.,* 2009]. The study of chondrocytes follows naturally from the work on fibroblasts where alginate was used as a 3D scaffold material. Furthermore, the effect of PLIUS on chondrocytes in 3D culture was investigated. The results could be beneficial for articular cartilage repair after damage by disease or trauma.

In the final chapter of the thesis, the overall conclusions will be described. These concern the effect of different alginate concentrations, different seeding densities, and PLIUS treatment (with or without growth factors) on several cells types, that produce ECM, in 3D culture in alginate gels.

2. BACKGROUND TO THE STUDY

2.1 Tissue engineering

The purpose of tissue engineering is to use living cells to produce replacements for tissue lost as a result of trauma or disease [Malafaya *et al.,* 2007, Marler *et al.,* 1998, Neidert *et al.,* 2002, Saltzman 2004]. Surgical techniques for dealing with tissue loss such as organ transplantation, autografts (tissue transfer from a healthy site to an affected site in the same individual) and replacement of tissue with mechanical devices have been developed [Marler *et al.,* 1998]. While these methods are very useful, they have limitations. For example, organ transplantation may be restricted by a shortage of donors, tissues transferred within an individual may be defective and transferring tissues to a different individual could lead to rejection [Eisenbarth 2007, Langer and Vacanti 1993, Marler *et al.,* 1998].

Mechanical devices also have problems: they may wear out in use and are not repaired by biological mechanisms [Marler *et al.,* 1998], they may induce inflammation and/or infection and may necessitate further treatment e.g. administration of anti-coagulants following heart-valve replacement [Marler *et al.,* 1998]. Additionally, these mechanical devices do not grow as the body of the recipient grows [Marler *et al.,* 1998]; this is an important consideration when repairing some tissues in children. Three principle strategies have been explored for creating new tissues: the injection of isolated cells or cultured cells to the damaged area, implantation of tissue that has been assembled *in vitro* from cells cultured in scaffolds and *in situ* tissue regeneration [Griffith and Naughton 2002, Marler *et al.,* 1998]. For cellular implantation, individual cells/small aggregates of cells from the patient or donor are injected

to the lesion area. For tissue implantation, a 3D culture system (cells and scaffold) is developed *in vitro* and implanted to the patient once the tissue reaches maturity. For *in situ* implantation, a scaffold is implanted directly in the damaged tissue and the body will stimulate its own cells to promote the repair [Griffith and Naughton 2002].

2.2 We want our cells to make ECM

2.2.1 ECM

ECM is an extracellular product secreted by cells that gives support and strength to the cells to perform their important roles in living systems [Badylak *et al.,* 2008, Hukins *et al.,* 1995]. ECM consist a mixture of components such as collagens, glycoproteins and glycosaminoglycan (GaG) [Badylak *et al.,* 2008, Harbers and Grainger 2006, Hukins *et al.,* 1995].

Since that the composition of ECM depends on the cells' location in the body, different tissues (like tendon, and cartilage) have different physical properties because they have different proportions of macromolecular components [Hukins *et al.,* 1995]. Additionally, cell-matrix interactions are important for the function of the tissue in living systems [Harbers and Grainger 2006, Hukins *et al.,* 1995]. For example, fibroblasts in tendon produce a high proportion of fibrillar collagens to provide tensile strength to the tissue. In contrast, cartilage has a high GaG concentration to bind water and so resist compression of the tissue [Hukins *et al.,* 1995].

2.2.2 Proteoglycan matrix components

GaGs are polymers that are composed of repeating sugar units. GaGs (except for hyaluronic acid) are covalently bound to proteins in ECM, the resulting molecules are called

proteoglycans [Hukins *et al.,* 1995, Ratner and Bryant 2004]. GaGs can be divided into four major group depending on their chemical composition: keratan sulfates, heparin sulfates and heparin, chondroitin sulfates and dermatan sulfates and hyaluronic acid [Hukins *et al.,* 1995, Ratner and Bryant 2004]. GaGs are negatively charged and so bind to water [Harbers and Grainger 2006, Saltzman 2004] with the result that cartilage, for example, consists of about 80% water [Hukins *et al.,* 1995]. When tissues are dehydrated their properties change dramatically; therefore, the water content of the tissue is important for maintaining its physical properties and functions [Hukins *et al.,* 1995].

As mentioned before, different tissue locations will have different compositions of ECM. Therefore, different types of GaG exist in the ECM of different tissues [Hukins *et al.,* 1995]. For example, chondroitin sulfates occur in skin for scarless wound repair in fetal [Adzick and Lorenz 1994, Hukins *et al.,* 1995]and cartilage and dermatan sulfates occurs in skin and tendon [Hukins *et al.,* 1995].

2.2.3 Non-proteoglycan matrix components

Components other than proteoglycans and hyaluronic acid include collagen and fibronectin. These non-proteoglycan matrix components have been widely used to encourage cell grow and differentiation *in vitro* and *in vivo* [Badylak 2007].

The most abundant protein in the body, and the main component (other than water) in the ECM of many tissues (e.g. tendon skin and ligaments) is collagen; it is secreted by fibroblasts, chondrocytes, tenocytes and endothelial cells [Doroski *et al.,* 2007, Saltzman 2004]. Collagen consists of a polypeptide chain with a repeating amino-acid sequence glycine-X-Y, where X is often proline and Y is often hydroxyproline [Badylak *et al.,* 2008, Hukins *et al.,* 1995]. The

most common types are I, II and III that form fibrils (i.e fibrillar collagen) that can be observed by electron microscopy [Doroski *et al.,* 2007, Hukins *et al.,* 1995]. An important characteristic of collagen is the ability to provide strength by resisting forces that tend to pull the tissue apart [Doroski *et al.,* 2007, Hukins and Aspden 1985, Hukins *et al.,* 1995]. In tissue engineering, collagen has been used as a scaffold since that it is from a natural source. Cells can grow/proliferate properly, because of their interactions with the collagen scaffold [Ungaro *et al.,* 2005]. There are many different types of collagen in the body that have different chemical compositions; the main types of collagen that form fibres to reinforce ECM are type I (in tendon) and type II (in many forms of cartilage, such as in finger, elbow and ankle joints) [Hukins *et al.,* 1995].

Hyaluronic acid is also known as hyaluronan or hyaluronate. It is an important GaG component of many connective tissues, especially soft or fluid tissues like synovial fluid [Drury and Mooney 2003, Mano *et al.,* 2007]. However because it is not bound to protein to form a proteoglycan, it differs from the other GaGs; also, unlike the others, it does not have sulfate groups [Saltzman 2004]. Hyaluronic acid is important for wound healing, embryonic development, transporting metabolites and nutrients, cell migration and cell proliferation [Lee and Mooney 2001, Saltzman 2004, Ungaro *et al.,* 2005]. In tissue engineering applications, it has shown to have excellent potential for artificial skin, wound healing and soft tissue augmentation [Lee and Mooney 2001]. However, it requires thorough purification to remove all of the impurities including toxins that could transmit diseases and cause immune response problems.

Fibronectin is a glycoprotein that mediates cell-ECM interactions [Hukins *et al.,* 1995, Minuth *et al.,* 2005]. The binding of fibronectin to collagen and heparin contributes to the organization of ECM and also affects cell morphology, migration and differentiation [Mano *et al.,* 2007, Saltzman 2004, Ungaro *et al.,* 2005]. Previous reports also mentioned that fibronectin plays an important role in cell adhesion by interacting with cells to promote cell attachment to ECM [Ruoslahti 1988]. This ability comes from a sequence of three amino acids, arginine-glycine-aspartic acid (abbreviated to RGD) that interacts with the cell surface [Badylak *et al.,* 2008, Hukins *et al.,* 1995].

2.3 Ideal scaffold material

Generally, materials that are implanted into the body cause an immune response because they do not exist there naturally [Jones 2005]. Many of them are toxic; however, there are also biocompatible materials that are suitable to be implanted in the body [Jones 2005].

Ideally, a scaffold should acts as a template for the cells to grow on and then, later, regenerate new tissue that can function well when implanted to the lesion area of the patient [Jones 2005]. The scaffold material should be non-toxic, biocompatible, and easy to sterilize; in addition, it should allow cellular interactions, and resemble mechanically the surrounding tissue [Eisenbarth 2007, Kim *et al.,* 2000, Mano *et al.,* 2007].

2.4 Natural polymer as a scaffolds

2.4.1 Alginate

Calcium alginate forms a hydrogel that has been widely used in tissue engineering due to its structural similarity to the GaGs in ECM, low toxicity, cost and spontaneous or mild gelation conditions that have no deleterious effect on the growth of a range of cell types [Khattak *et*

al., 2006, Wan *et al.,* 2008]. Alginate is abundant in nature and can be found in marine brown algae and some soil bacteria [Simpson *et al.,* 2004]. Sodium alginate solution turns into a hydrogel when binds with divalent cations such as Ca^{2+}, resulting in a three dimensional network described by the "egg-box model" (Figure 2.1) [Braccini and Perez 2001, Grant *et al.,* 1973, Simpson *et al.,* 2004]. Furthermore, the physical properties of alginate also vary widely depending on its molecular weight [Draget *et al.,* 1994] and proportion of guluronic and mannuronic acid residues; these are the two sugar residues that are repeated in the polysaccharide chain and guluronic acid is responsible for Ca^{2+} ions binding in the egg-box model [Simpson *et al.,* 2004]. For example, by changing the Ca^{2+} ions concentration the strength of the gel network of alginates with a high guluronic acid content can be altered but alginates with high in mannuronic acid content are less sensitive to such changes [Simpson *et al.,* 2004, Wan *et al.,* 2008]. By doing this, the growth of encapsulated cells can be controlled [Simpson *et al.,* 2004].

However, the disadvantages of ionically crosslinked alginate hydrogels is that they undergo slow, uncontrolled dissolution [Drury and Mooney 2003, Lee and Mooney 2001]. When placed in media, monovalent ions such as Na^{+} ions can also compete with Ca^{2+} ions and so disrupt the gel over time [LeRoux *et al.,* 1999, Segeren *et al.,* 1975, Wan *et al.,* 2008]. To overcome this limitation, barium and copper-crosslinked alginate gels are more stable in aqueous environment but these cations are toxic to cells [Kuo and Ma 2008].

Another potential limitation in using alginate gels in tissue engineering is the lack of cellular interaction since alginate is known to discourage protein adsorption and is unable to specifically interact with mammalian cells [Lee and Mooney 2001, Rowley *et al.,* 1999, Shih-

Feng *et al.,* 2010, Smetana 1993]. Furthermore, previous studies also reported that viability or proliferation of the cells is limited due to lack of adhesion molecules available within the alginate matrix for the cells to adhere to [Shih-Feng *et al.,* 2010]. Those cells that seek attachment will attach to one another to form cell spheroids/agglomerates as proliferation occurs [Bartold and Page 1985, Glicklis *et al.,* 2004]. To overcome this, there are varieties of short amino acid sequences found in the ECM molecules that can mediate cell adhesion. As mentioned in section 2.2.3, the fibronectin-derived adhesion peptide (RGD) is commonly coupled with the alginate and it has been shown that muscle cells adhere and proliferate when in contact with RGD-modified alginate [Augst *et al.,* 2006].

This study shows that RGD-modified alginate contributes to cell proliferation and attachment but RGD itself is very expensive and so does not provide a practical route for large scale production in tissue engineering. Alternatively, growth factor tethering and incorporation are other avenues by which hydrogels can be modified to regulate the functions of interacting cells [Mann *et al.,* 2001]. However, there is no literature cited in this thesis, discussing that lack of cell adhesion could also have a bad impact on the ECM production. Consequently, it is worth trying unmodified alginate to encapsulate cells and induce them to produce ECM in culture.

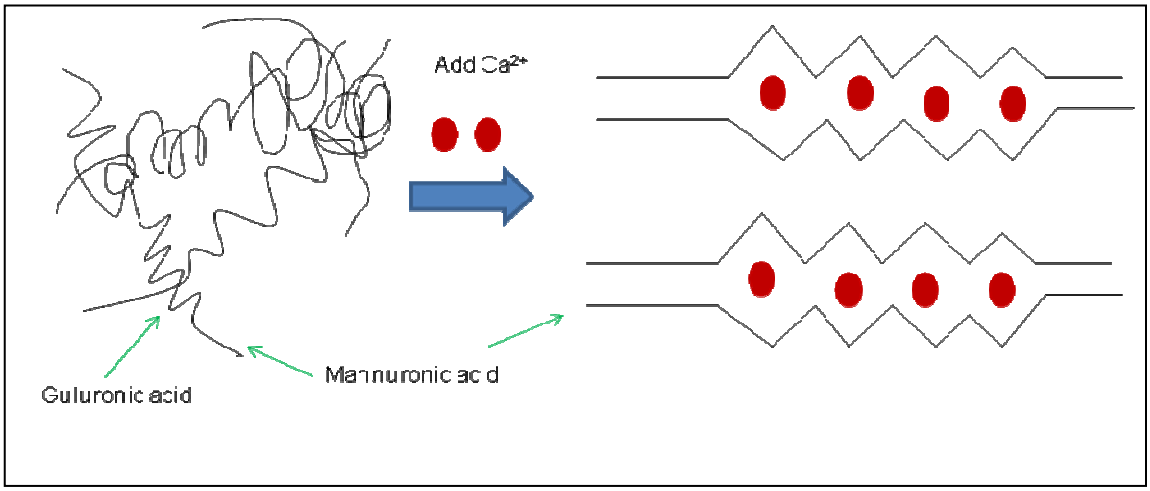


Figure 2.1: A schematic figure of the "egg-box" model. Gelation of alginate solution with the addition of Ca^{2+} ions, based on a figure in Wan *et al*. (2008).

2.4.2 Other natural polymers

Chitosan can be prepared by chemical treatment of the polysaccharide chitin [Lee and Mooney 2001] that can be widely found in many insect exoskeletons, mollusc shells, fungi and crustacean shells [Riddle and Mooney 2004]. It has been used in many biomedical applications especially in tissue engineering because it has biocompatibility, low toxicity, structural similarity to GaGs, and the ability to be degraded by enzymes such as chitosanase and lysozyme [Lee and Mooney 2001, Singh and Ray 2000]. However, it has been reported that chitosan supports almost no fibroblast proliferation [Mori *et al.,* 1997, Ueno *et al.,* 2001] or ECM production by fibroblasts *in vitro* [Ueno *et al.,* 2001].

It has been explained in the previous section (2.2.3) that collagen is an important component of ECM [Burdick and Stevens 2005, Mano *et al.,* 2007]. Since it is naturally derived, many studies have used it as a scaffold material in tissue engineering, usually denatured when it

forms a hydrogel [Burdick and Stevens 2005]. However, collagen is mechanically weak and can quickly degrade *in vitro* [Ding *et al.,* 2008, Lee and Mooney 2001].

Since there are some drawback in using collagen hydrogels, fibrin hydrogels can be used as an alternative [Grassl and Tranquillo 2006]. Fibrin is important in wound healing and homeostasis (the processes whereby the body/cell maintains the equilibrium of its internal environment when there is a change in the external environment) and has also been used as a sealant in surgery [Lee and Mooney 2001, Malafaya *et al.,* 2007, Mano *et al.,* 2007]. It can be sourced from the patient's own blood and so reduce immune rejection problems [Lee and Mooney 2001, Mano *et al.,* 2007]. However, fibrin also has its limitation in tissue engineering applications. They are limited mechanical strength, shrinkage of the gel during flat sheet formation and rapid degradation before proper tissue formation occurs [Ahmed *et al.,* 2008, Jockenhoevel *et al.,* 2001, Lee and Mooney 2001, Mol *et al.,* 2005].

2.5 Why use alginate as a scaffold material?

Hydrogels, both synthetic and natural, are promising tissue engineering scaffolds for three reasons: their water content resembles that of ECM; they can be formed *in situ* to make implantation easier; and they can encapsulate cells during gel formation [Rowley *et al.,* 1999, Wang *et al.,* 2003a]. Any hydrogel scaffold must initially be sufficiently strong to survive in the *in vivo* environment and protect encapsulated cells and new tissue while eventually degrading to enable new tissue to form [Wang *et al.,* 2003a]. The new tissue can then integrate into the surrounding tissues while the hydrogel scaffold degrades, eliminating any need for further surgery [Abbah *et al.,* 2008].

When compared to other type of hydrogel, alginate has several advantages in tissue engineering applications. Because of the mild gelation conditions, alginate has been shown to support the growth of a range of different cell types in tissue engineering applications, including: pancreatic islet cells [Lim and Sun 1980, Peirone *et al.,* 1998], fibroblasts, and chondrocytes [Hunt *et al.,* 2009, Lee *et al.,* 2003, Pokrywczynska *et al.,* 2008]. More recent work has involved the encapsulation and differentiation of mesenchymal stem cells to cartilage-forming chondrocytes [Gold 1981, Lee *et al.,* 2007, Ma *et al.,* 2003].

In contrast, previous findings reported that encapsulation in chitosan almost suppresses fibroblast proliferation [Mori *et al.,* 1997, Ueno *et al.,* 2001]. Encapsulation in alginate preserves chondrocyte phenotype and increases proteoglycan accumulation gradually; this does not happen in collagen gels [vanSusante *et al.,* 1995]. Furthermore, a previous study reported that fibroblasts encapsulated in alginate can be maintained in culture for up to 33 days [Hunt *et al.,* 2009]; this is an advantage of using alginate as compared to fibrin that degrades rapidly in culture [Ahmed *et al.,* 2008, Jockenhoevel *et al.,* 2001, Mol *et al.,* 2005].

Using alginate as a scaffold material to culture chondrocytes has several advantages over culture in monolayer. A previous study on culturing chondrocytes in alginate beads, reported that the cells are normally associated with ECM, and as a consequence, may require a scaffold [Masuda *et al.,* 2003]. The expansion of chondrocytes in alginate beads was also shown to provide a potential culture system for cartilage repair using cells cultured from the person's own tissue [Lee *et al.,* 2003]. This is one of the promising effects of alginate when using chondrocytes in this study.

2.6 The cells

Cells that are used in tissue engineering can be obtained from a variety of donors such as from primary tissues or cell lines [Palsson and Bhatia 2003]. Primary tissues can be from the same individual (autologous), from the same species but a different individual (allogenic), from a genetically identical donor such as a homozygous (identical) twin (syngeneic) and from different species (xenogenic). Cell lines are cells that can proliferate indefinitely after they have been genetically modified [Marler *et al.,* 1998, Palsson and Bhatia 2003].

Figure 2.2, shows how specific cells types can be maintained and proliferated in a 2D culture system. The specific cell types can be incorporated into a suitable scaffolds where these scaffolds must be compatible with the cells in *in vitro* or/and *in vivo* [Vacanti and Vacanti 2000]. As the cells grow in the scaffold, they produce their own ECM. Then, a tissue-like structure is formed and when this material reaches maturity it can be transplanted to the patient [Khademhosseini and Langer 2007].

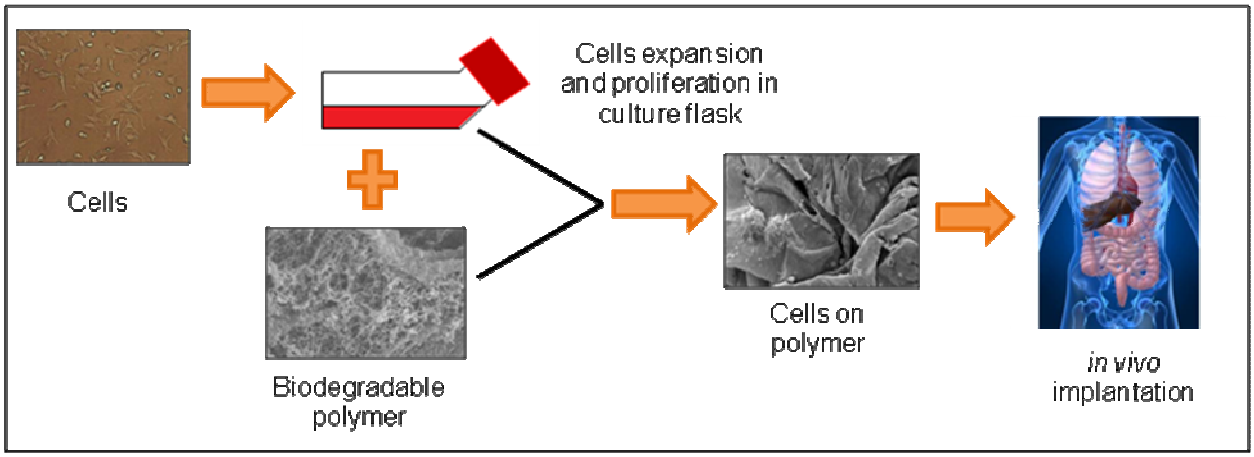


Figure 2.2: The tissue engineering approach to developing organ or tissue replacement using cultured cells. Based on an idea from Lee and Mooney (2001).

2.6.1 Fibroblasts

The most common cell types in connective tissue ECM are fibroblasts. They synthesize collagen and other components of ECM (e.g. glycoproteins and GaGs) to maintain and support a structural framework in connective tissue such in tendons and ligaments [Doroski *et al.,* 2007, Salem *et al.,* 2002].

As mentioned in section 2.2.3 and 2.4.2, collagen is an ECM component that has been used widely in tissue engineering applications. Fibroblasts (Figure 2.3) are one of the cells that synthesize collagen and can easily be cultured in simple media as long as serum is present [Freshney 2005]. Collagen type I is most common type made by fibroblasts [Freshney 2005]. Furthermore, in the literature, only one previous study [Pokrywczynska *et al.,* 2008] has attempted to encapsulate fibroblasts in alginate. Hence, observing the cell behaviour in the alginate, is a good starting point, as a preliminary study. This will be discussed in detail in Chapter 3 and Chapter 4.

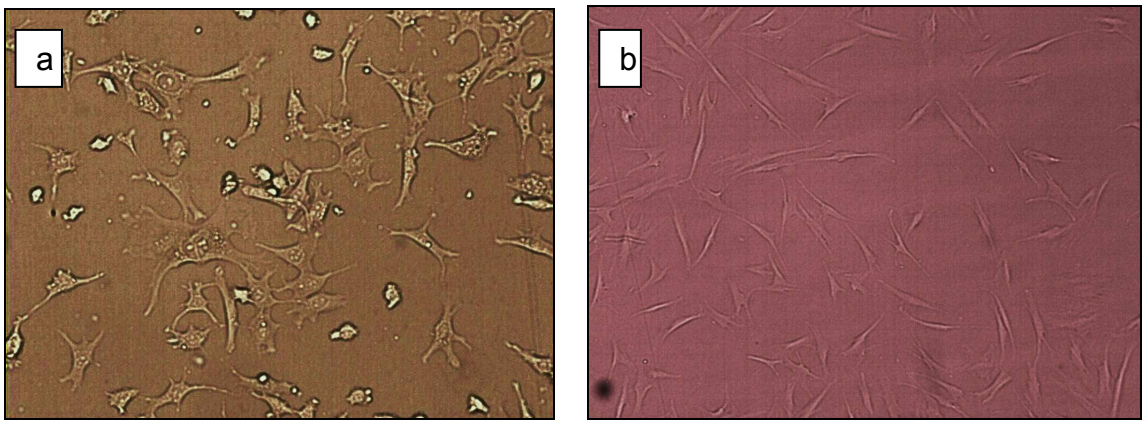


Figure 2.3: Fibroblasts. (a): 3T3 mouse fibroblasts (see section 4.3.1 for a detailed description) (b): Human dermal fibroblasts (see section 6.3.2 for a detailed description).

2.6.2 Calf chondrocytes

Chondrocytes (Figure 2.4) are a specialized type of fibroblast and act as one of the cellular components of articular cartilage [Hukins *et al.,* 1995, Noriega *et al.,* 2007]. These cells synthesize collagen type II and GaG for mechanical support, and growth of articular cartilage [Archer and Francis-West 2003, Archer *et al.,* 1990, Choi *et al.,* 2006].

Several studies have addressed the effect of PLIUS on cartilage at the cellular level (chondrocytes) by showing positive effects on cell proliferation and the production of ECM [Hsu *et al.,* 2006, Parvizi *et al.,* 1999, Zhang *et al.,* 2003]. Because of this, many efforts have been made to develop efficient methods for expanding the isolated chondrocytes *in vitro* without hampering their phenotypic characteristics [Choi *et al.,* 2006, Hunziker 2002].

In addition, chondrocytes need to be cultured in 3D (scaffold) form to maintain their rounded and cartilage-forming phenotypes as this is critical for the regeneration of cartilage *in vitro* [Lee *et al.,* 2003]. Furthermore, chondrocytes that are cultured in 2D (monolayer) have a tendency to dedifferentiate to fibroblasts [Bittencourt *et al.,* 2009, Choi *et al.,* 2006, Lee *et al.,* 2003].

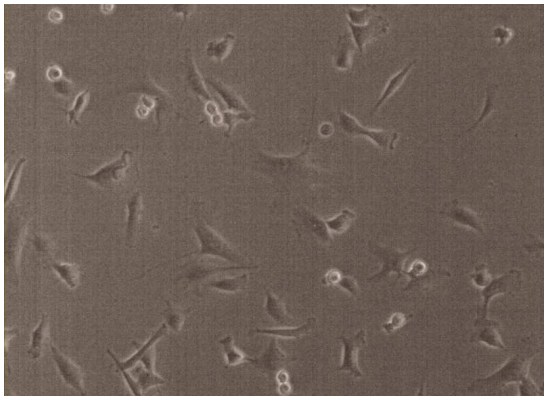


Figure 2.4: Calf chondrocytes (see section 7.3.1 for a detailed description).

2.7 Growth factors

2.7.1 Ascorbic acid

A previous report stated that ascorbic acid is a growth factor that is involved in collagen gene expression by fibroblasts [Chojkier *et al.,* 1989]. It functions as a cofactor for specific enzymes (lysyl hydroxylase and prolyl hydroxylase) that are essential for collagen synthesis. It is involved in the hydroxylation process where proline forms hydroxyproline and lysine forms hydroxylysine [Pinnell 1985, Tajima and Pinnell 1982]. Hydroxyproline is one of the amino acids in the collagen sequence and is needed for collagen helix formation [Badylak *et al.,* 2008, Hukins *et al.,* 1995, Pinnell 1985]. Furthermore, collagen cross-linking would be weak without hydroxylysine [Pinnell 1985]. Previous studies also mentioned that ascorbic acid has an effect on the translation and transcription of genetic information into the collagen amino acid sequence [Pinnell 1985, Tajima and Pinnell 1982].

Previous studies also reported that ascorbic acid increases ECM synthesis in chondrocytes, human fibroblasts and human aortic smooth muscle cells [Bittencourt *et al.,* 2009, Chojkier *et al.,* 1989, Kim *et al.,* 2009].

2.7.2 Transforming growth factor beta 1 (TGF-β1)

TGF-β1 was reported to be involved in stimulating collagen and GaG biosynthesis by chondrocytes, and fibroblasts in cultured rabbit marrow mesenchymal stem cells and neonatal rat smooth muscle cells [Clark *et al.,* 1995, McAnulty 2007, Neidert *et al.,* 2002, Park *et al.,* 2009, Redini *et al.,* 1988, Ross and Tranquillo 2003].

However, it is most common in regulation of ECM production by fibroblasts and in wound healing [McAnulty 2007]. In wound healing, it is involved in differentiation of fibroblasts into specialised cells called myofibroblasts [Hashimoto *et al.,* 2001, McAnulty 2007, Petrov *et al.,* 2002]. If fibroblast function is impaired or inappropriate, diseases resulting from diminished or excess ECM deposition can occur [Hashimoto *et al.,* 2001, McAnulty 2007, Phan 2008].

2.8 Therapeutic Ultrasound

2.8.1 Ultrasound

Ultrasound is sound with a frequency (greater than about 20 kHz) which is too high for human hearing [Doan *et al.,* 1999]. Therapeutic ultrasound is used in medicine for tissue repair [Dalecki 2004]. It usually has a frequency between 1 and 3 MHz with an intensity range of 0.1 to 2.0 W/cm^2 [Doan *et al.,* 1999]. It is a wave that transports mechanical energy; although it causes heat; this is simply a consequence of supplying mechanical energy at this frequency, rather than being anything additional or special [Doan *et al.,* 1999, Ozgonenel *et al.,* 2009].

2.8.2 Pulsed-low intensity ultrasound (PLIUS)

(PLIUS) or pulsed low intensity (less than 1 W/cm^2) ultrasound whose intensity is low enough to have minimal thermal or destructive properties [Min *et al.,* 2007, Takayama *et al.,* 2007]. This was confirmed from the PLIUS characterization in my work that showed that the temperature rise was ≤ 0.3°C within a 5 min exposure (Appendix A2). PLIUS is known to accelerate bone regeneration, increase cell proliferation, protein synthesis and cytokine production by fibroblasts, osteoblasts and monocytes in culture [Doan *et al.,* 1999, Ikeda *et al.,* 2006, Takayama *et al.,* 2007]. PLIUS stimulation also has been shown to effect proliferation and matrix deposition of human chondrocytes [Hsu *et al.,* 2006] and increase proteoglycan synthesis in a human nucleus pulposus (the central region of the intervertebral disc) cell line [Kobayashi *et al.,* 2009]. There were also reports showing that PLIUS does not affect cell viability and can increase proteglycan synthesis by cells encapsulated in alginate [Iwashina *et al.,* 2006, Miyamoto *et al.,* 2005, Zhang *et al.,* 2003]. However, studies on PLIUS with frequency and intensity used in the present study on alginate encapsulated 3T3 fibroblast, HDF and calf chondrocytes have not been reported before. Although ultrasound has widespread use in medicine, there is little scientific evidence about what it does [Tsai *et al.,* 2006]. The present study provides a good opportunity to investigate how PLIUS effect on different cells types from different sources.

2.9 Assay of cell behaviour in culture

2.9.1 Hydroxyproline assay

Hydroxyproline is formed from the hydroxylation of proline by the enzyme prolyl hydroxylase during collagen synthesis [Pinnell 1985, Tajima and Pinnell 1982]. As mentioned in section 2.2.3, hydroxyproline is a major component of and is always present in collagen

[Badylak *et al.,* 2008, Hukins *et al.,* 1995]. Consequently, measuring hydroxyproline content can directly related to collagen content [Reddy and Enwemeka 1996]. Hydroxyproline can be assayed by oxidation to a compound related to pyrrole, which may then form a coloured compound with p-dimethylaminobenzaldehyde (P-DMBA) [Kyaw and Hlape 1973].

2.9.2 Glycosaminoglycan (GaG) assay

1,9 dimethylmethylene blue dye (DMB) is used for GaG detection. This assay relies on the changes to the DMB when this cationic dye bind to the anionic sulfate and carboxyl groups in the GaG [Enobakhare *et al.,* 1996]. Many previous studies used DMB dye at pH 3.0 and this resulted in complications when using alginate as a scaffold material. This is because, alginate also has a carboxyl group and at pH 3.0, DMB dye binds to both carboxyl groups (alginate and GaG) as well as to sulfate groups in the GaG [Enobakhare *et al.,* 1996]. However, by using a pH of 1.5, the dye reaction with alginate can be minimized [Chang *et al.,* 2001]. The absorbance of the test samples was measured with light of wavelength 600 nm in the dark to maintain the condition of the dye [Farndale *et al.,* 1986].

2.10 Rheology

"Rheology is the study of deformation and flow" [Cogswell 1981]. It means that a liquid will change in shape and/or volume (i.e. it will deform) when the liquid molecules move past or towards to each other (i.e. when they flow) [Barnes 2000, Harris 1977]. Flow of liquid layers past each other is called shear [Barnes 2000]. Flow is initiated by shear stress, as shown in Figure 2.3 [Brydson 1981].

The flow of a liquid is restricted by its viscosity [Barnes 2000]. In the case of alginate solutions, the thicker the solution (i.e. the higher the alginate concentration), the more difficult

for the solution to flow/move and deform [Cogswell 1981]. So when stress is applied to the alginate, the material will continues to deform and when the solution become thicker the stress that is applied to it needs to be increased to deform and move the alginate solution [Cogswell 1981]. A rheometer measures the stress required to induce flow at a controlled shear rate [Cogswell 1981].

There are several factors that affect the viscosity of polymer solutions. These include temperature, pressure and molecular weight of the dissolved polymer [Brydson 1981]. The effect of temperature on alginate viscosity is demonstrated in chapter 3 (section 3.5.1) and Appendix (A1). Molecular entanglement of the polymers also plays an important role [Barnes *et al.,* 1989, Cogswell 1981]. In polymer solutions at high concentration, molecular entanglement is expected to be higher than in a solution at lower concentration because there is less water present. Molecular entanglement also increases when the molecular weight of the polymer increases. Thus high molecular weight polymers and high concentrations lead to increase the shear stress that applied to the polymer solutions.

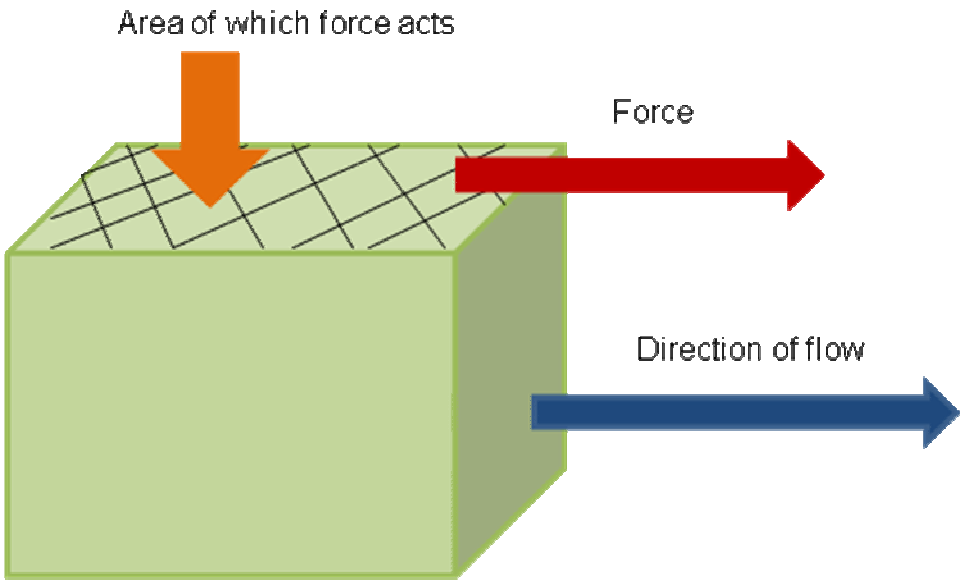


Figure 2.3: The shear stress which initiates the flow is the force divided by the shaded area. Based on an idea by Brydson (1981).

2.11 Research questions

- What is the rheology behaviour of alginate solutions with different concentrations?
- Is the viability and proliferation of alginate encapsulated cells influenced by different concentrations of alginate solutions?
- Does PLIUS alone, or PLIUS in conjunction with growth factors, enhance cell proliferation and ECM production on 3T3 cells, HDF cells and chondrocytes encapsulated in alginate gels?

3. INVESTIGATING ALGINATE PROPERTIES FOR CELL ENCAPSULATION

3.1 Introduction

As discussed in section 2.4.1, alginate solution turns into a hydrogel when the guluronic acid residues bind to divalent cations. Its physical properties also can change markedly depending on the molecular weight and proportion of guluronic acid. Since calcium alginate has been widely used in cell delivery and tissue engineering applications, it is important to reduce the possibility that it degrades before the cells can perform their required functions.

In this chapter, the viscosity of alginate solutions to be used for cell encapsulation were measured. A decrease in viscosity was used as a measure of alginate degradation (see section 2.10). The mechanical degradation of the different concentrations of alginate gel were determined when maintained in supplemented cell culture medium for up to 28 days. This study was carried out to determine whether the alginate concentrations are likely to have an influence on the encapsulated cells (discussed later on the Chapter 4 section 4.6). To identify the mechanical integrity, viscosity measurements, ageing of alginate solutions (section 3.3.2 and 3.3.3) and a degradation study (section 3.3.5) were undertaken.

3.2 Materials

Sodium alginate from kelp (brown seaweed) (viscosity = 20-40 cP = 0.02-0.04 Pa.s for a 2% solution at 25ºC Cat no: 180947, Batch no: MKBB8173, M_w 120 000-190 000, mannuronic/guluronic ratio 1.56), phosphate-buffered saline [(PBS) (0.01M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4)] and Dulbecco's modified Eagle's Medium (DMEM), were obtained from Sigma-Aldrich (Poole, Dorset, UK).

3.3 Methods

3.3.1 Preparing alginate solution

Sodium alginate solutions (0.5, 1, 2, and 5 wt/vol% made with double distilled water) for the degradation study were autoclaved at 121°C for 15 min to sterilise before being used [Hunt *et al.,* 2010]. For the viscosity and ageing studies, sodium alginate solutions (0.5, 1, 2 and 5 wt/vol%) were prepared with double distilled water without sterilisation. All of the alginate solutions were kept at room temperature for the whole experiment.

3.3.2 Viscosity measurements

The viscosity of alginate solution (0.5, 1, 2 and 5 %) was measured using a rotating rheometer (AR 1000, Jencons Scientific LTD, Lutterworth, Leicestershire, UK) fitted with a 40 mm 2° steel cone-plate geometry [Furth *et al.,* 2008, Rezende *et al.,* 2009, Roopa and Bhattacharya 2009] with a shear rate up to 1000 (1/s). The rotational speed depended on the sample viscosity, computed from means of stress and shear rate (TA Instruments Ltd, Crawley, west Sussex, UK). Experiments were run on all of the alginate samples at two different temperatures (25°C in Appendix A1 and 37°C). This was done in order to compare whether temperature changes have a dramatic effect on the alginate solution.

3.3.3 Ageing of alginate solutions

The ageing (viscosity) of alginate solution (0.5, 1, 2 and 5%) was measured (section 3.3.2) for up to 28 days. All of the solutions were kept at room temperature (see section 3.3.2 for a detailed description).

3.3.4 Preparing alginate discs

Alginate discs were prepared by following Hunt et al's [Hunt *et al.,* 2010] method, but some slight modifications were made for this study. 1 ml of sodium alginate solutions (0.5, 1, 2, and 5 %) were pipette into a well in a twelve-well plate containing a filter paper that was soaked in sterile $CaCl_2$ solution (100 mM), that was placed at the bottom of the well. The alginate solution was covered with another filter paper that was also soaked in sterile $CaCl_2$ solution. The well was then filled with sterile $CaCl_2$ solution to gel the alginate [Khattak *et al.,* 2006]. The resulting suspensions were incubated for 2 h at 37°C with 5% CO_2. The resulting discs, with an average diameter of 17.25 mm (Figure 3.1) were transferred to a six-well plate, and then washed three times with PBS. DMEM (5 ml) was added to each well and the constructs incubated at 37°C with 5% CO_2; the medium was changed every 3 days for a period of up to 28 days. When compared to other forms of alginate, the disc is the most appropriate shape to achieve cell encapsulation and obtain the number of cell densities per each disc, accurately.

Figure 3.1: Alginate disc.

3.3.5 Hydrogel degradation study

The hydrogels were degraded for a period of up to 28 days at 37°C with supplemented DMEM. In order to observe the degradation process of the discs at different alginate concentrations, they (3 samples each) were removed from the media at each specified day and freeze dried for 2 days. The dry mass (D_s) of the alginate disc was measured and the fractional loss was determined by dividing D_s by the mass of the sample on day 0 (D_0).

3.4 Statistical analyses

The Shapiro-Wilk test [Pallant 2007] showed that all of the results were normally distributed except on days 2 and day 3 for the alginate disc degradation study. As a consequence, results were compared using a one-way ANOVA and Kruskal Wallis tests [Pallant 2007]. Differences were considered to be significant if the probability $p < 0.05$.

3.5 Results

3.5.1 Viscosity of different concentration alginate solutions

Figure 3.2a and 3.2b shows that the viscosity decreases with increasing shear rate which indicated shear-thinning behaviour [Rezende *et al.,* 2009]. This is clearly observed with a high concentration of alginate solution but the shear thinning behaviour is not apparent for alginate concentration less than 5%. Theoretically, increasing the alginate concentration increases both the viscosity and stress for a specific value of shear rate [Rezende *et al.,* 2009].

3.5.2 Ageing of alginate solutions

As mentioned in section 3.5.1, alginate solution tends to decrease in viscosity when the shear rate is increased. The study was prolonged for up to 4 weeks to see if there was any possibility that ageing could influence the viscosity of various alginate concentrations. As

seen in figures 3.3a, 3.3b, 3.3c and 3.3d, all of the alginate concentrations show decreased viscosity over 4 weeks. The most marked reduction was seen with the 5 % alginate concentration; while the others only decreased slightly.

3.5.3 Degradation in different concentrations of alginate disc

Figure 3.4 shows the dissolution process for an alginate disc up to 28 days in the supplemented media [Hunt *et al.,* 2010]. The figure represents the dissolution pattern for all of the alginate concentrations and the process can clearly been seen from day 1 up to day 4 but then slows down by day 28. As mentioned in section 2.4, this can be explained by the presence of monovalent ions that compete with the Ca^{2+} ions. The high molecular weight of the alginate also contributes to the degradation process (section 2.10). When the alginate disc was highly concentrated, more Ca^{2+} ions are exchanged with the monovalent ions. As a consequence, the degradation process can be seen clearly when compared to the low concentration of alginate disc.

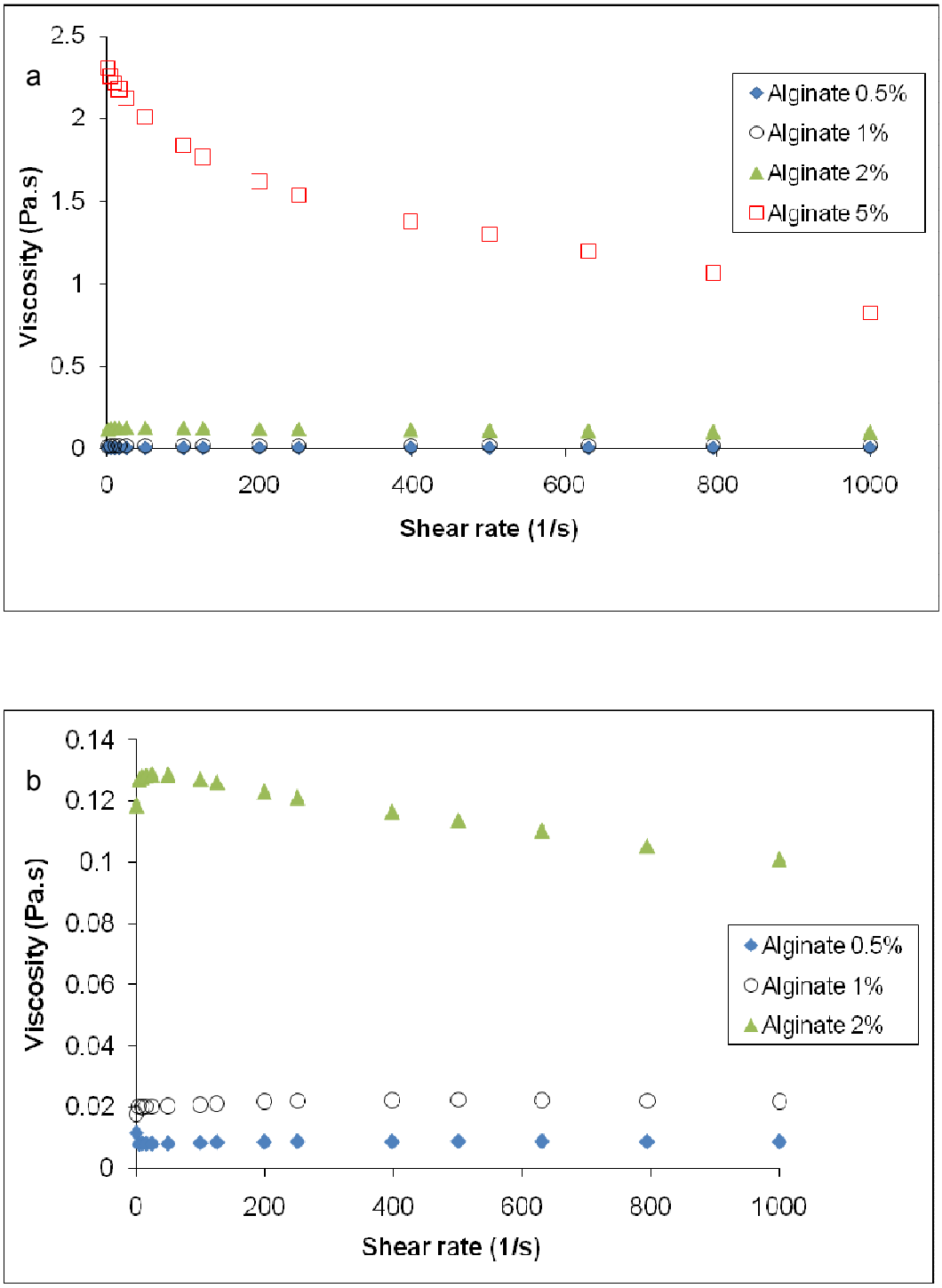
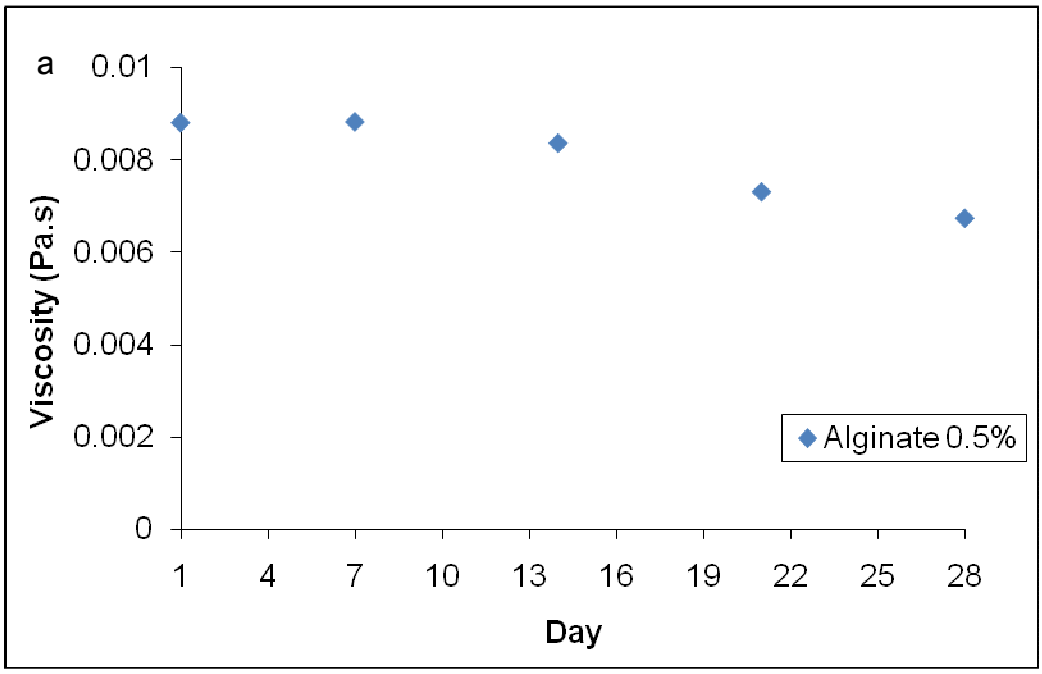
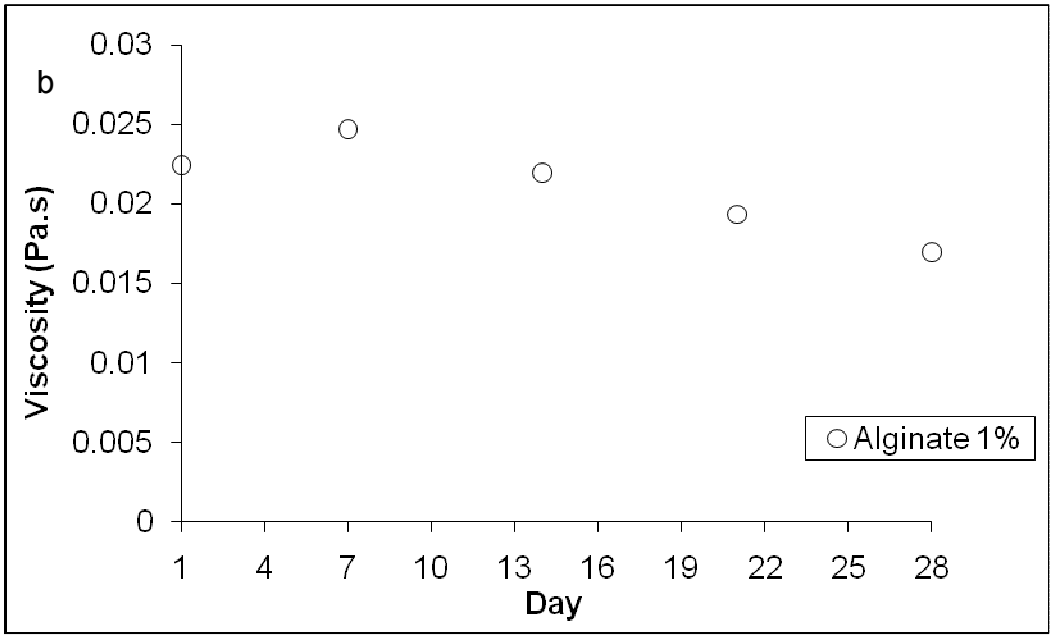


Figure 3.2: Viscosity variation for different alginate solutions at a temperature of 37°C (a) for all concentrations and (b) for lower concentrations.


a
0.01
0.008
0.006
0.004
0.002
0
Viscosity (Pa.s)
1
4
7
10
13
16
19
22
25
28
Day
Alginate 0.5%


b
0.03
0.025
0.02
0.015
0.01
0.005
0
Viscosity (Pa.s)
1
4
7
10
13
16
19
22
25
28
Day
Alginate 1%

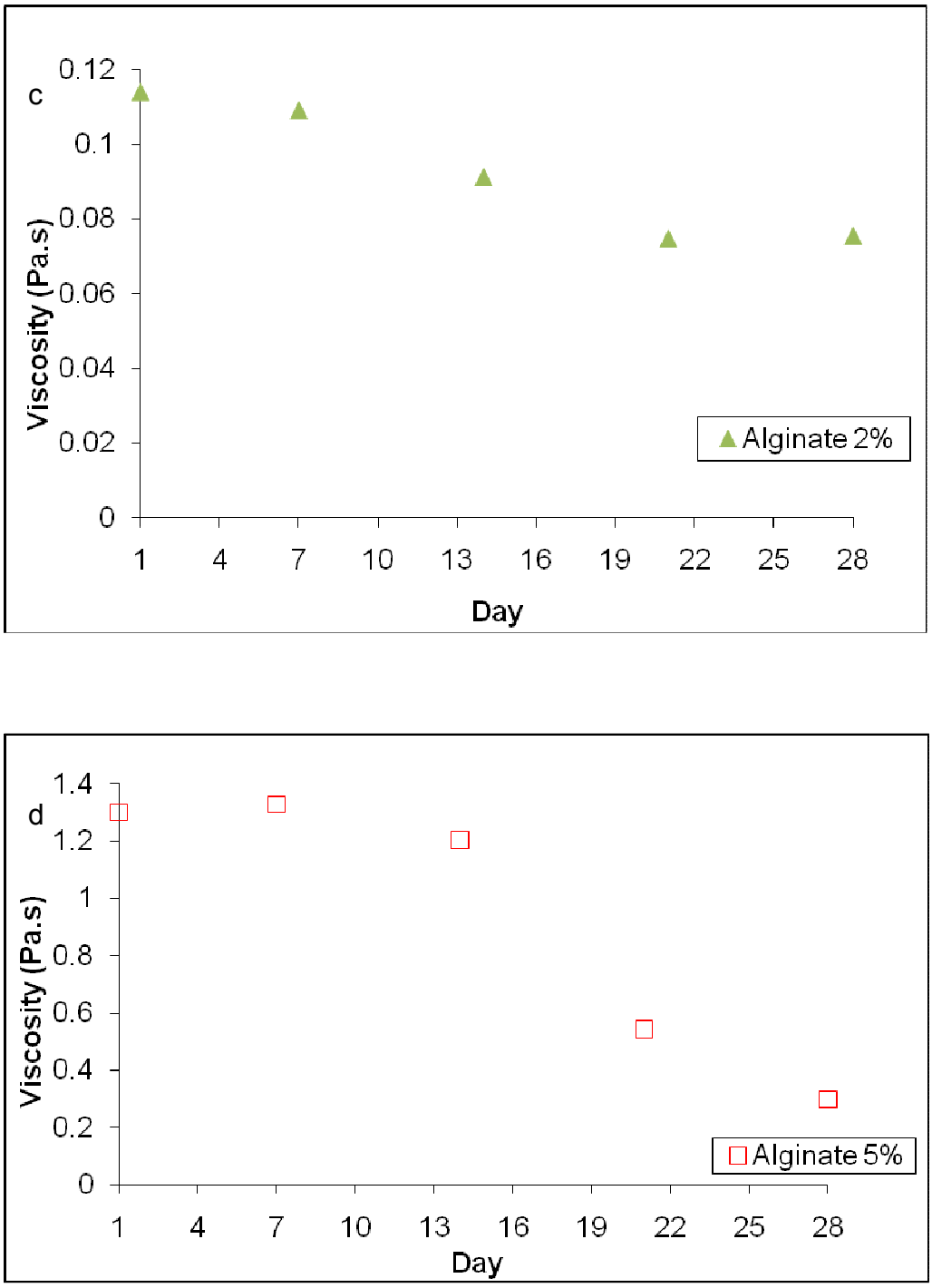


Figure 3.3: Viscosity reduction of (a) 0.5%, (b) 1%, (c) 2% and (d) 5% alginate solutions up to 28 days at a shear rate 500 s^{-1}.

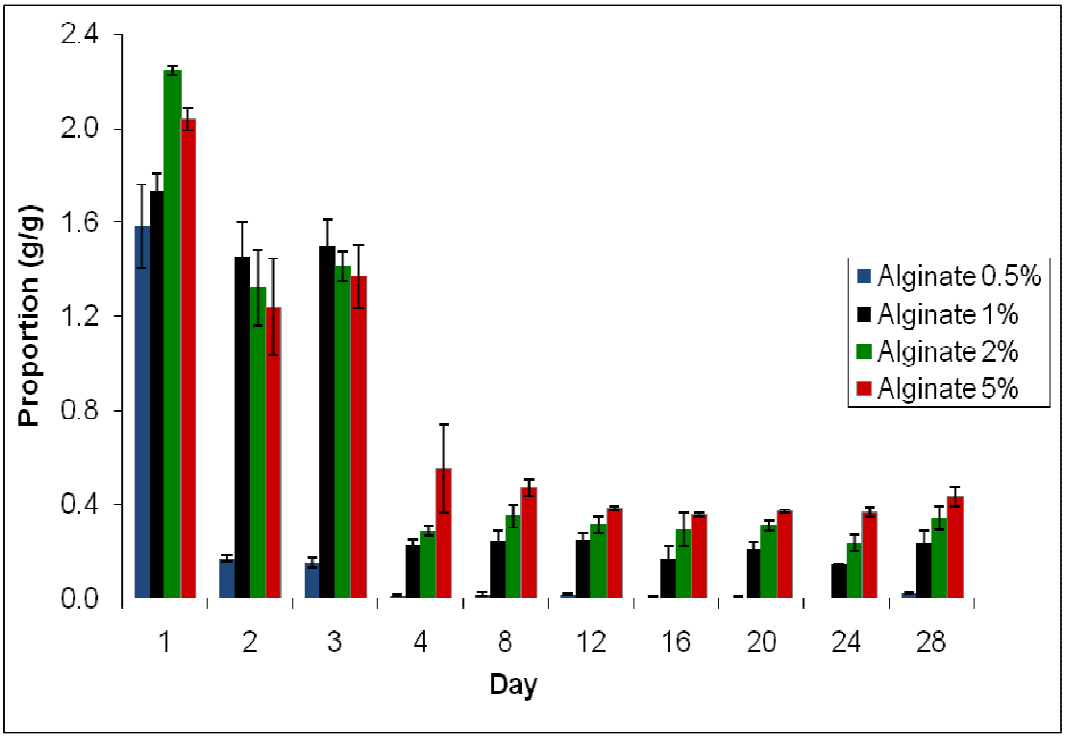


Figure 3.4: Degradation of alginate discs of various concentrations up to 28 days. All of the alginate concentrations have a significant effect at each time points, except for day 2 and 3. Each result is the mean of three observations. Error bars represent standard deviations**.**

3.6 Discussion

Viscosity is defined as resistance to flow [Barnes *et al.,* 1989]. Less viscous solutions flow more easily when subjected to a shear stress [Barnes *et al.,* 1989]. This can be seen when using a high concentration of alginate, (Figure 3.2a and 3.2b) when shear thinning is also observed. A possible explanation is because of the intermolecular association, known as entanglement, of the polymer chains being disrupted by application of shear stress [Barnes *et al.,* 1989]. High viscosity may not be desirable in terms of maintaining cell viability before gel formation, as a high solution viscosity would lead to cells being exposed to high shear stress during mixing. Also, cell membranes are highly sensitive to shear stress, and the mixing can lead to cell death [Chisti 2001].

The ageing process has a greater effect at higher alginate concentration (5%) (Figure 3.3d) rather than at lower concentration (Figure 3.3a, Figure 3.3b and Figure 3.3c). This can be explained by molecular entanglement being higher in 5% alginate solution. As mentioned in section 2.10, if application of shear stress reduces entanglement, the effect will then be more noticeable at higher concentrations. Also, the storage temperature for the alginate solution contributes to this ageing process. This is shown in the viscosity study at two different temperatures, 37 °C (as shown in Figure 3.2a and 3.2b) and 25°C in Appendix A1.

Gelation of alginate depends on the affinity of alginate or specifically the guluronic acid residues towards ions that bind to it [Draget *et al.,* 1997]. In this study, the alginate that was used had a higher mannuroric than guluronic acid residue content and so will form a relatively weak gel in $CaCl_2$ solution. As explained before, in section 2.4, it is known that alginate gel maintained in an aqueous environment, or specifically in tissue culture medium, has a

tendency to swell because of loss of crosslinking Ca^{2+} ions [Drury and Mooney 2003, Kuo and Ma 2008]. With regard to alginate concentration, higher polymer concentration may lead to a degradation tendency over time (swelling) because of the ability of alginate molecules to attract water [Drury and Mooney 2003, Kuo and Ma 2008]. As shown in figure 3.4, the degradation process can be clearly seen within 4 days in culture for all concentrations of alginate. Furthermore, with high intermolecular association, the swelling or degradation process takes longer. This explains why gel degradation can be seen more clearly at high concentration of alginate than at low concentration.

By increasing the ionic binding, the mechanical rigidity of the gel could be enhanced but it could also lead to a greater concentration of counterions within the gel resulting in increased diffusion of water into the gel [Kuo and Ma 2008]. Furthermore, using a high concentration $CaCl_2$ solution (100 mM) would be deleterious to the cells during the encapsulation process but no problem was observed in alginate that was rich in mannuronic acid residues [Simpson *et al.,* 2004]. Indeed, for the past 20 years 100 mM $CaCl_2$ solution has been used as a standard solution for alginate encapsulation and no deleterious effects have been reported. Low concentration $CaCl_2$ solution tends to form a fragile gel. Also when there is a low concentration of $CaCl_2$, the gel may degrade because there are few Ca^{2+} ions in it to replace those that diffused out. As stated in a previous study, a homogenous gelling process or slow cross-linking is essential to reduce the degradation process but these conditions are not suitable for cell encapsulation studies as they could have adverse effects on cell viability [Chisti 2001]. So, in the present study alginate with a high mannuronic acid content was used as this alginate was not sensitive to changes in the strength of the alginate gel network and tends to form spaces for the cells to grow into during the swelling or degradation processes

that occur in culture. This was supported by previous studies [Simpson *et al.,* 2004, Wan *et al.,* 2008] that showed that the strength of the alginate network also affects the growth of encapsulated cells. A weak alginate gel network is advantageous in tissue engineering constructs, or areas within a construct, when cell growth and expansion are required but not when they are not [Simpson *et al.,* 2004].

4. EFFECT OF CALCIUM ALGINATE CONCENTRATION ON VIABILITY AND PROLIFERATION OF ENCAPSULATED 3T3 MOUSE FIBROBLASTS

4.1 Introduction

There have been contrasting reports on the behaviour of certain cell types, such as fibroblast, βTC3 cells, hADAS cells, chondrocytes and bone marrow cells [Awad *et al.,* 2004, Chia *et al.,* 2005, Pokrywczynska *et al.,* 2008, Simpson *et al.,* 2004, Wang *et al.,* 2003a] in alginate. For example, some authors report no significant change in cell numbers when compared to monolayer culture [Mosahebi *et al.,* 2001], whereas others report proliferation and subsequent formation of agglomerates known as "spheroids" [Bartold and Page 1985]. The apparent variability of the reported cell behaviour may be attributed to a range of factors, including cell seeding density, alginate concentration and media type. Many researchers seed cells in alginate at densities of around 1×10^6 cells/ml [Choi *et al.,* 2006, Iwashina *et al.,* 2006, Lee *et al.,* 2006, Pokrywczynska *et al.,* 2008] but there has been little published work on modifying the alginate concentrations at specific cell densities.

As explained in Chapter 3, an increase in the viscosity also increased the shear thinning behaviour and led to cells being exposed to high shear stresses during the mixing process. Consequently, this chapter presents the first detailed study of the influence of different alginate concentrations (and seeding densities on cell (section 4.3.3) viability, proliferation

and spheroid formation (section 4.3.4) for 3T3 fibroblasts. This was done to further elucidate the roles that these variables play in influencing 3T3 fibroblast behaviour when treated with PLIUS (Chapter 5)

4.2 Materials

Alginate (see section 3.2), Dulbecco's Modified Eagle's Medium (DMEM), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) powder, and the DNAQF DNA Quantification Kit, were purchased from Sigma-Aldrich (Poole, Dorset, UK). Foetal calf serum was purchased from PAA Laboratories (Farnborough, Hampshire, UK) and the Live/Dead fluorescence assay kit (calcein AM and propidium iodide) was obtained from Invitrogen (Paisley, UK). All other materials that not mentioned here were obtained from Sigma-Aldrich.

4.3 Methods

4.3.1 3T3 mouse fibroblasts

Fibroblasts were from a commercially available cell line (NIH 3T3 murine fibroblasts, ATCC, Middlesex, UK). Cells were maintained in DMEM supplemented with calf serum (10 v/v%), penicillin/streptomycin (1 v/v%), L-glutamine (2.4%), and HEPES (2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid) (2.4 v/v%). Cells were stored in sterile conditions at 37ºC and 5% CO_2 and media was refreshed every three days.

4.3.2 Cell encapsulation

Cells were detached from the flask in which they were cultured with trypsin (0.05%) and EDTA (0.2 g/l) and resuspended in DMEM. Viable cell numbers were determined by staining with trypan blue and counting in a haemocytometer, which is essential in quantification of the

growth properties of the cells [Freshney 2005]. After that, sodium alginate solutions (0.5, 1, 2, and 5 % made with double distilled water) were seeded with cells at low (0.76×10^5 cells/ml) and intermediate (0.76×10^6 cells/ml) densities. The cell suspension was transferred to the wells in twelve-well plates and incubated in a bath of sterile $CaCl_2$ solution for 2 h [Khattak *et al.,* 2006]. The resulting discs of calcium alginate with encapsulated cells were washed three times with sterile PBS (phosphate-buffered saline solution) and transferred to six-well plate; each well contained supplemented DMEM (5 ml) which was refreshed every 3 days.

4.3.3. Live and Dead assay

Fluorescence microscopy was used to examine fibroblasts encapsulated in calcium alginate. A slice of the construct (< 1.0 mm thickness) was incubated in DMEM (1 ml) with propidium iodide (25 µl, 150 µM) and calcein AM (2µl, 100 µM) for 30 min. It was then examined using a fluorescence microscope at 100× magnification (Axiolab, Zeiss, Oberkochen, Germany). Under these conditions, viable cells are green while dead cells (that contained propidium iodide) are red [Khademhosseini *et al.,* 2006]. The percentages of viable cells (live/dead) were assessed from the photomicrograph obtained with a digital camera (Powershot G5, Canon, Tokyo, Japan) [Bunger *et al.,* 2002]. The percentage of viable cells was measured every 4 days; all measurements were in triplicate.

4.3.4 MTT assay

The MTT assay was used to determine the number of viable cells in a sample; in this technique, living cells convert the MTT to formazan [Uludag and Sefton 1990]. MTT solution (5 mg/ml) was prepared in PBS (100 mM). MTT solution (500 µl) was added to the DMEM containing the encapsulated cells. The MTT was then left to stand for 20 h in an incubator at 37ºC in an atmosphere containing 5% CO_2 to allow for optimum formazan production

[Uludag and Sefton 1990]. The alginate discs were then removed from the wells and immersed in a solution of HCl in isopropanol (2 ml, 100 mM). The alginate was then placed into a shaker incubator for 1 h to allow the formazan to fully diffuse from the disc into the solvent. The absorbance of the coloured solution was determined at a wavelength of 570 nm using a spectrophotometer (Cecil CE 1020, Cecil Instrument, Cambridge, UK) in order to determine the formazan concentration; absorbance measurements from discs without cells were subtracted from these results. The cell numbers were calculated from a standard curve of MTT concentration against cell number [Uludag and Sefton 1990]. The assays were undertaken at four day intervals and measurements were made in triplicate.

4.3.5 Cell content

Since the MTT assay is only known to be a reliable indicator of cell number when the samples compared exhibit identical metabolic rates, the results were validated using the Hoechst assay. Constructs, that had been cultured up to 10 days, were freeze-dried for 2 days, then digested in papain solution (1 ml; 125 μg/ml) [Hoemann *et al.,* 2002]. Total cell numbers were determined by a Hoechst assay using a DNAQF DNA Quantitation Kit [Elder *et al.,* 2006]. Hoechst 33258 dye (200 μl; 2 μg/ml) was added to samples (10 μl) in a 96 well plate. Fluorescence (excited at a wavelength of 360 nm) was measured using a spectrophotometer (Promega glomax, Promega, Southampton, UK) at a wavelength of 460 nm, at ambient temperature. The total cell numbers were obtained from a calibration curve of fluorescence intensity plotted against results from known densities (2.0–36.0 × 10^4 cells/ml) of 3T3 fibroblasts cells.

4.4. Statistical analyses

The Shapiro-Wilk test [Pallant 2007] showed that the results were not normally distributed. As a consequence results were compared using the Mann-Whitney and Kruskal Wallis tests [Pallant 2007]. Differences were considered to be significant if the probability $p < 0.05$; as shown in the figures, significant differences are marked with an asterisk.

4.5 Results

When seeded at a concentration of 0.76×10^6 cells/ml, at day 1, there were appreciable numbers of dead cells (Figure 4.1a) in the intermediate concentrations of alginates (5 and 2 %) but not at the lower concentrations (1 and 0.5 %) (Figure 4.1b). However, the majority of cells appeared to be viable in the higher concentrations of alginate at day 16 and day 28. The cells appeared to grow in spheroids rather than being evenly dispersed thorough the gel; these spheroids can be clearly seen at day 28 (2 and 5 % concentrations of alginate) and day 16 (0.5 and 1% concentrations of alginate). The tendency of the cells to grow in spheroids made it difficult to reliably count numbers from the micrographs. The first time-point at which the formation of these cell spheroids was noted appeared to be related to the concentration of alginate from which the gel was formed (Figure 4.2). When the gel was formed from 0.5% concentration alginate solution, for example, spheroid formation was noted after 8 days in culture, in comparison spheroid formation was not noted until after 20 days in culture in the 5 % alginate gel (Figure 4.2).

Quantitative results from fluorescence microscopy are shown in Figure 4.3. For cells encapsulated at a seeding density of 0.76×10^6 cells/ml, in 5 % alginate solution, only 50 ± 4.5% (± standard deviation) of the original cells were shown to be viable one day after

establishing the culture and, in the case of the 2% alginate, 70 ± 4.0 % cells were shown to be viable after day one. In both of these conditions, cell viability was shown to improve to greater than 90% after only 4 days of culture (Figure 4.3a). Although there were significant differences between cell numbers in 5 and 2 % gels at day 12 and day 20, there was no consistent trend. In the case of the cells cultured in 0.5 or 1% of alginate solution, cell viability was greater than 90% (Figure 4.3b) after the first day of culture; however, with both of these gels, after 8 days of culture the gel discs start to soften in the medium.

When the alginate gels were used to encapsulate cells at a lower concentration, the percentage alginate seemed to have a more marked influence on the viability of the encapsulated cells. After one day in culture, a proportion of cells fluoresced red in the Live/Dead assay indicating that the cells were dead (Figure 4.4). From the quantitative analysis, the influence of alginate concentration appeared to be significant on days 16, 20 and 28 in the 2% concentration alginate gel (Figure 4.5). Percentage of viable cells shows not much difference when using low (0.76×10^5cells/ml) and intermediate (0.76×10^6cells/ml) seeding cell densities (Figure 4.6).

To further quantify the influence of alginate concentration on cell number, cell number was also determined using the MTT assay (Figure 4.7). The results from the MTT assay further confirmed the observation that after 1 day of culture, in 5 % alginate, there were fewer viable cells. In the case of the 2% alginate, although cells were seeded at a density of 0.76×10^6 cells/ml, after one day of culture, there were only 0.22×10^6 cells/ml actively metabolising. After four days in culture, cell number increased in all conditions. In 0.5% alginate, cell number was shown to increase 5 fold in a period of 4 days. For the remainder of the

experiment, cell number increased in all conditions, although cell number reached a plateau in the 2 % alginate after 20 days. Since the MTT assay can only be used as an indicator of cell number when the cells exhibit a constant metabolic rate, the Hoechst assay was used to verify cell number at days 1, 9 and 18. Cell number, as determined using the Hoechst assay at these time-points, was shown to be within 10% of that determined using the MTT assay (Figure 4.8).

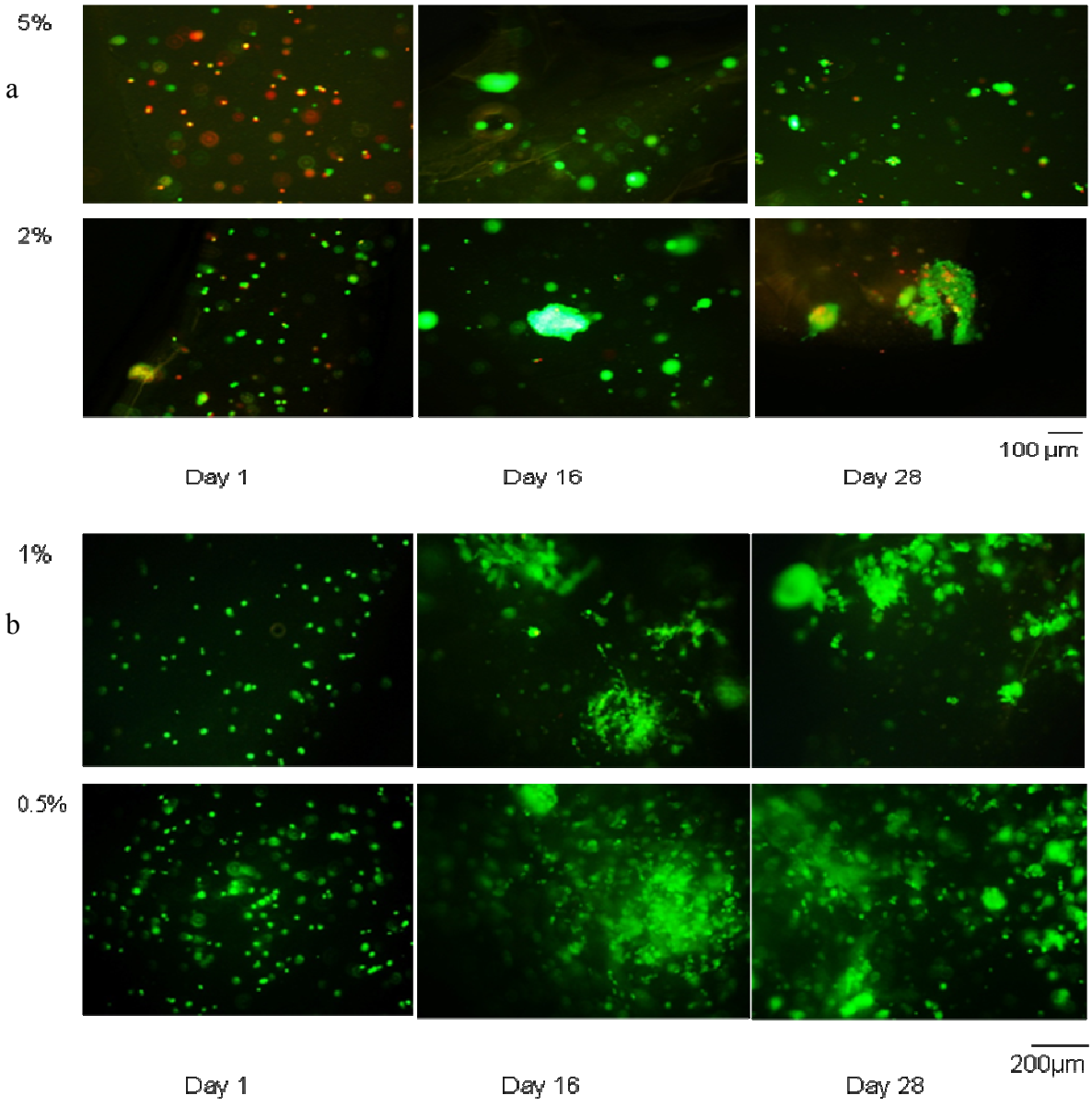


Figure 4.1: Fluorescence micrographs showing viable (green) and dead (red) cells in sections alginate discs for the intermediate seeding cells density (0.76×10^6 cells/ml). Micrographs were obtained after 1, 16 and 28 days for (a): 5 and 2%, (b) 1 and 0.5 % of alginate concentrations.

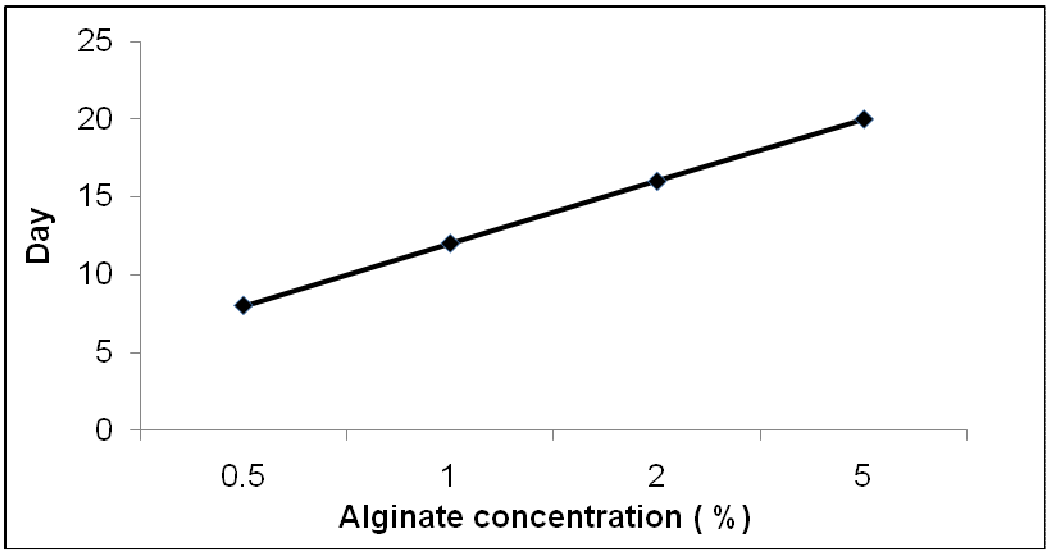
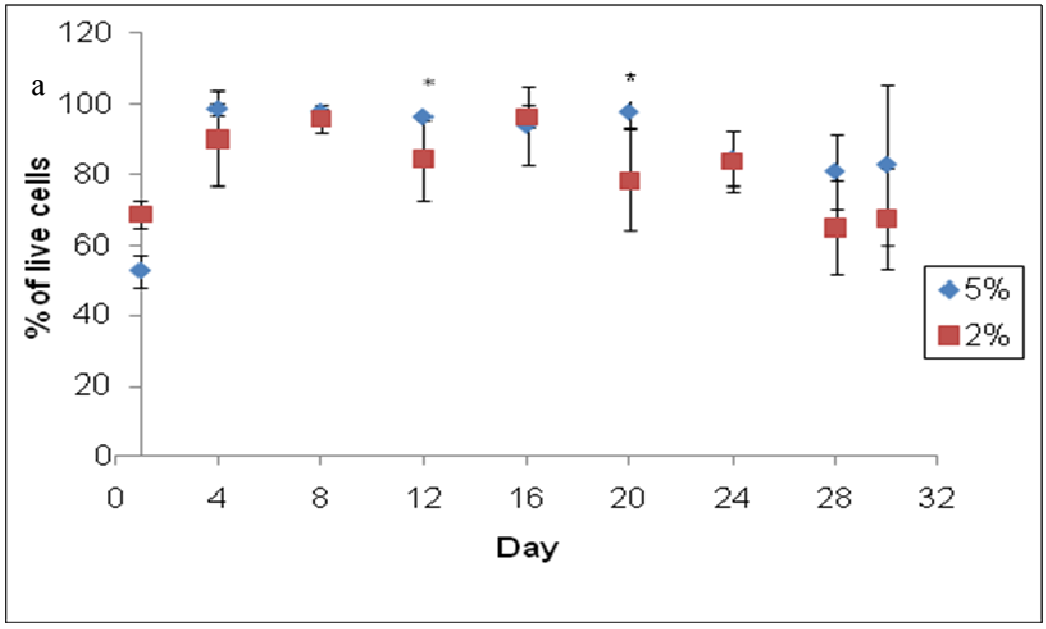


Figure 4.2: First day at which spheroid formation was detected. Specimens corresponding to the intermediate seeding density, were stained with propidium iodide and calcein AM and examined by fluorescence microscopy.



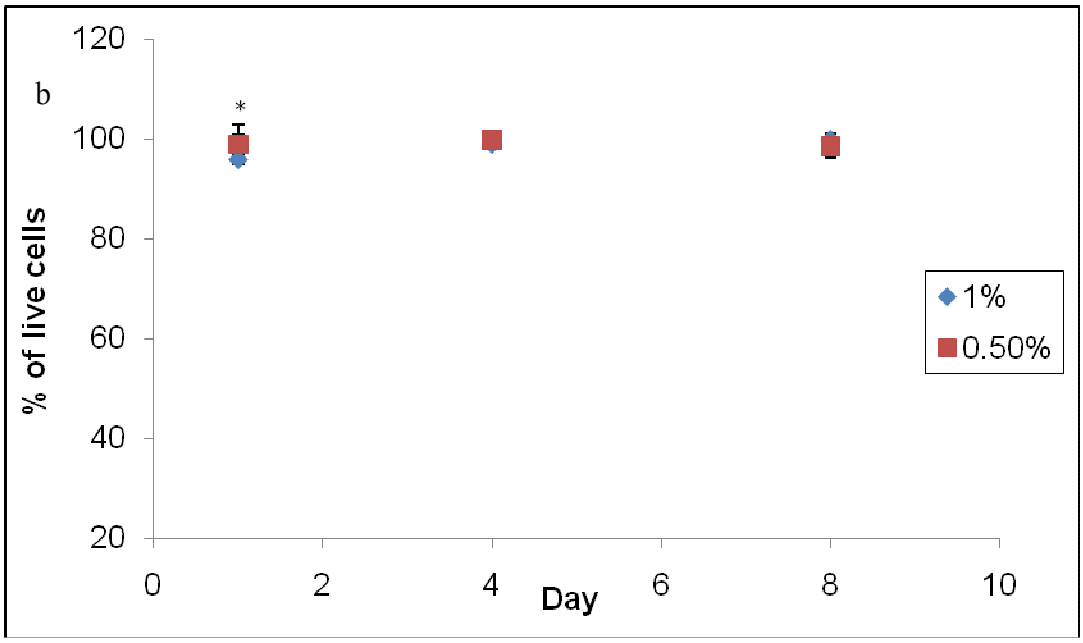


Figure 4.3: Number of viable cells, measured by fluorescence microscopy from specimens stained with propidium iodide and calcein AM. Results are shown for the intermediate seeding density of fibroblasts (0.76×10^6 cells/ml) in discs containing (a) 5 and 2 % and (b) 1 and 0.5 % alginate concentrations. Each result is the mean of three observations. Error bars represent standard deviations. Results that are significantly different ($p < 0.05$) are marked with an asterisk.

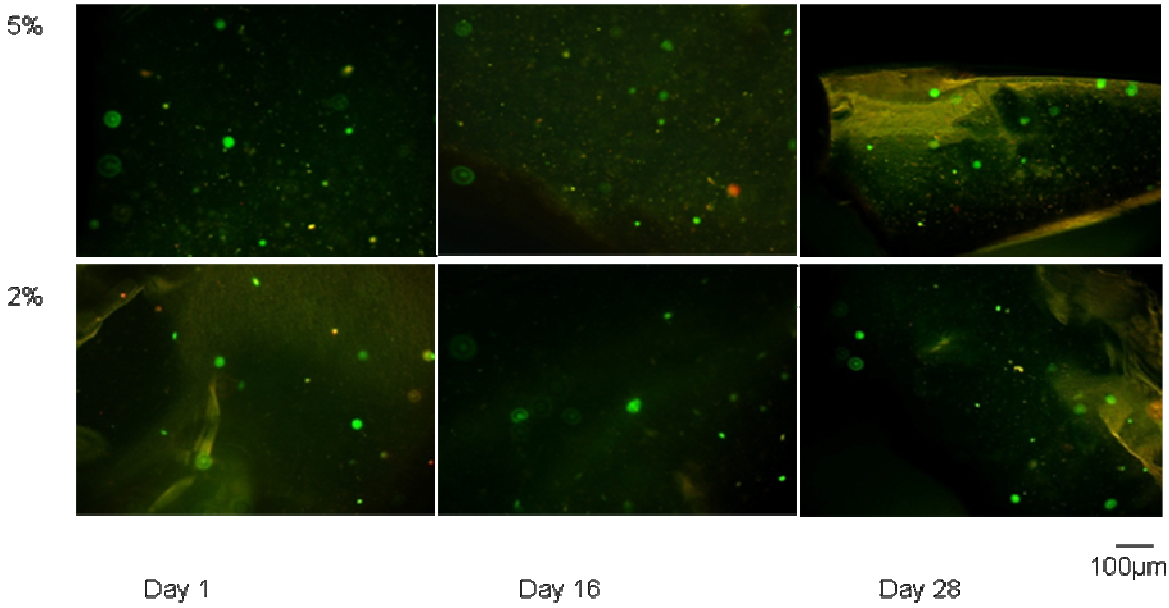


Figure 4.4: Fluoresence micrographs showing viable (green) and dead (red) cells in sections of alginate discs for the low seeding cells density (0.76×10^5 cells/ml). Micrographs were obtained after 1, 16 and 28 days.

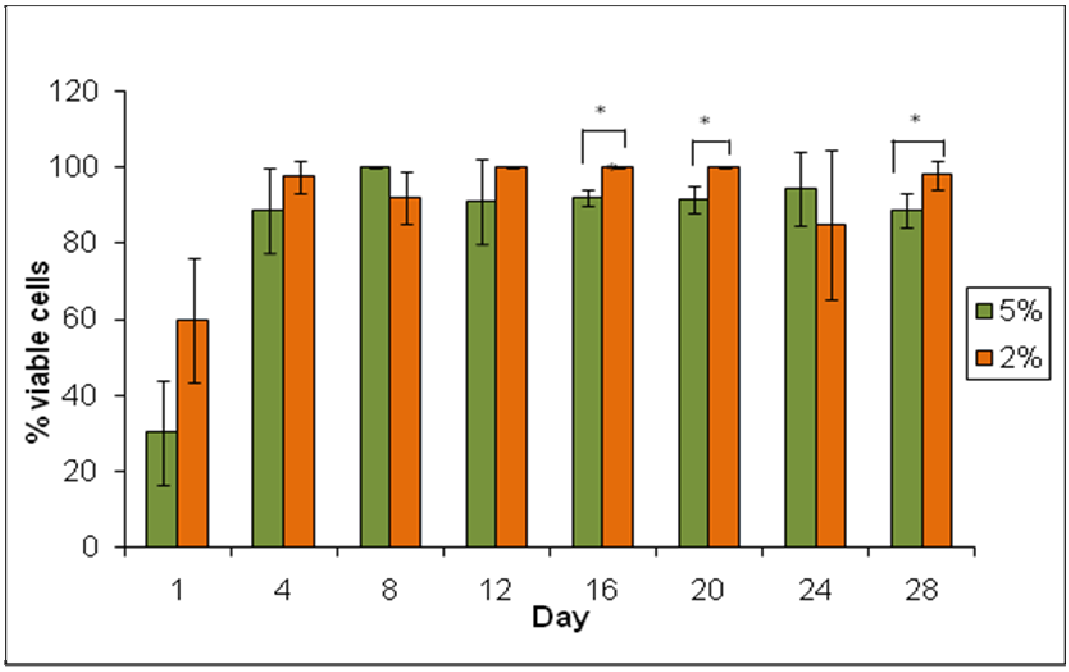


Figure 4.5: Number of viable cells, measured by fluorescence microscopy from specimens stained with propidium iodide and calcein AM. Results are show for the low seeding density of fibroblasts (0.76×10^5 cells/ml) in discs containing 2 and 5% alginate concentrations. Each result is the mean of three observations. Error bars represent standard deviations. Results that are significantly different ($p < 0.05$) are marked with an asterisk.

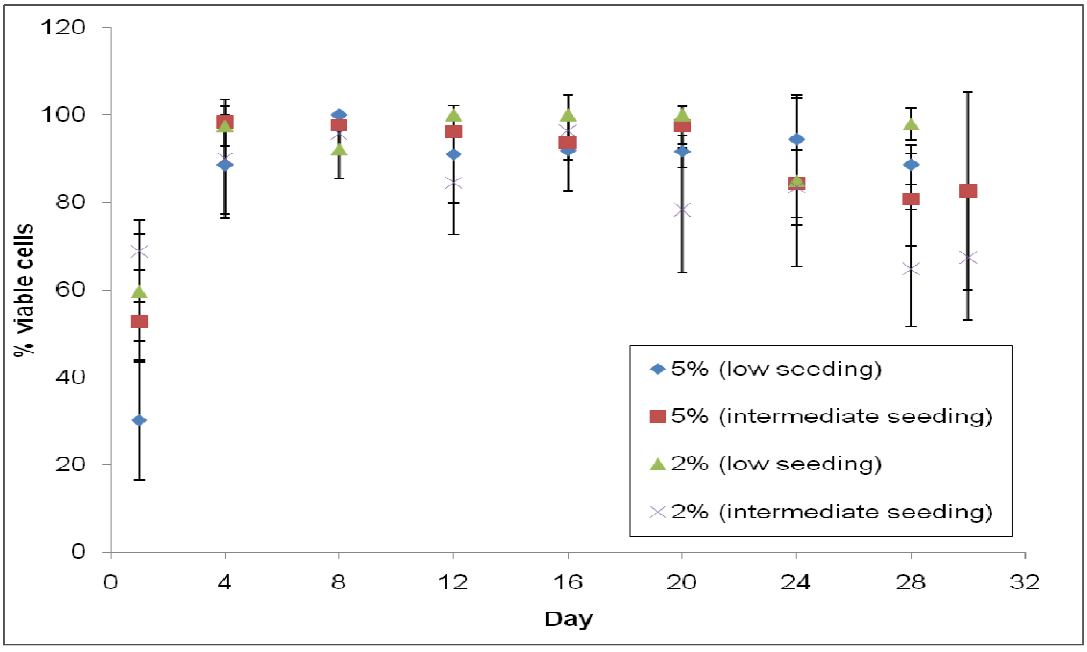


Figure 4.6: Number of viable cells, measured by fluorescence microscopy from specimens stained with propidium iodide and calcein AM. Results are show for the low and intermediate seeding densities of fibroblasts in discs containing 2 and 5% alginate concentrations. Each result is the mean of three observations. Error bars represent standard deviations.

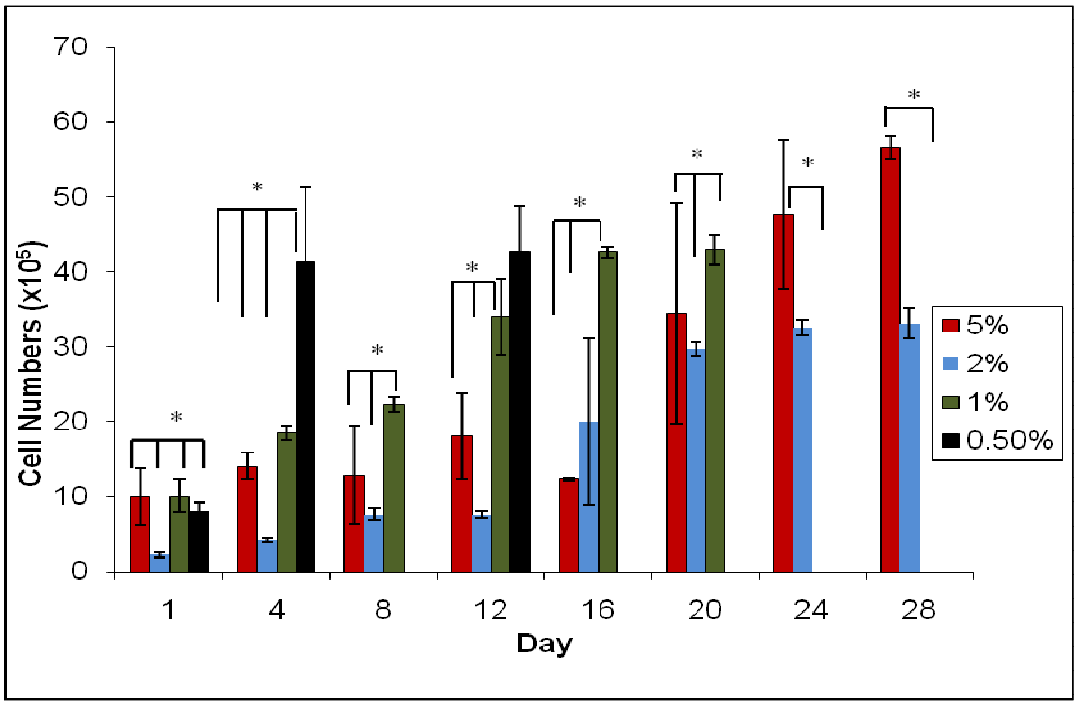


Figure 4.7: MTT Assay with different concentration of alginate for the intermediate seeding density (0.76×10^6 cells/ml). Each result is the mean of three observations. Error bars represent standard deviations**.** Results that are significantly different ($p < 0.05$) are marked with an asterisk.

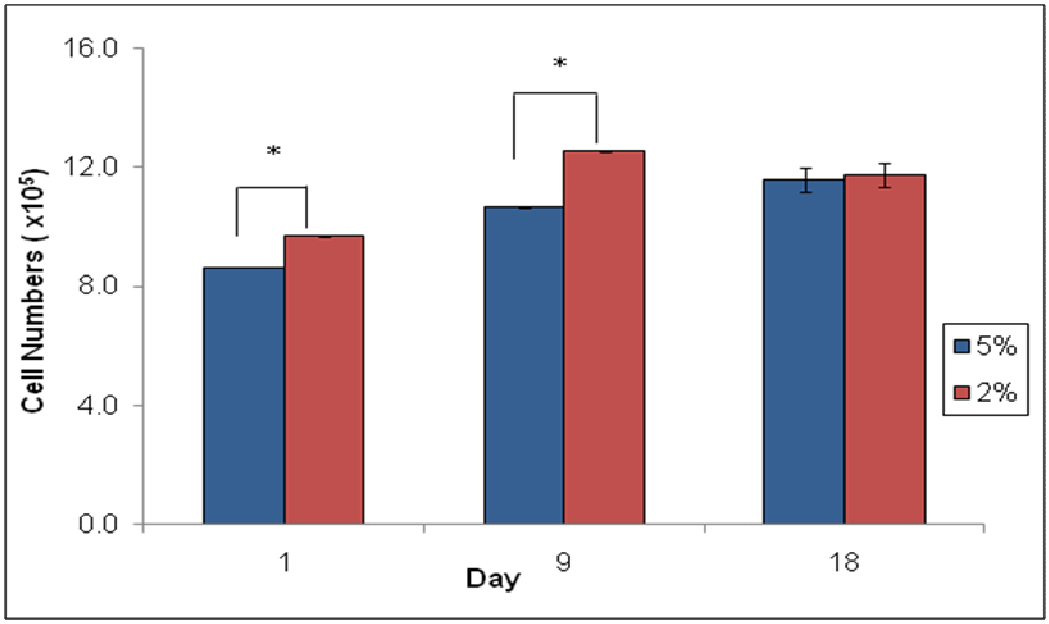


Figure 4.8: Total cell numbers measured by the Hoechst 33258 assay at day 1, 9 and 18. Each result is the mean of three observations. Error bars represent standard deviations. Results that are significantly different ($p < 0.05$) are marked with an asterisk.

4.6 Discussion

For over 30 years, alginate has been used to encapsulate prokaryotic cells [Kierstan and Bucke 1977]. Since the early reports, it has been used as a scaffold for engineering tissues *in vitro* or for the delivery of cells *in vivo* [Eiselt *et al.,* 2000]. This early work has led to research on the use of alginate to encapsulate a range of cell types. Although, on the whole, there is agreement that alginate provides a matrix in which cells can survive, different authors have reported contrasting cell responses to encapsulation. For example, some report the formation of cell spheroids within the alginate and, therefore, a continuous increase in cell number, whereas others report no significant change in cell number with time [Bartold and Page 1985, Mosahebi *et al.,* 2001]. Here it was shown that the rate of cell proliferation and also the formation of cell spheroids within the scaffold material were dependent upon the concentration of alginate used to encapsulate the cells and also the density at which the cells were seeded into the scaffold material. At lower alginate concentrations (Figure 4.3b), there was a higher rate of cell proliferation than at comparatively higher concentrations (Figure 4.3a). Furthermore there was a relationship between the first observation of cell spheroids within the scaffold and alginate concentration (Figure 4.2).

Since chemically, the different concentrations of alginate are identical, it is likely that cell number was influenced by the mechanical confinement of the cells within the structure of the scaffold [Peirone *et al.,* 1998]. *In vitro*, as the scaffold degrades there is little or no tissue ingrowth to reinforce the structure and consequently the alginate gel will become considerably weaker and will disintegrate [Drury and Mooney 2003, LeRoux *et al.,* 1999]. Disintegration will remove the mechanical constraint of the matrix and enable cell proliferation. As mentioned in section 2.4, ionically cross-linked gels, such as alginate,

normally undergo dissolution at a rate that is influenced heavily by the localised ionic environment in which the scaffold is placed [Drury and Mooney 2003, LeRoux *et al.,* 1999]. This phenomenon occurred more quickly at 0.5-1% alginate than in the range 2-5% and provides a possible explanation for the inverse relationship between the rate of cell [McConell *et al.,* 2004] proliferation and alginate concentration.

As stated in section 2.4, unmodified alginates do not encourage cellular interaction and caused the cells to form spheroids. Whether spheroid formation within the tissue engineering scaffolds is desirable or not depends on the application. For the formation of a homogeneous tissue structure, spheroid formation is not desirable since necrotic regions within the spheroids are formed probably due to insufficient nutrients and oxygen perfusion to their centre [McConell *et al.,* 2004]. In other cases, however, the formation of spheroids in the hydrogel is a requirement for cell viability and for the maintenance of cellular phenotype [Yan *et al.,* 2005, Yang *et al.,* 2002]. In the case of hepatocytes, for example, alginate has been galactosylated in order to encourage spheroid formation and hence improved liver function [Yang *et al.,* 2002]. Here, as the gel degraded with time in culture, more space would have been available that would have enabled the formation of cell spheroids (Figure 4.2). Previous work has noted that murine 3T3 fibroblasts mixed with alginate (1% concentration) at different high cells densities (2×10^6, 4×10^6 and 6×10^6 cells/ml) showed spheroid formation after two weeks in culture [Pokrywczynska *et al.,* 2008]. Human foetal kidney 293 cells encapsulated in 1.5% of alginate also formed spheroids after three weeks in culture [Read *et al.,* 2001]. Whether or not spheroids form could also be determined by the proliferation rate of the encapsulated cell population. Rapidly proliferating cells have been shown to form spheroids more quickly than those that proliferate more slowly

[Pokrywczynska *et al.,* 2008]. The findings presented in this thesis suggest that it is possible to control the cell spheroid formation process by modifying both the alginate concentration and the cell seeding density.

In addition to the influence that the alginate gel has on cell characteristics, it is essential that the alginate exhibits sufficient mechanical integrity to make it sufficiently robust for clinical application. As the alginate concentration is increased, the alginate gel will become stiffer and more durable when immersed in culture medium. A compromise must be made, however, between mechanical properties and the effect that the viscosity of the alginate solution (before gel formation) can have on the viability of the encapsulated cells. As the concentration of alginate was increased to 2 and 5 %, there were a large proportion of dead cells found within the hydrogel matrix at day one (Figure 4.1a) as consequence of increasing the viscosity. A possible explanation is that the higher concentration solutions of alginate damage the cells. As explained in section 2.10 and Chapter 3, increase in viscosity will increase the resistant of a solution to flow. As a result, it will also increase the shear stress at the cell surface and so affect the cells when they are homogenised in alginate solution.

Interestingly, although cell death in the gel matrix was apparent after one day of culture, the cell population appeared to recover quickly and levels of cell viability of >90% were reported in all conditions thereafter. This may suggest that the cells are able to rapidly proliferate into the spaces left by the dead cells in the alginate matrix, or that the propidium iodide based assay gave false positive results due to cell membrane damage that was insufficient to cause actual cell death.

5. PULSED-LOW INTENSITY ULTRASOUND STIMULATION OF 3T3 MOUSE FIBROBLASTS

5.1 Introduction

As described in section 2.8.2, the intensity of PLIUS is low enough to have minimal destructive effects on the treated area. Indeed, PLIUS does not harm cells because it has a low intensity and that the heating effect is negligible (appendix A2). This chapter is about PLIUS treatment of 3T3 mouse fibroblast encapsulated in alginate. The effect of PLIUS treatment on encapsulated cells supplemented with growth factors (ascorbic acid and TGF-β1) is also investigated.

In this study, 3T3 mouse fibroblasts were cultured in alginate discs and the effect of PLIUS on cell number (measured by Hoechst 33258 assays), collagen synthesis (measured by hydroxyproline assays), and GaG content were evaluated. The spatial distribution of GaG deposition was determined using Alcian blue staining. SEM was used for observation of fibroblast morphology.

5.2 Materials

Alginate (see section 3.2), Dulbecco's Modified Eagle Medium (DMEM), Alcian blue dye, chloramine T, 1,9 dimethylmethylene blue dye (DMB), p-dimethylaminobenzaldehyde (P-DMBA), and a DNAQF DNA Quantification Kit, were purchased from Sigma-Aldrich (Poole, Dorset, UK). Foetal calf serum was purchased from PAA Laboratories (Farnborough,

Hampshire, UK). All other materials that not mentioned here were obtained from Sigma-Aldrich.

5.3 Methods

5.3.3 Cell encapsulation

3T3 fibroblasts were cultured accordance with the method in section 4.3.3.

5.3.4 Encapsulated cells supplemented with growth factors

Cells were cultured in accordance with the protocol in section 4.3.3, but some cultures were supplemented with 50μg/ml ascorbic acid [Neidert *et al.,* 2002] and 2.5ng/ml transforming growth factor beta-1(TGF-β1) [Ross and Tranquillo 2003] to encourage extracellular matrix production.

5.3.5 PLIUS treatment

A Sonopuls 491 (Enraf-Nonius, Rotterdam, Amsterdam) ultrasound source was used (Figure 5.1). Its transducer was immersed in a water bath filled with deionized water maintained at 37°C [Doan *et al.,* 1999] and treated with a chemical solution (Sigma Clean Water bath) to keep it free from bacterial and fungal growth. The deionized water was changed every week. The six-well plate containing constructs to be treated was placed on top of the transducer; the control group was maintained in the same conditions without being exposed to ultrasound. In order to reach to a suitable treatment setting, PLIUS treatment on the cells were optimised using several different parameters (data not shown here). In this study, the ultrasound stimulation was performed 5 min everyday for 10 days at a frequency of 1 MHz and an intensity of 0.2 W/cm^2 with a 20% duty cycle (i.e. 20 pulses were emitted in 1 s, so that each

pulse had a duration of 0.05 s). Treated and control samples were analysed, at regular intervals, to determine cell numbers, collagen content and GaG content.

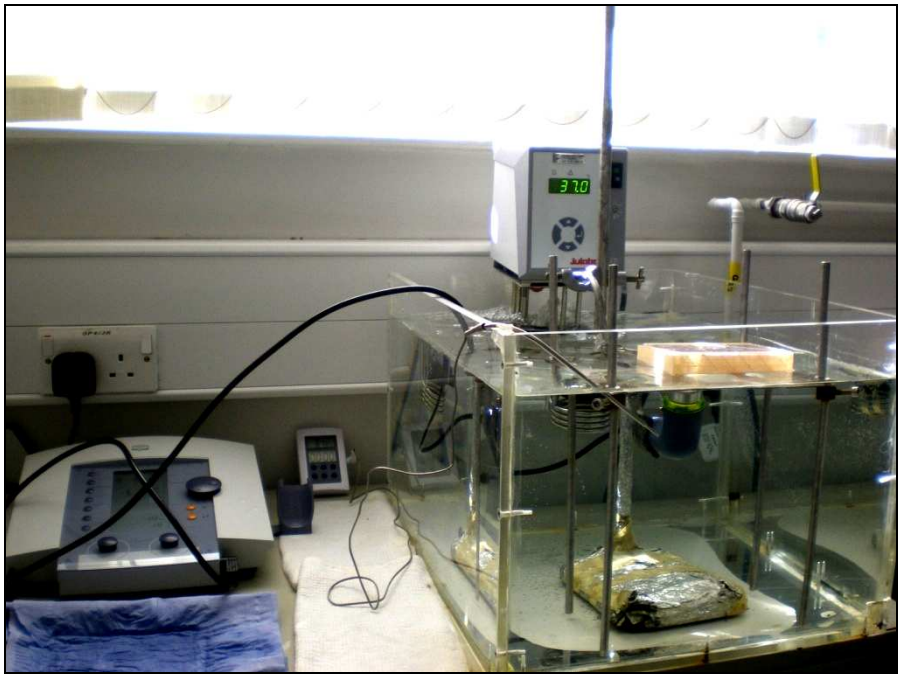


Figure 5.1: Ultrasound machine set up with the transducer immersed in the water bath at 37°C. The encapsulated cells that maintain in six-well plate containing DMEM media and were placed on top of the transducer.

5.3.6. Cell content

The method to determine the total cell number of 3T3 mouse fibroblasts was described in section 4.3.5.

5.3.7 Hydroxyproline assay (Method A)

Collagen production was quantified using a hydroxyproline assay [Awad *et al.,* 2004, Jamall *et al.,* 1981] performed on samples cultured for 4, 6, 8 and 10 days. A suspension (500 µl) of the sample that had been digested with papain was hydrolyzed with HCl (12 M) at 110°C for 20 h and then left in a vacuum incubator overnight to let all of the HCl evaporate [Reddy and Enwemeka 1996, Stegeman and Stalder 1967]. The samples were reconstituted in 50%

isopropanol with activated charcoal [Awad *et al.,* 2004, Ross and Tranquillo 2003], then filtered through filter paper (QL100, medium/fast flow rate, Fisherbrand Loughborough, Leicestershire, UK). Chloramine-T solution and p-dimethylaminobenzaldehyde (P-DMBA) solution (Ehrlich's reagent) were sequentially added to each filtered sample. The optical densities of the samples were measured using a spectrophotometer (Cecil CE 1020, Cecil Instrument, Cambridge, UK) at a wavelength of 550 nm. The hydroxyproline content of the samples was determined from a regression line of absorbance plotted against concentration (0-12 µg/ml) for trans-4-hydroxy-L-proline.

5.3.8 Hydroxyproline assay (Method B)

A second method was used for the quantification of hydroxyproline content using a method previously described [Awad *et al.,* 2004, Neidert *et al.,* 2002, Stegeman.H and Stalder 1967] with some modification. This method was cheaper and less time-consuming for running the experiment when compared to the method in section 5.3.7. With this method, 50µl of papain digested samples were hydrolyzed in HCl (6 M) at 110°C for 20 h and then kept in the vacuum desiccator overnight to allow evaporation of the remaining HCl. The dried samples were then reconstituted with 200 µl assay buffer [5g/l citric acid (monohydrate), 12 g/l sodium acetate (trihydrate), 3.4g/l sodium hydroxide, and 1.2ml/l glacial acetic acid in distilled water, at pH 6.0 [Awad *et al.,* 2004]. The reconstituted samples were then mixed with activated charcoal and left in ambient conditions for 30 min. The samples were then centrifuged at 14 000 rpm for 6 min. 50 µl of the clear samples were then mixed with chloramine T solution (62 mM) and then incubated at room temperature for 15 min to allow oxidation to occur. Then the samples were mixed with 50 µl (0.94 M) p-DMBA solution [Neidert *et al.,* 2002] and incubated at 60°C for 30 min. The absorbance of the samples was measured at 550 nm using a microplate reader (Promega glomax, Promega, Southampton,

UK). The hydroxyproline contents of the samples were determined from a regression line of absorbance plotted against concentration (0-1 µg/ml) for trans-4-hydroxy-L-proline.

5.3.9 Glycosaminoglycan (GaG) assay

The GaG content was quantified by a previously described method [Enobakhare *et al.,* 1996] with a slight modification. Briefly, 40 µl of papain digested samples were added to 250 µl DMB dye at pH 1.5. Absorbance was measured at 600 nm using a spectrophotometer. (Promega glomax, Promega, Southamton, UK). The GaG content of the samples was determined by calibrating the absorbance measurements using results from a standard solution of shark cartilage chondroitin 6 sulfate (0-100 µg/ml) from Sigma-Aldrich (Poole, Dorset, UK).

5.3.10 Alcian blue staining

Alginate discs containing cells were stained using Alcian blue dye [Shakibaei and DeSouza 1997, Stevens *et al.,* 2004]. The encapsulated cells were fixed with 10% formalin for 20 min then washed with PBS. The discs were then stained with Alcian blue dye for 48 h [0.05% Alcian blue in 3% acetic acid, pH 1.5 and 0.3M $MgCl_2$] [Shakibaei and DeSouza 1997]. The alginate/cells were then washed sequentially with 3% acetic acid, 3% acetic acid and 25% ethanol, 3% acetic acid and 50% ethanol and 70% ethanol. The encapsulated cell were observed using a light microscope (Axiolab, Zeiss, Oberkochen, Germany) [Shakibaei and DeSouza 1997, Stevens *et al.,* 2004].

5.3.11 Sample preparation for SEM

3T3 mouse fibroblasts encapsulated in alginate disc were fixed with 2.5% glutaraldehyde in 0.1M phosphate buffer for 1 h. After that the samples were dehydrated in alcohol (50%, 70%, 90%, 100%) twice for 15 min for each respective alcohol change. Then the samples were

dried using critical point CO_2 and the dried samples were mounted on a stub and coated with platinum. The samples were then viewed using scanning electron microscopy (Philips XL30 ESEM FEG, Netherlands).

5.4 Statistical analyses

The Shapiro-Wilk test [Pallant 2007] for the normality test was done for all of the data presented here. The data were analyzed using the Independent t-test for normal data and Mann-Whitney test for non-normal data [Pallant 2007]. Differences were considered to be significant if the probability $p < 0.05$.

5.5 Result

5.5.1 Total cell number

The total number of cells encapsulated in alginate was determined after 4, 6, 8 and 10 days of PLIUS treatment using the Hoechst 33258 assay. Using this method, PLIUS did not show any consistent effect on the number of encapsulated cells when compared to the control group (Figure 5.2 and Figure 5.3) or when supplemented with ascorbic acid and TGF-β1 (Figure 5.4).

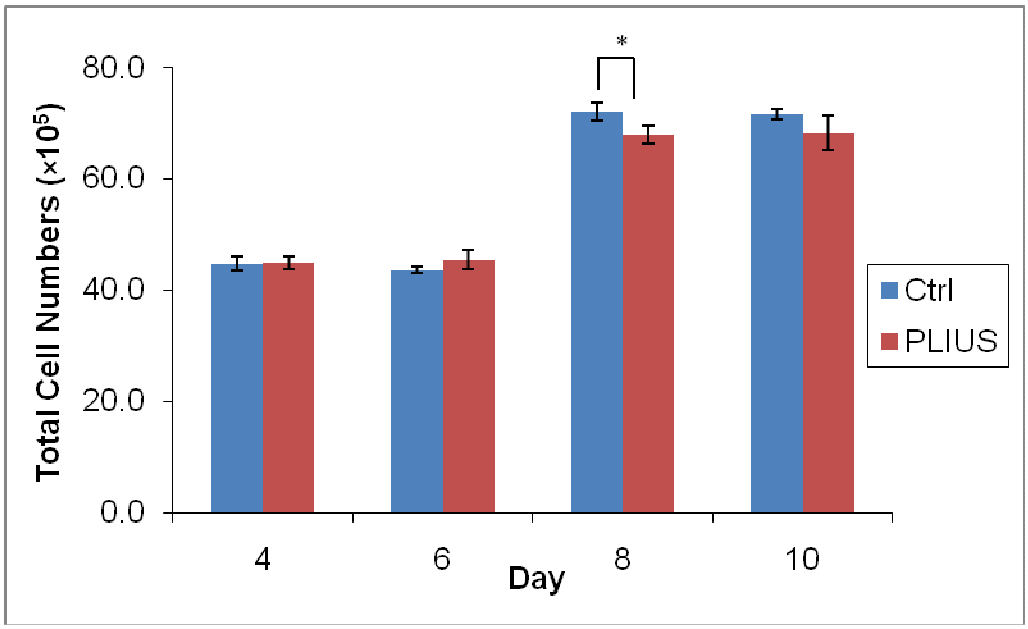


Figure 5.2: Cell proliferation study with intermediate seeding density (0.76×10^6 cells/ml) in control (Ctrl) and treated group (PLIUS) after exposure with ultrasound in culture. Each result is the mean of three observations. Error bars represent standard deviations**.** Results that are significantly different ($p < 0.05$) are marked with an asterisk.

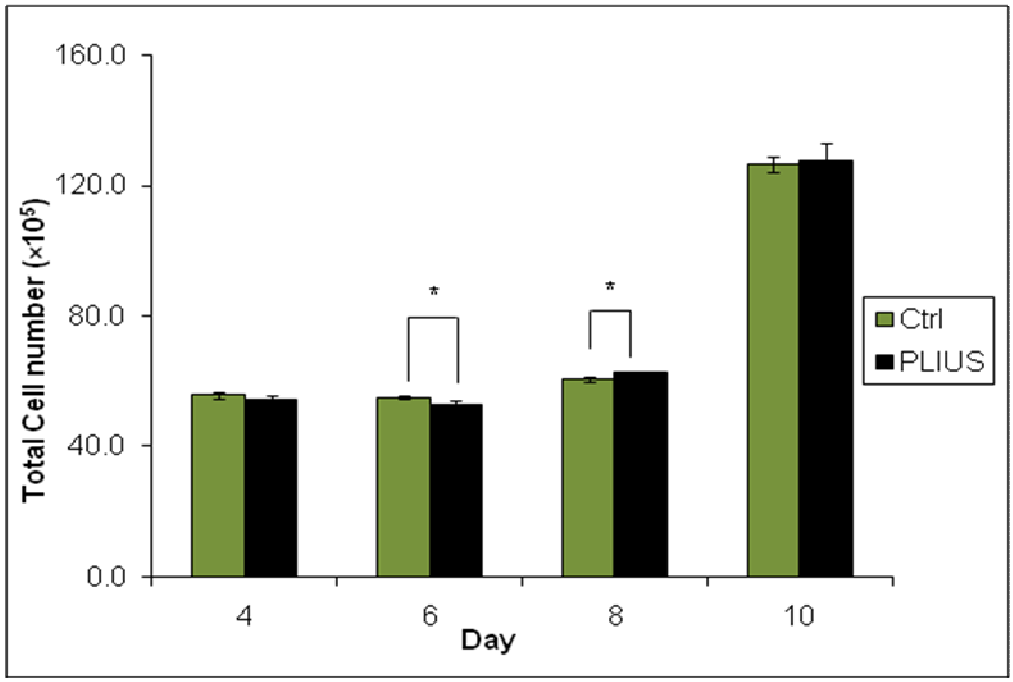


Figure 5.3: Cell proliferation study with high seeding density (2.0×10^6 cells/ml) in control (Ctrl) and treated group (PLIUS) after exposure with ultrasound in culture. Each result is the mean of three observations. Error bars represent standard deviations**.** Results that are significantly different ($p < 0.05$) are marked with an asterisk.

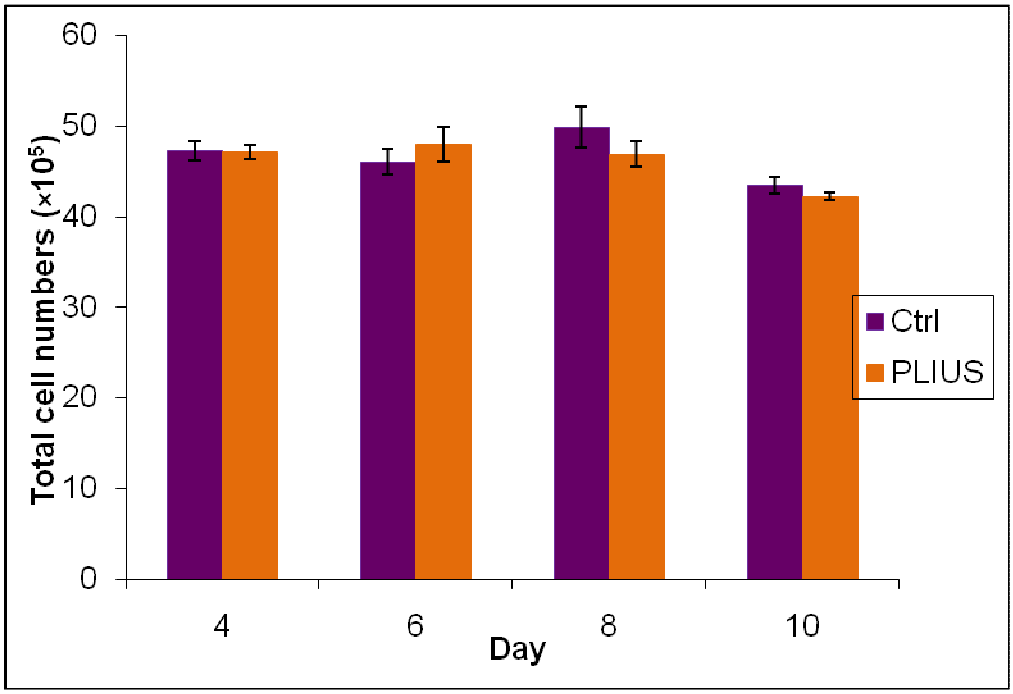


Figure 5.4: Cells proliferation study with high seeding density (2.0×10^6 cells/ml) and supplemented with growth factors (ascorbic acid and TGF-β1) in control (Ctrl) and treated group (PLIUS) after exposure with ultrasound in culture. Each result is the mean of three observations. Error bars represent standard deviations.

5.5.2. Collagen content

A hydroxyproline assay was undertaken to assess the ability of PLIUS to stimulate collagen production of the encapsulated cells. The experiments were done for two different seeding densities (0.76×10^6 cells/ml and 2.0×10^6 cells/ml) with two different medium conditions (with or without the growth factors).

When seeded at 0.76×10^6 cells/ml, as shown in figure 5.5, the group treated with PLIUS appeared to produce collagen and reaches statistical significance ($p < 0.05$) at day 8 only. Consequently, the PLIUS effect at seeding density 0.76×10^6 cells/ml appears to be negligible.

At the higher cell density (2.0×10^6 cells/ml) (Figure 5.6) collagen production increased significantly ($p < 0.05$) for the treated group (PLIUS) at day 6 and day 10 in culture.

It has been described in section 2.7 that growth factors (ascorbic acid and TGF-β1) are involved in collagen production. Here, (Figure 5.7), it shows that the hydroxyproline content of encapsulated cells (2.0×10^6 cells/ml) treated with PLIUS in a medium supplemented with growth factors (ascorbic acid and TGF-β1) appeared to increase the accumulation of collagen from day 4 to day 6 when compared to the control group. Comparing the total amounts of hydroxyproline with (Figure 5.7) and without the growth factors (Figure 5.5 and Figure 5.6), it seemed that encapsulated cells treated with growth factors and PLIUS might have more beneficial effect on increasing collagen content than the PLIUS alone on 3T3 fibroblasts.

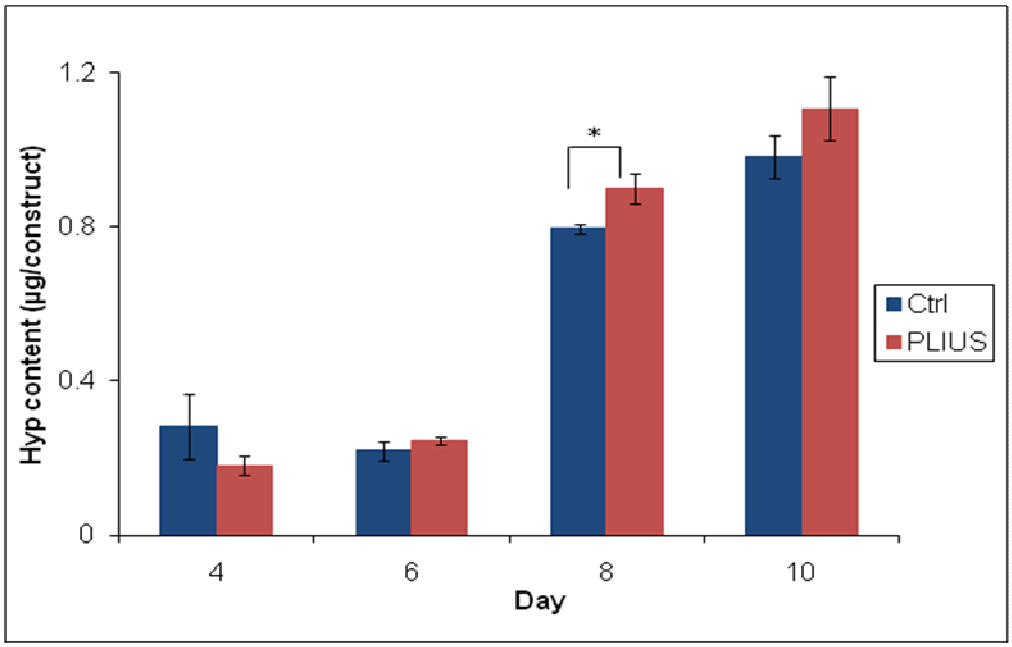


Figure 5.5: Hydroxyproline (Hyp) content by using Method A with intermediate seeding density (0.76×10^6 cells/ml) in control (Ctrl) and treated group (PLIUS) after exposure with ultrasound in culture. Each result is the mean of three observations. Error bars represent standard deviations. Results that are significantly different (p <0.05) are marked with an asterisk.

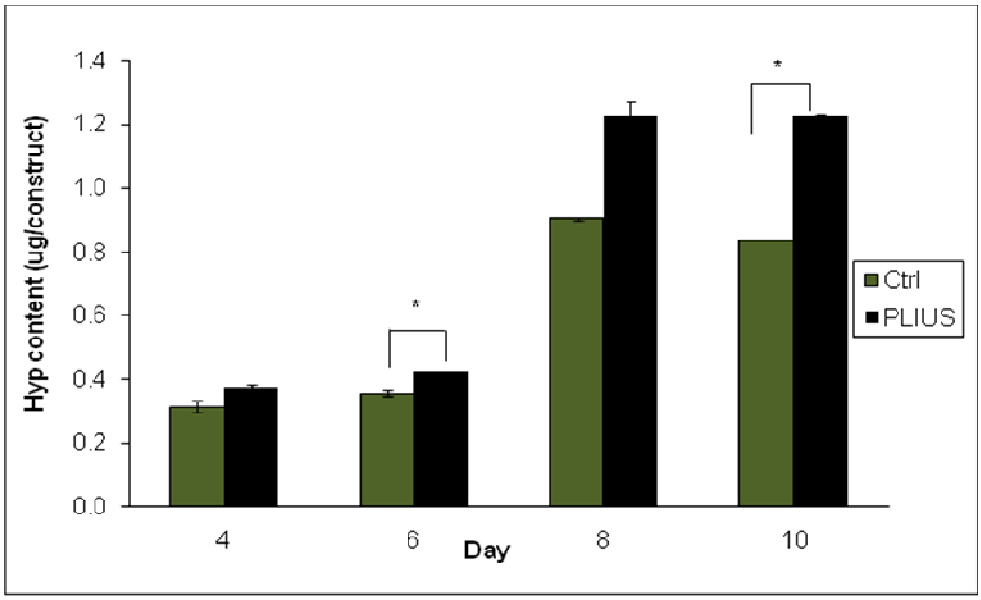


Figure 5.6: Hydroxyproline (Hyp) content by using Method A with high seeding density (2.0×10^6 cells/ml) in control (Ctrl) and treated group (PLIUS) after exposure with ultrasound in culture. Each result is the mean of three observations. Error bars represent standard deviations. Results that are significantly different (p <0.05) are marked with an asterisk. However, no significance value on day 8 (p = 0.05).

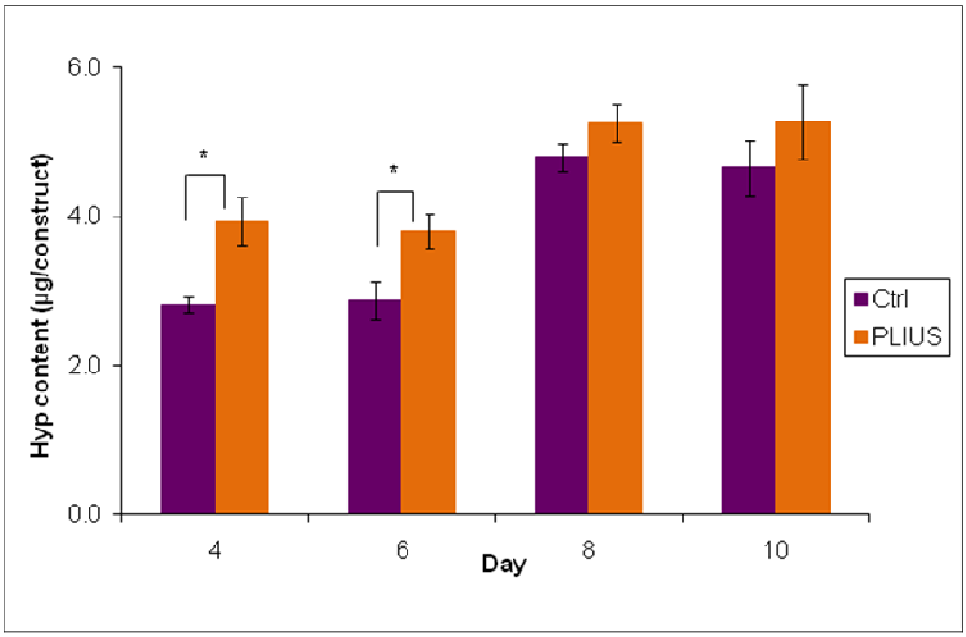


Figure 5.7: Hydroxyproline (Hyp) content by using Method B with high seeding density (2.0×10^6 cells/ml) supplemented with growth factors (ascorbic acid and TGF-β1) in control (Ctrl) and treated group (PLIUS) after exposure with ultrasound in culture. Each result is the mean of three observations. Error bars represent standard deviations**.** Results that are significantly different (p <0.05) are marked with an asterisk.

5.5.3 Glycosaminoglycan (GaG) detection

To further investigate the effect of PLIUS on the encapsulated cells, the DMB assay was undertaken to quantify GaG production for up to 10 days. The encapsulated cells (Figure 5.8 and Figure 5.10) that were treated with PLIUS did not produce a significantly higher quantity of GaG when compared to the control at intermediate seeding density (0.76×10^6 cells/ml) and high seeding density (2.0×10^6 cells/ml) supplemented with ascorbic acid and TGF-β1.

Encapsulated cells at a seeding density of 2.0×10^6 cells/ml (Figure 5.9) produced significantly higher GaG levels when compared to the control group after 10 days of treatment ($p < 0.05$). But the GaG levels did not vary markedly when compared to the control at days 4, 6 and day 8. Furthermore, the results shown that PLIUS, alone (Figure 5.8 and 5.9) or in conjunction with the growth factors (ascorbic acid and TGF-β1) (Figure 5.10), shows a fluctuating pattern of stimulating the GaG productions.

GaG accumulation in the encapsulated fibroblasts was examined using Alcian blue staining. On day 10, as shown in Figure 5.11, the stained sample showed the formation of proteoglycan- rich matrix around the fibroblast cells indicating the cells had begun to produce GaGs.

Alcian blue staining revealed the accumulation of GaG for the whole experimental period. From these images, there are no clear difference in GaG production for control and treated (PLIUS) groups.

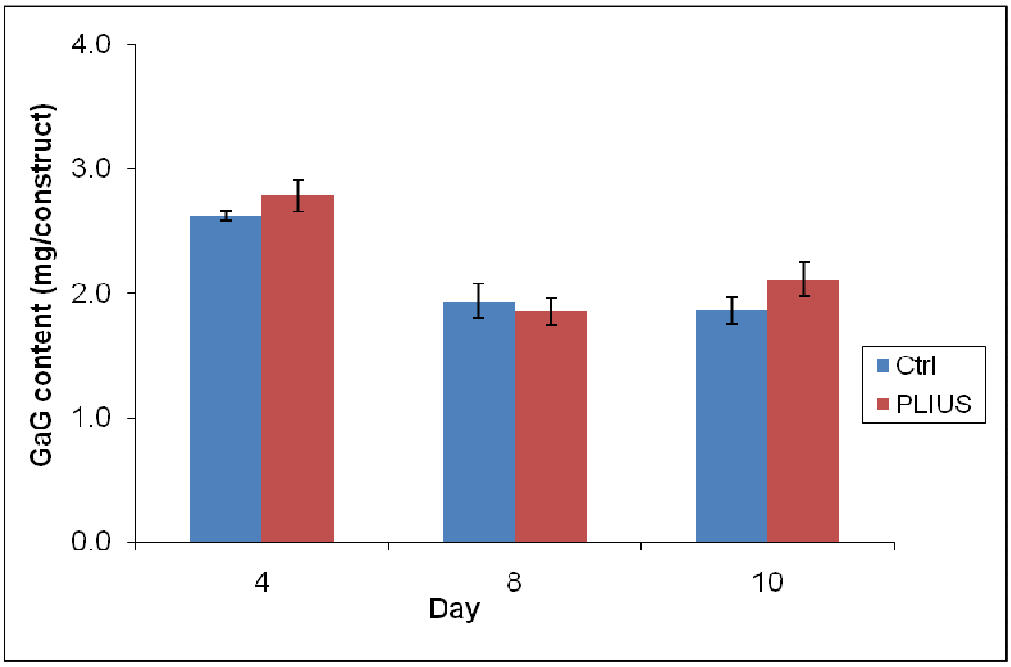


Figure 5.8: Glycosaminoglycan (GaG) content with intermediate seeding density (0.76×10^6 cells/ml) in control (Ctrl) and treated group (PLIUS) after exposure with ultrasound in culture. Each result is the mean of three observations. Error bars represent standard deviations.

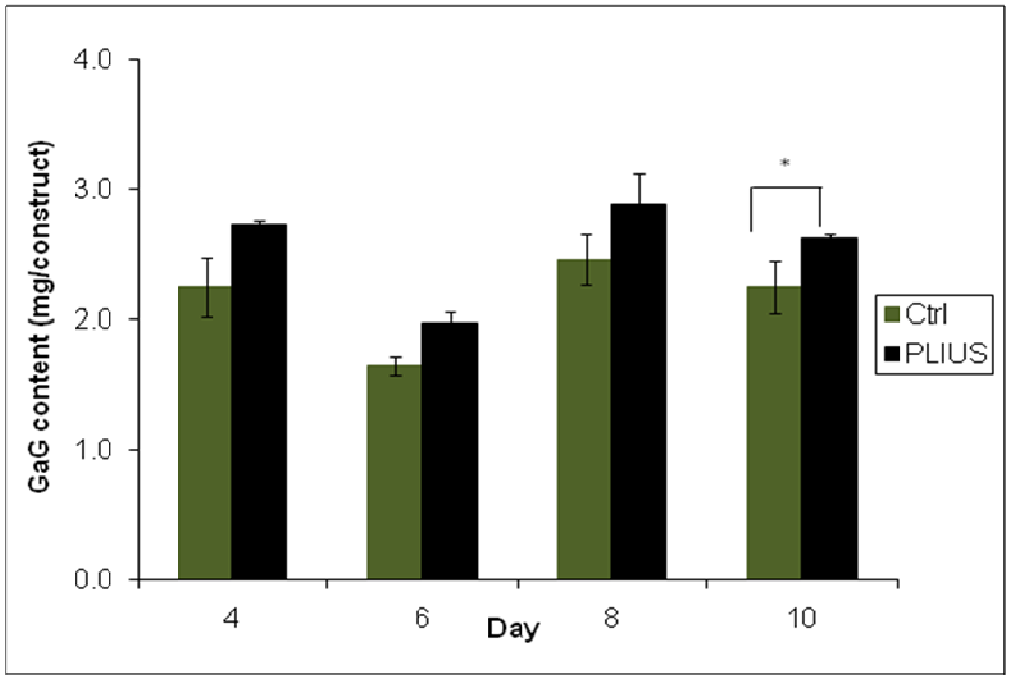


Figure 5.9: Glycosaminoglycan (GaG) content with high seeding density (2.0×10^6 cells/ml) in control (Ctrl) and treated group (PLIUS) after exposure with ultrasound in culture. Each result is the mean of three observations. Error bars represent standard deviations**.** Results that are significantly different ($p < 0.05$) are marked with an asterisk.

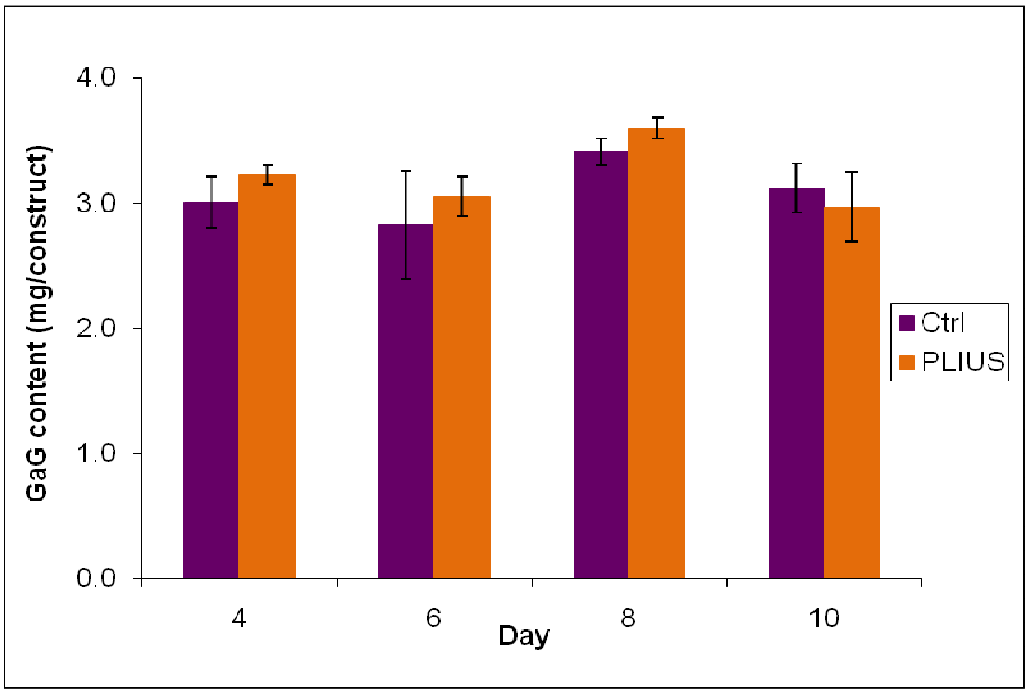


Figure 5.10: Glycosaminoglycan (GaG) content with high seeding density (2.0×10^6 cells/ml) supplemented with growth factors (ascorbic acid and TGF-β1) in control (Ctrl) and treated group (PLIUS) after exposure with ultrasound in culture. Each result is the mean of three observations. Error bars represent standard deviations.

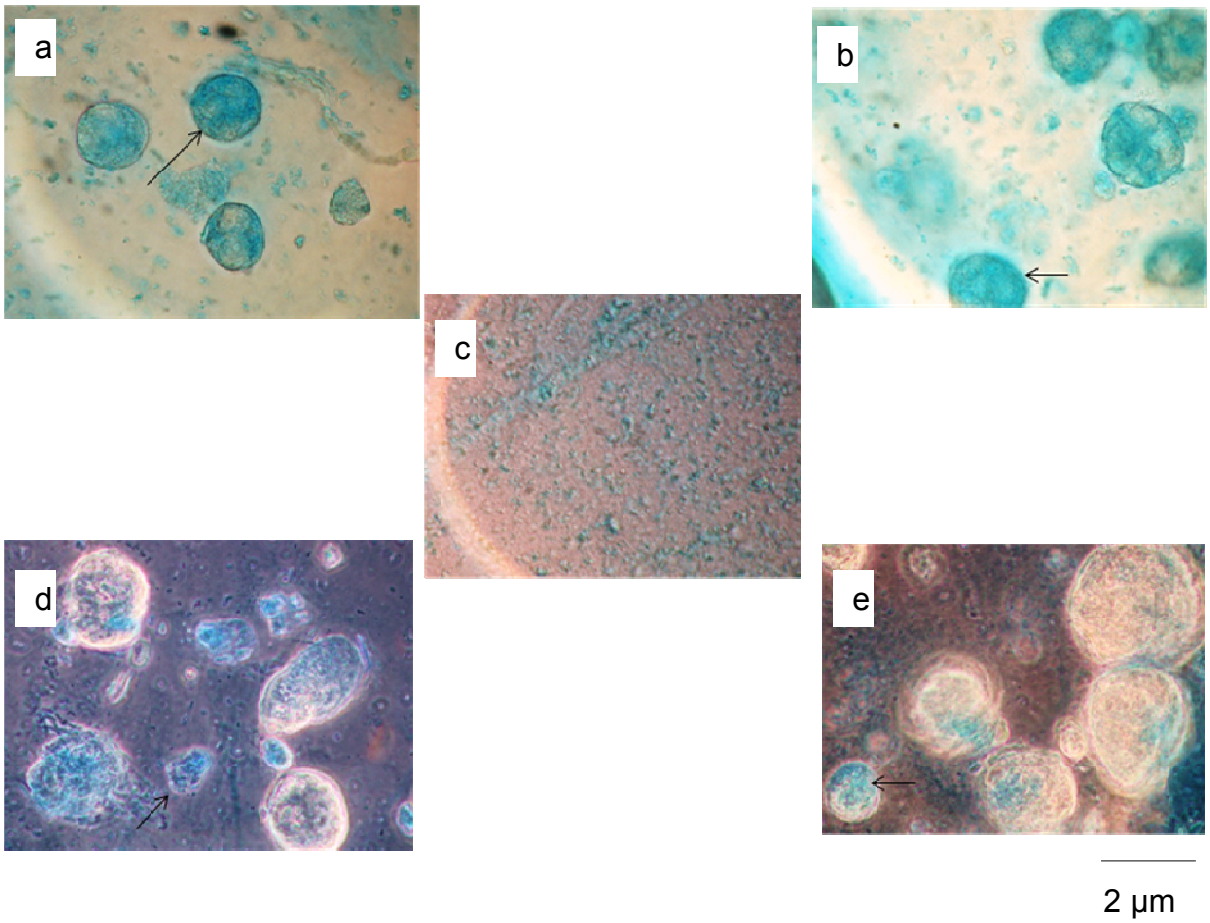


Figure 5.11: Alcian blue staining of 3T3 mouse fibroblast encapsulated alginate after 8 days in culture. The blue dark surrounding the cells shows detection of GaG (arrow). (a and b): Control and treated group with seeding density 2.0×10^6 cells/ml, (c): Alginate disc only, (d and e) : Control and treated group with seeding density 2.0×10^6 cells/ml supplemented with growth factors. All micrograph are at the same magnification.

5.5.4 SEM

Figure 5.2, 5.3 and 5.4, show that PLIUS has no effect on the viability/proliferation of 3T3 mouse fibroblasts. This is probably due to lack of adhesion between 3T3 cells and alginate. This was confirmed by SEM images (Figure 5.12) which show floating/aggregation of cells encapsulated in alginate discs. Indeed, as explained in section 2.4.1, alginate does not interact with mammalian cells unless it is chemically modified.

In this study, even without modification, alginate itself was able to maintain cell viability and enhanced collagen productions when treated with PLIUS alone (Figure 5.5 and Figure 5.6). Additionally, when the cells were encapsulated in alginate containing growth factors (ascorbic acid and TGF-β1) as shown in figure 5.7, the encapsulated cells show more collagen production than the cells encapsulated in alginate without growth factors (Figure 5.6). This is supported by a previous study; when transforming growth factor (TGF β) was tethered to PEG, (polyethylene glycol) it enhanced ECM of smooth muscle cell [Mann *et al.,* 2001]. Furthermore, cells encapsulated with growth factor (ascorbic acid and TGF-β1) and treated by PLIUS exposure (Figure 5.7), produce more collagen than with PLIUS exposure alone (Figure 5.5 and 5.6).

Nevertheless, this study shows that the cells still survive in alginate even without modification or growth factors.

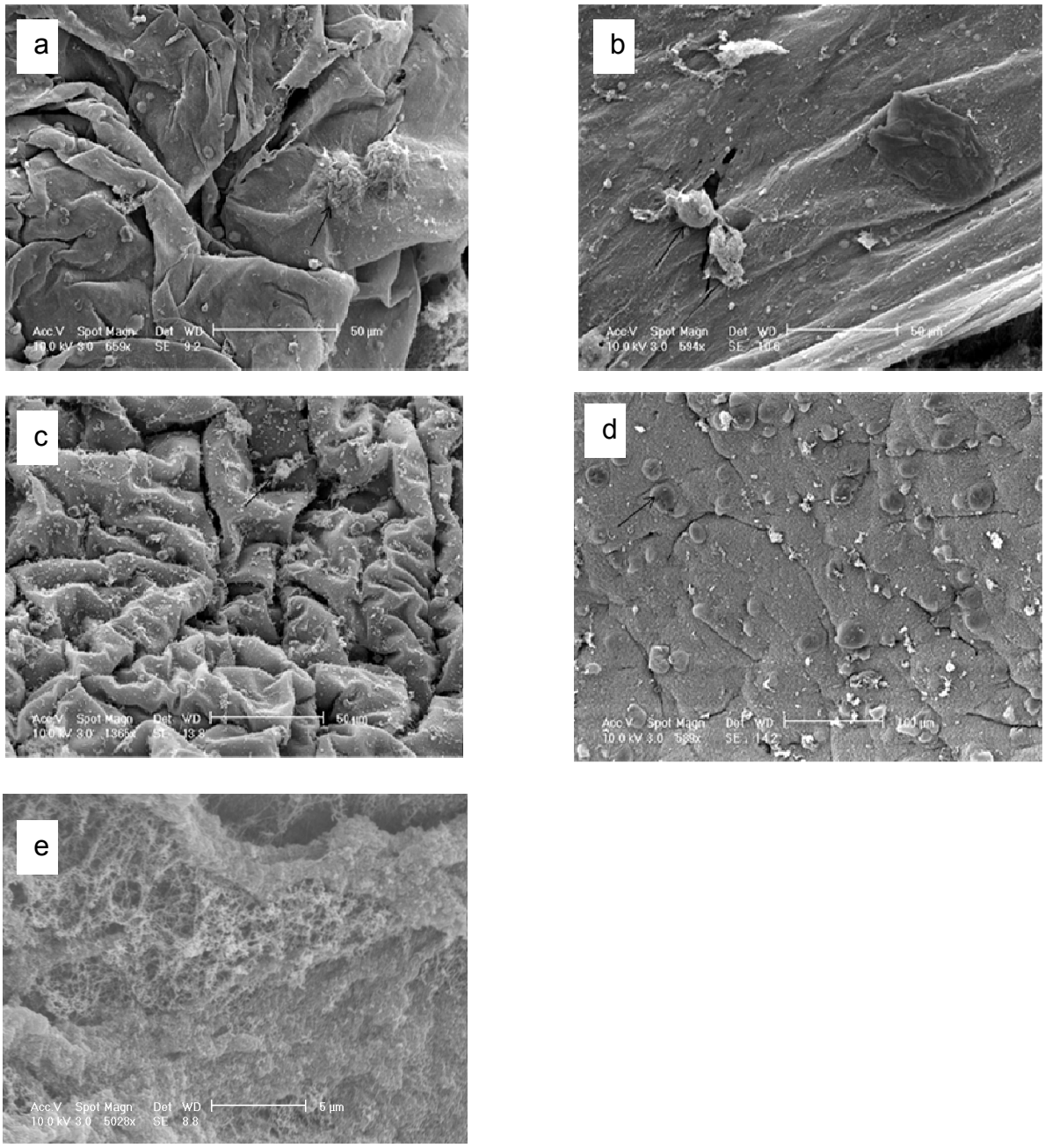


Figure 5.12: Scanning electron micrographs of a cross section of alginate disc showing 3T3 mouse fibroblast cells encapsulated in alginate disc after 10 days in culture. Viable cells on the surface of alginate disc (arrow). (a): Control group and (b): Treated group at seeding density 2.0×10^6 cells/ml, (c): Control group and (d): Treated group at seeding density 2.0×10^6 cells/ml supplemented with ascorbic acid and TGF-β1, (e): Alginate disc only.

5.6 Discussion

The results presented here suggest that PLIUS has no appreciable effect on 3T3 fibroblasts proliferation. This finding was supported by a previous study by Miyamoto *et al*. (2005) who showed that bovine intervertebral disc cells encapsulated in alginate did not show any significant increase in cell number. However, there were also contradictory results that suggested that PLIUS can increase DNA synthesis (i.e. cell proliferation) in monolayers and when encapsulated [Doan *et al.,* 1999, Iwashina *et al.,* 2006]. Nevertheless, there are many factors that may influence these contradictory results. The PLIUS frequency and intensity, cell types and cell conditions that were used in the present study were totally different to those used in previous studies [Doan *et al.,* 1999, Iwashina *et al.,* 2006]. There is no suggestion, in the work reported here or any of the papers cited, that treating the cells with PLIUS decreases the number of cells, i.e. the cells are not harmed by treatment with ultrasound under these conditions.

Still, ultrasound has proved to be beneficial in increasing ECM production. Therapeutically, non-thermal effects play a primary role in many previous findings. Specifically PLIUS or non-pulsed low intensity ultrasound stimulates collagen synthesis in fibroblasts [Doan *et al.,* 1999, Harvey *et al.,* 1975, Ramirez *et al.,* 1997, Reher *et al.,* 1998, Tsai *et al.,* 2006, Webster *et al.,* 1980]. As in this study, the PLIUS treatment that has been used shows non-thermal effects (Appendix A2) and, as reported before, non-thermal effects can be divided into cavitation and acoustic streaming [Baker *et al.,* 2001, Johns 2002, ter Haar 2007]. Previous work demonstrated that cavitation occurs when fibroblasts are exposed to PLIUS *in vitro* and stimulates collagen synthesis [Webster *et al.,* 1980]. Contrary to the previous study, the non-thermal effect in the present study involved none of these. As mentioned in Appendix (A2),

cavitation is a formation and collapsing microscopic bubbles that increases the temperature and causes damage or kills the cells [Johns 2002]. However, none of these phenomena appear to be seen in this study. As in Appendix A2, the temperature rise was low enough to be considered negligible and the cells also showed no damage (section 5.5.4) and also proliferated (section 5.5.1) when exposed to PLIUS.

Indeed, treatment of fibroblasts, cultured in alginate gels, with PLIUS or in conjunction with growth factors (ascorbic acid and TGF-β1) increases collagen production. This conclusion is clear from comparison of results of total cell number studies and collagen production observations. Although treatment with PLIUS alone or with growth factor supplements (ascorbic acid and TGF-β1) does not have an appreciable effect on the numbers of cells, more collagen is produced by the treated (PLIUS with or without growth factors supplement) than the control group. This result can only be explained by treated cells producing more collagen. This result is consistent with observations on a range of cell types reported in the literature. In monolayer culture, there is an increase in collagen production on exposure to PLIUS [Doan *et al.,* 1999, Reher *et al.,* 1998, Tsai *et al.,* 2006]. Moreover, cells in scaffolds also showed increased in collagen deposition when treated with PLIUS [Iwashina *et al.,* 2006, Miyamoto *et al.,* 2005, Schumann *et al.,* 2006, Zhang *et al.,* 2003].

It has also been noted that conditions for ultrasound stimulation that lead to increased cell proliferation in monolayer culture may also reduce collagen production [Doan *et al.,* 1999]. However, the negative effect on cellular proliferation following treatment with PLIUS on encapsulated cells has not been reported previously. The PLIUS treated group supplemented with growth factors (Figure 5.4) has fewer cells when compared with the PLIUS-treated

group alone (Figure 5.2 and Figure 5.3). However, the results of the treated group (PLIUS supplemented with growth factors) show more collagen production (Figure 5.7) than the results without the growth factor supplements (Figure 5.5 and Figure 5.6). It seems that fibroblasts, or specifically 3T3 fibroblasts, without growth factor supplements, are involved more in cell proliferation rather than in collagen production.

This result was supported by previous findings that growth factors accelerate ECM accumulation [Bittencourt *et al.,* 2009, Chojkier *et al.,* 1989, Clark *et al.,* 1995, Kim *et al.,* 2009, Neidert *et al.,* 2002, Park *et al.,* 2009, Ross and Tranquillo 2003]. In section 2.7, it was explained that ascorbic acid is important in functioning as a cofactor for collagen synthesis while TGF-β1 is involved in fibroblasts differentiating into myofibroblasts during wound healing. The results presented here show that the growth factors being used in this study have the effect of boosting collagen production with the aid of PLIUS.

In the GaG study, the amount of GaG in the treated group (PLIUS with or without growth factor supplements) showed no significant difference when compared to the control group (Figure 5.8 and Figure 5.10) and an apparently significant difference only after 10 days of treatment in figure 5.9 (PLIUS alone). As mentioned in a previous study [Enobakhare *et al.,* 1996], positive results in detection of GaG may be a result of interference by the alginate (as mentioned in section 2.9.2) if using DMB dye at pH 3.0. In this study, the interference was minimized by using the DMB dye at pH 1.5. [Chang *et al.,* 2001]. However, Alcian blue staining (Figure 5.10) showed that the encapsulated cells produced GaG in culture.

As seen in SEM images, cells encapsulated within the alginate disc did not adhere to the surrounding molecules (Figure 5.12). However, the lack of cell adhesion that prevents cell spreading and proliferation is not necessarily detrimental to the cells (Figure 5.2, Figure 5.3, and Figure 5.4) since that the viable cells within the matrix can maintain their viability [Shih-Feng *et al.,* 2010]. Moreover, collagen production in this study shows an increase on exposure to PLIUS alone or in conjunction with growth factors. This finding showed that the cells are involved more in producing ECM when treated with PLIUS with or without the ascorbic acid and TGF β-1 but not in cell division.

Treatment with PLIUS may be beneficial in tissue engineering for inducing fibroblasts to make stronger ECM perhaps leading to more effective tissue regeneration and repair. As explained before (section 2.2.1), ECM composition depends on the location of the cells that made them, resulting in different in their physical properties. Hukins and Aspden (1985) also mentioned that collagen reinforces ECM and, therefore, connective tissues leading, to increased stiffness and strength. Therefore, increased collagen production, as a result of ultrasound treatment, is likely to result in stronger tissues. It has been suggested previously that PLIUS, or therapeutic ultrasound generally, can enhance bone repair [Lirani-Galvao *et al.,* 2006, Malizos *et al.,* 2006], help in biological repair of intervertebral disc [Iwashina *et al.,* 2006] and shorten the time of cartilage formation in bioreactors [Hsu *et al.,* 2006]. Collagen production can also be enhanced by TGF β1, insulin and ascorbic acid [Awad *et al.,* 2003, Chojkier *et al.,* 1989, Kim *et al.,* 2009]. Physical stimulation with/without biochemical stimulation has shown a potential effect in stimulating collagen production without harming cell viability.

In future, studies on the effect of different PLIUS intensities on 3T3 mouse fibroblasts would be useful for understanding the specific conditions that can be beneficial to or harm cell viability/proliferation and ECM production.

6. PULSED –LOW INTENSITY ULTRASOUND EFFECT ON HUMAN DERMAL FIBROBLASTS

6.1 Introduction

Human dermal fibroblasts have been successfully used for treatment of sun-damaged-skin [Varani *et al.,* 1994] and fetal skin repair [Lin *et al.,* 1995]. Safe and non-invasive exposure using pulsed low-intensity ultrasound (PLIUS) may then be a useful method, alone or in conjunction with biochemical stimulation, for increasing ECM production and cell proliferation in dermatological applications. PLIUS could also be beneficial to improve wound healing for people suffering from diabetes mellitus, venous insufficiency and excessive pressure [Lai and Pittelkow 2007].

As described in Chapter 5, PLIUS was used to successfully stimulate collagen production by 3T3 mouse fibroblasts. In this chapter, the work is extended to HDF cells.

6.2 Materials

The materials that used in this study were exactly the same as those described in sections 3.2 and 5.2.

6.3 Methods

6.3.1 Human dermal fibroblasts

Fibroblasts were from a commercially available cell line (adult human dermal fibroblast (HDF), HPA Culture Collections, Porton Down, Salisbury, UK). Cells were maintained in DMEM supplemented with calf serum (10 v/v%), penicillin/streptomycin (1 v/v%), L-glutamine (2.4%), and HEPES (2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid) (2.4 v/v%). Cells were stored in sterile conditions at 37ºC and 5% CO_2 and the media was refreshed every three days.

6.3.2 Other methods

All other techniques are described in the appropriate sections: cells encapsulation method (section 4.3.3), cell encapsulation method with growth factor supplements (section 5.3.4), PLIUS treatment (section 5.3.6), cell content measurement (section 4.3.5), hydroxyproline assay method B (section 5.3.7), glycosaminoglycan (GaG) assay (section 5.3.9), Alcian blue staining (section 5.3.10) and sample preparation for SEM (section 5.3.11).

6.4 Statistical Analyses

The analyses are described in section 5.4

6.5 Results

6.5.1 Total cell number

To examine the total cell number for human dermal fibroblasts in alginate, the encapsulated cells were assayed using Hoechst 33258 dye after 4, 6, 8 and 10 days of PLIUS treatment. Throughout the experimental period, cells remained viable and showed proliferation in both groups (Figure 6.1 and 6.2). However, the group treated with PLIUS (Figure 6.1) showed

higher proliferation with a significant value at days 4 and 8 when compared to the control group.

Figure 6.2, it shows that the control group has a significance difference ($p < 0.05$) in culture supplemented with the growth factors (ascorbic acid and TGF-β1), when compared to the treated group, at day 6 only. From this result, it can be assumed that PLIUS treatment in conjunction with the growth factors (ascorbic acid and TGF-β1) did not stimulate the cells to proliferate more in culture.

As shown in figure 6.1, up until 8 days of treatment, the treated group significantly increased by a factor of two (128×10^5 cells/ml) compared with the control group (83×10^5 cells/ml) in cell number. When both experiments were compared, as shown in figure 6.1 and figure 6.2, PLIUS treatment alone (treated group) represents better stimulation of the encapsulated HDF cells than when they were supplemented with the growth factors (ascorbic acid and TGF-β1). When comparing the cell number for the PLIUS treated group (Figure 6.1) with the PLIUS with growth factors (treated group) in figure 6.2, it can be seen that PLIUS alone stimulates cell proliferation by a factor of almost three (128×10^5 cells/ml) when compared with PLIUS with growth factors (45×10^5 cells/ml). In conclusion, supplementation with growth factors (ascorbic acid and TGF-β1) does not stimulate cell proliferation when combined with PLIUS.

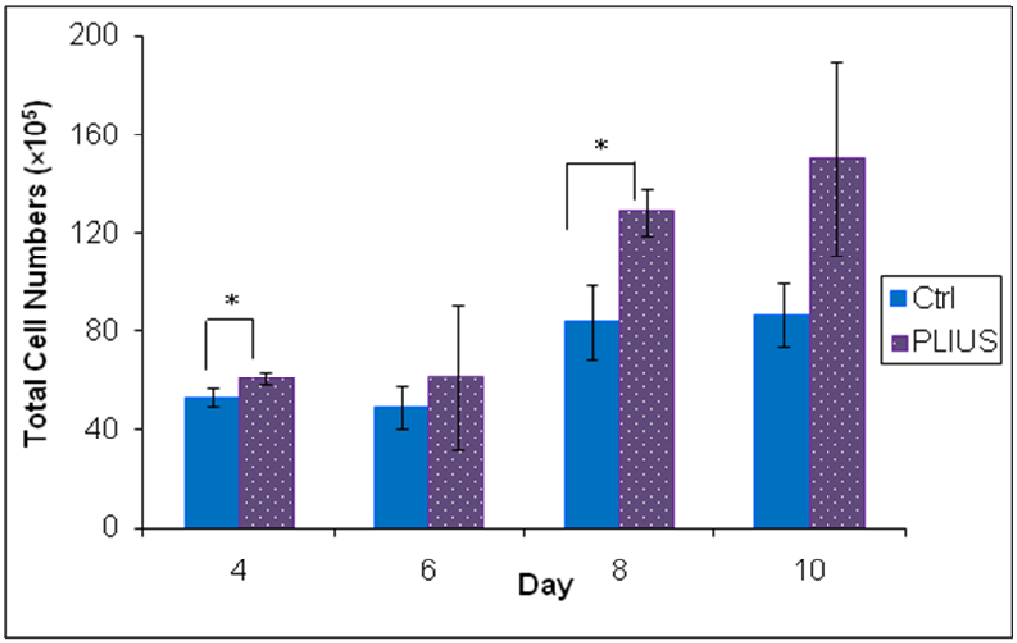


Figure 6.1: Cell proliferation study with high seeding density (2.0×10^6 cells/ml) in control (Ctrl) and treated group (PLIUS) after exposure with ultrasound in culture. Each result is the mean of three observations. Error bars represent standard deviations**.** Results that are significantly different ($p < 0.05$) are marked with an asterisk.

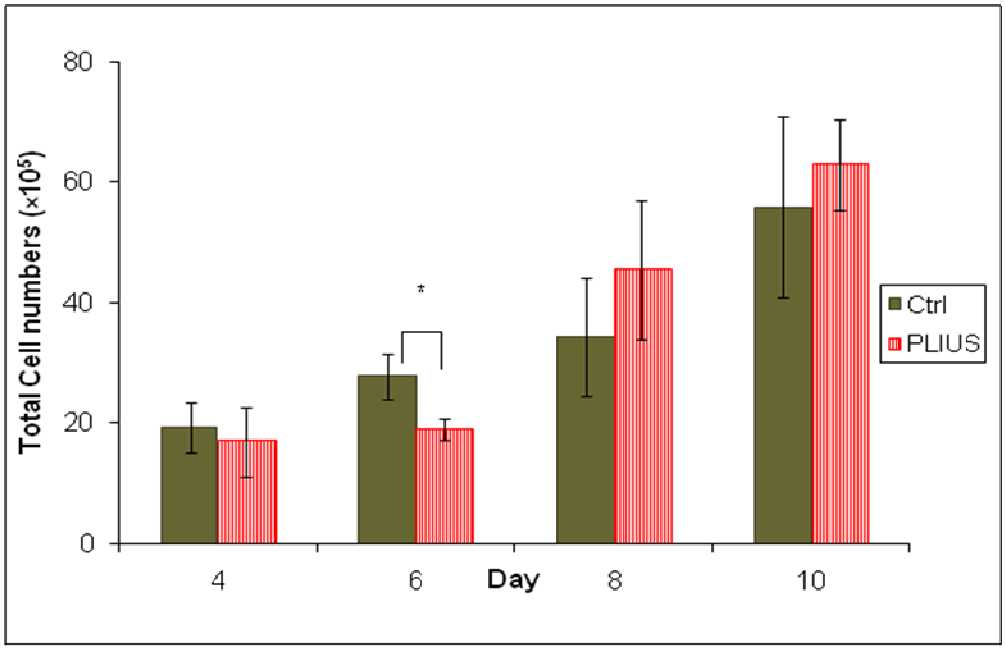


Figure 6.2: Cells proliferation study with high seeding density (2.0×10^6 cells/ml) and supplemented with growth factors (ascorbic acid and TGF-β1) in control (Ctrl) and treated group (PLIUS) after exposure with ultrasound in culture. Each result is the mean of three observations. Error bars represent standard deviations. Results that are significantly different ($p < 0.05$) are marked with an asterisk.

6.5.2. Collagen content

This assay was done to assess the ability of PLIUS, alone or in conjunction with growth factors (ascorbic acid and TGF-β1), in stimulating collagen production in the encapsulated cells. The encapsulated cells that were treated with PLIUS alone showed a significant increase in hydroxyproline content ranging from 3.5µg to 6.7µg after 4 ($p < 0.01$), 6, 8 and 10 days ($p < 0.05$) of treatment (Figure 6.3).

In contrast to the result in figure 6.3, the experiment in conjunction with the growth factors showed a fluctuating pattern (Figure 6.4) with hydroxyproline content ranging from 2.9 µg to 5.8 µg, although this was only significant ($p < 0.05$) at day 6 for the whole experimental period. From these results, it appears that PLIUS alone is able to promote collagen production in HDF cells encapsulated in alginate.

6.5.3 Glycosaminoglycan (GaG) detection

The GaG content was quantified by the DMB assay. With this assay, the application of PLIUS alone has no significant effect on the treated group (Figure 6.5) for up to 10 days of treatment. When the growth media were supplemented with growth factors (Figure 6.6), a significant effect ($p < 0.01$) was obtained at day 8 but not at any other time, so there was no consistent significant result.

GaG accumulation in the encapsulated HDF cells was examined by Alcian blue staining. After 8 days as shown in Figure 6.7, the stained sample showed halos of Alcian blue around the cells indicating that they had begun to produce GaGs. Alcian blue staining revealed the formation of GaGs for the whole experimental period. Although the results were qualitative, they showed no difference in GaG production for control and treated groups.

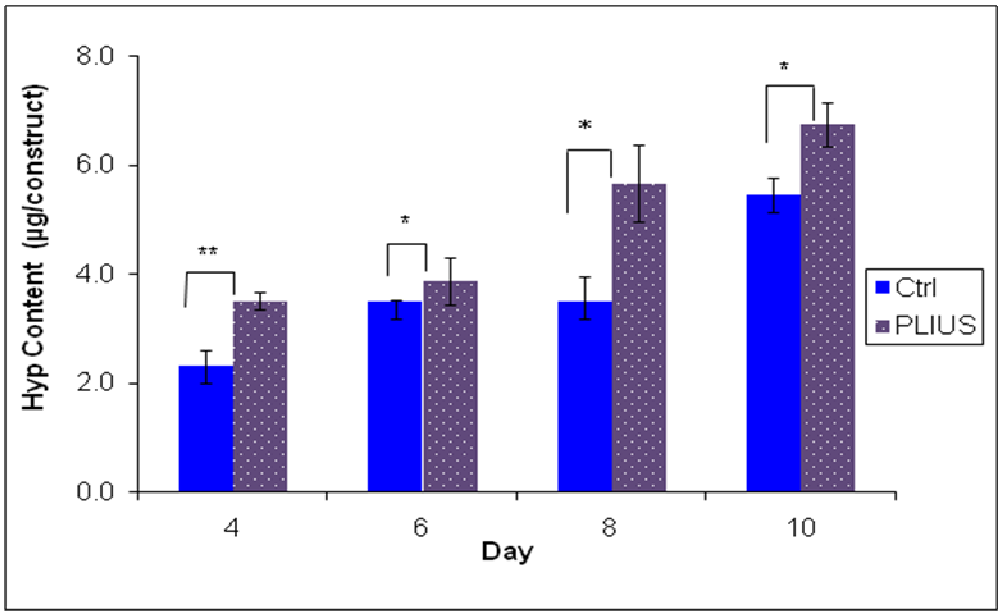


Figure 6.3: Hydroxyproline (Hyp) content with high seeding density (2.0×10^6 cells/ml) in control (Ctrl) and treated group (PLIUS) after exposure with ultrasound in culture. Each result is the mean of three observations. Error bars represent standard deviations. Results that are significantly different ($p < 0.05$,<0.01) are marked with an asterisk.

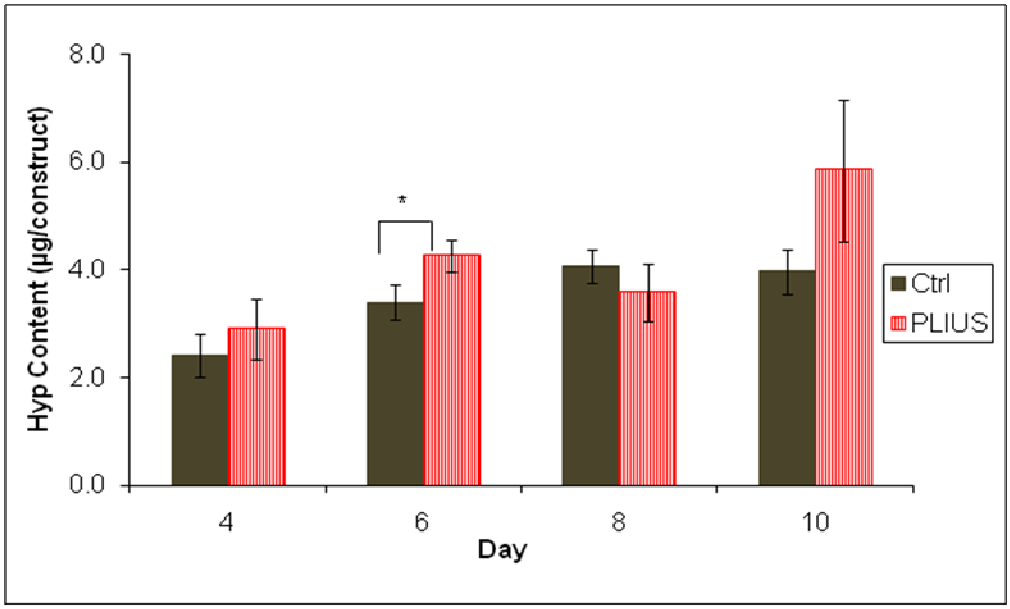


Figure 6.4: Hydroxyproline (Hyp) content with high seeding density (2.0×10^6 cells/ml) supplemented with growth factors (ascorbic acid and TGF-β1) in control (Ctrl) and treated group (PLIUS) after exposure with ultrasound in culture. Each result is the mean of three observations. Error bars represent standard deviations**.** Results that are significantly different ($p < 0.05$) are marked with an asterisk.

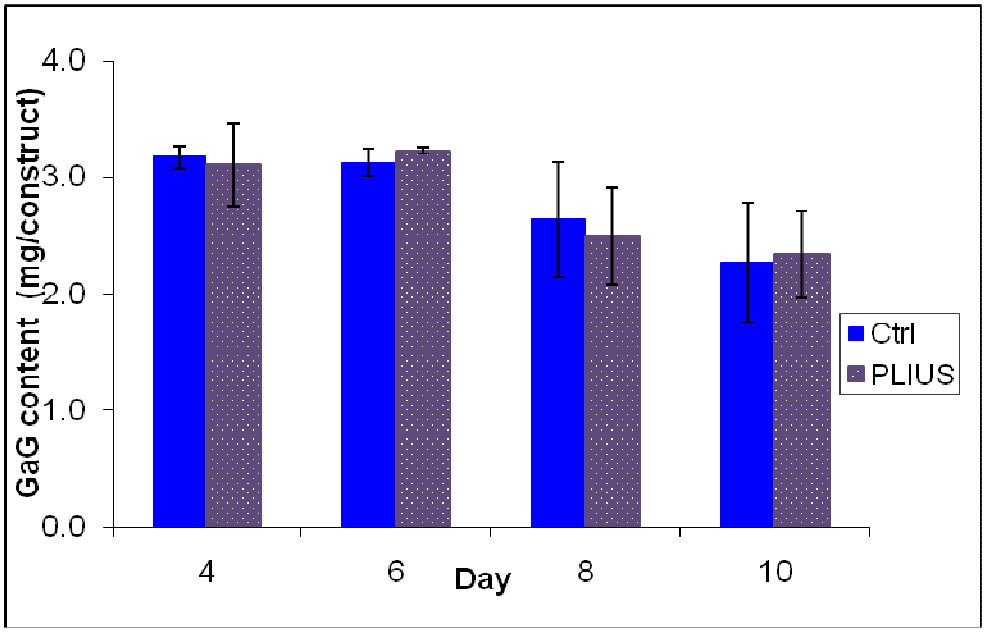


Figure 6.5: Glycosaminoglycan (GaG) content with high seeding density (2.0×10^6 cells/ml) in control (Ctrl) and treated group (PLIUS) after exposure with ultrasound in culture. Each result is the mean of three observations. Error bars represent standard deviations.

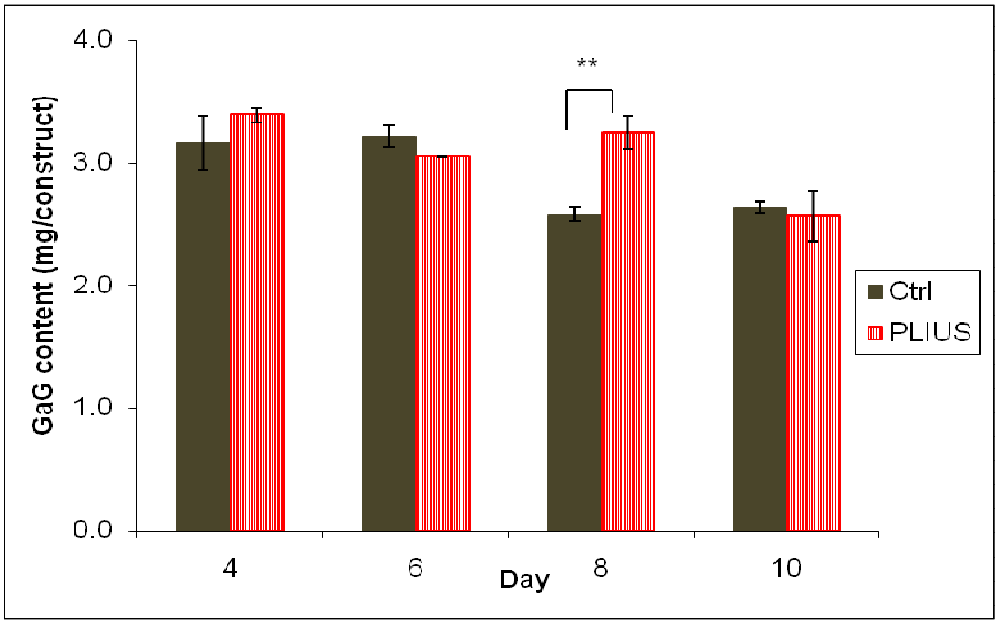


Figure 6.6: Glycosaminoglycan (GaG) content with high seeding density (2.0×10^6 cells/ml) supplemented with growth factors (ascorbic acid and TGF-β1) in control (Ctrl) and treated group (PLIUS) after exposure with ultrasound in culture. Each result is the mean of three observations. Error bars represent standard deviations**.** Results that are significantly different ($p < 0.01$) are marked with an asterisk.

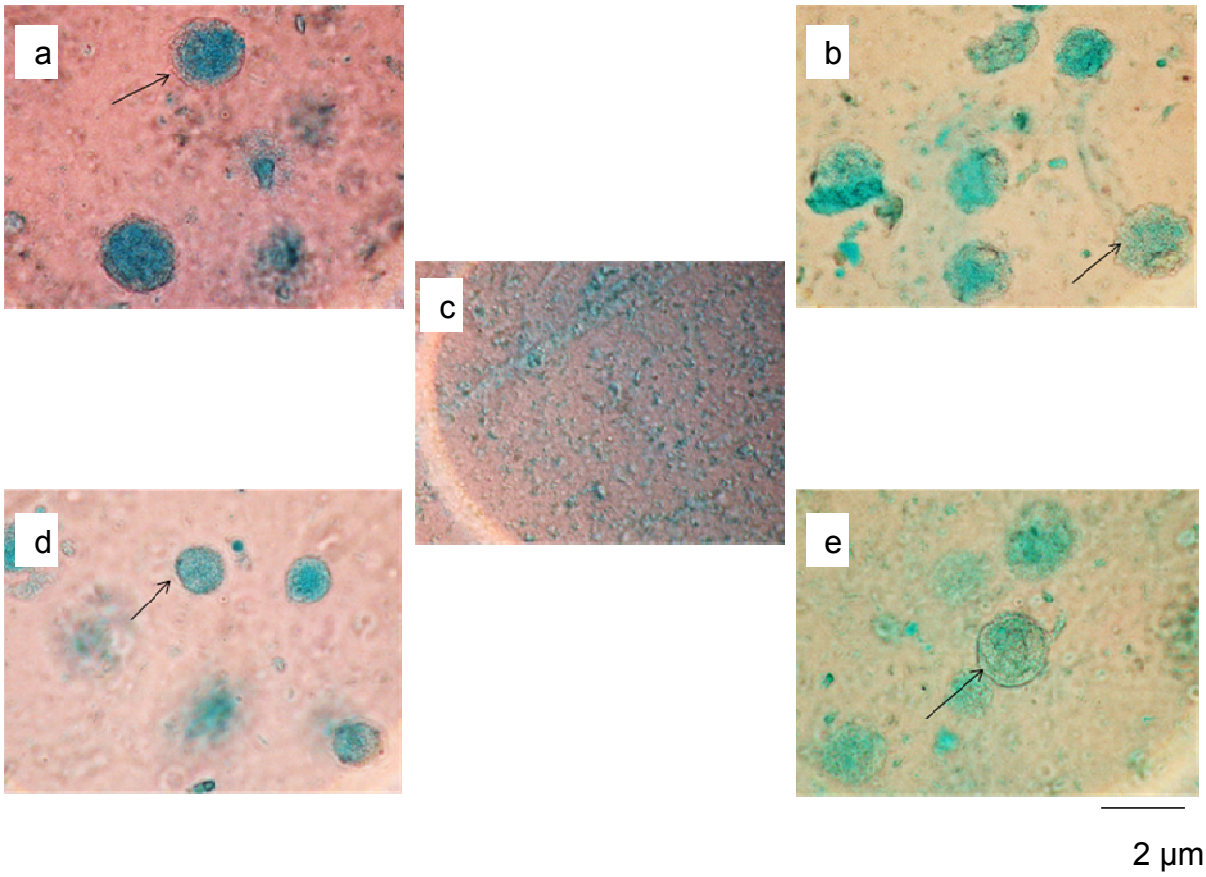


Figure 6.7: Alcian blue staining of human dermal fibroblast encapsulated alginate after 8 days in culture. The blue dark surrounding the cells shows detection of GaG (arrow). (a and b): Control and treated group with seeding density 2.0×10^6 cells/ml, (c): Alginate disc only (d and e) Control and treated group with seeding density 2.0×10^6 cells/ml supplemented with growth factors. All micrograph are at the same magnification.

6.5.4 SEM

As shown in figure 6.8, there were no apparent damage to the cells for the PLIUS treated group when compared to the control group. These results are the same as for the 3T3 cells in section 5.5.4. Indeed, the cells did not spread, into a fibroblast-like shape because alginate is lacking in cell attachments. However, this does not appear to be a detrimental effect because the HDF cells maintained their viability and proliferated in culture.

6.6 Discussion

Many previous studies have shown a positive effect of therapeutic ultrasound, at different doses, on different types of human fibroblasts [Doan *et al.,* 1999, Harvey *et al.,* 1975, Lai and Pittelkow 2007, Reher *et al.,* 1998, Wang *et al.,* 2003b, Webster *et al.,* 1980]. However, previous studies were all performed on monolayers of cells; this is the first study that the cells were maintained in 3D culture.

It is clear from the results presented here that therapeutic doses of PLIUS have a positive effect on HDF cells. With or without growth factors, the treated cells proliferate for up to 10 days of treatment (Figure 6.1 and Figure 6.2). This finding is supported by previous studies that showed that therapeutic ultrasound increased the cell numbers for human fibroblasts [Doan *et al.,* 1999, Reher *et al.,* 1998, Wang *et al.,* 2003b]. As discussed in the previous chapter, PLIUS does not stimulate the proliferation of 3T3 cells. However, in the case of HDF cells, the results presented in this chapter show that PLIUS did stimulate cell proliferation. This might be due to the different source of the fibroblasts that were being exposed to PLIUS. Doan *et al.* (1999) stated that if the cell numbers increased because of PLIUS treatment; this could be interpreted as a deleterious effect because the cells are involved in cell division but not in matrix production.

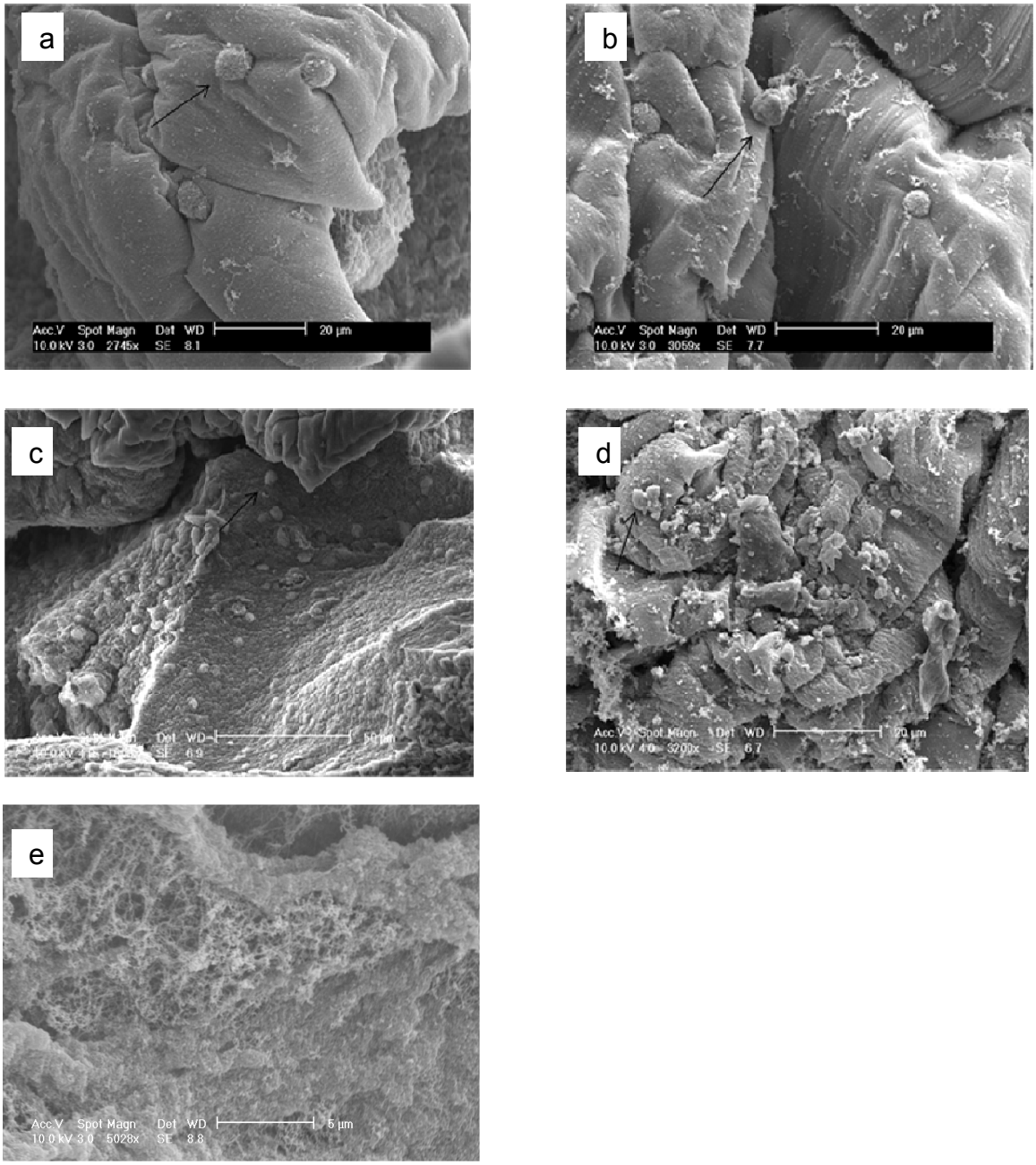


Figure 6.8: Scanning electron micrographs of a cross section of alginate disc showing human dermal fibroblast cells encapsulated in alginate disc after 10 days in culture. Viable cells on the surface of alginate disc (arrow). (a): Control group and (b): Treated group at seeding density 2.0×10^6 cells/ml, (c): Control group and (d): Treated group at seeding density 2.0×10^6 cells/ml supplemented with ascorbic acid and TGF-β1, (e): alginate disc only.

However, the data presented here were not totally in agreement with the results of Doan *et al.* (1999).

As shown in figure 6.3, the hydroxyproline content increased for up to 10 days of treatment but when the cells were supplemented with growth factor (Figure 6.4) the results show a fluctuating pattern. As discussed in Chapter 5, 3T3 cells treated with PLIUS and a growth factor supplement (ascorbic acid and TGF-β1) produced more collagen (Figure 5.7) than the control group. However, HDF cells maintained in culture supplemented with growth factor (Figure 6.4) show no increase in hydroxyproline when treated with PLIUS. It is well known that growth factors function by accelerating matrix accumulation and show positive effects to many types of cells [Bittencourt *et al.,* 2009, Chojkier *et al.,* 1989, Clark *et al.,* 1995, Kim *et al.,* 2009, Neidert *et al.,* 2002, Park *et al.,* 2009, Ross and Tranquillo 2003] However, the results presented in this chapter are inconsistent with these published observations. This may have been due to the type of cells and scaffold being used, and the culture conditions to maintain the cells [Bittencourt *et al.,* 2009, Chojkier *et al.,* 1989, Clark *et al.,* 1995, Kim *et al.,* 2009, Neidert *et al.,* 2002, Park *et al.,* 2009, Ross and Tranquillo 2003]. Furthermore, this study involved exposure to PLIUS but the cited studies did not use any physical stimulation to enhance ECM accumulation.

Treatment with PLIUS alone appeared to have no effect on GaG production (Figure 6.5) but shows a significant result (3.2 mg/construct) only at day 8 when in conjunction with growth factors (Figure 6.6) but not at other times during the experimental period.

Nevertheless, Alcian blue staining (Figure 6.7) showed that the HDF cells/alginate construct produced GaG with or without the growth factor supplements. The results were very similar to those described for 3T3 cells in Chapter 5.

SEM shows that cells encapsulated within an alginate disc did not adhere to the surrounding alginate (Figure 6.8). This was supported by previous studies showing that cells did not adhere to the alginate gel [Augst *et al.,* 2006, Lee and Mooney 2001] unless the alginate was chemically modified [Augst *et al.,* 2006, Rowley *et al.,* 1999].

Furthermore, alginate is a highly porous material and so is likely to entrap cells within the matrix rather than their adhering to the alginate molecular chains [Shih-Feng *et al.,* 2010]. However, lack of cell adhesion did not prevent the HDF cells proliferating, even though the cells did not attach to the alginate (Figure 6.1 and Figure 6.2). Moreover, the collagen content also increased significantly for the whole experimental period as a result of PLIUS treatment alone (Figure 6.3) but not when it was combined with growth factors (Figure 6.4).This shows that the cells can produce more collagen when treated with PLIUS alone.

It is noteworthy that, under the conditions used in these experiments, PLIUS treatment did not show any deleterious effect on the cells. This is consistent with recent work in which PLIUS with a frequency of 1 MHz and intensities in range of 0.75 to 1mW/cm^2 with 10% -20% duty cycles did not inhibit cell viability and proliferation [de Oliveira *et al.,* 2008, Doan *et al.,* 1999, Ramirez *et al.,* 1997, Reher *et al.,* 1998, Wang *et al.,* 2003b]. Indeed, there is a possibility that the PLIUS conditions used in this experiment could be beneficial in wound healing [Lai and Pittelkow 2007] and dermatological applications.

7. EFFECT OF PULSED-LOW INTENSITY ULTRASOUND EXPOSURE ON CALF CHONDROCYTES

7.1 Introduction

Articular cartilage possess little capacity for self-repair, after being damaged by disease or trauma, due to the low mitotic activity of its cells [Hsu *et al.,* 2006, Hunziker 2002, Zhang *et al.,* 2003]. This low mitotic activity can potentially be ascribed to the low metabolic and biosynthetic activities of mature chondrocytes and, at least in part, to the lack of chondrogenic cells available for repair [Buckwalter and Mankin 1997, Fragonas *et al.,* 2000]. This is because articular cartilage is not similar to many other connective tissue, because it is avascular, aneural and has no lymphatic system. As a consequence, nutrients and waste products are delivered through diffusion process by pumping action that occurs during compression of cartilage [Furukawa *et al.,* 2008, Maroudas 1976].

In spite of the extensive published studies documenting the response of chondrocytes to PLIUS treatment [Hsu *et al.,* 2006, Parvizi *et al.,* 1999, Zhang *et al.,* 2003], in this chapter the exact conditions that have been used to treat 3T3 mouse fibroblasts (Chapter 5) and human dermal fibroblasts (Chapter 6) were used to test the effects on calf chondrocytes.

7.2 Materials

The materials that used in this study were exactly the same as those described in sections 3.2 and 5.2 except for the DMEM media from Invitrogen (Paisley, UK).

7.3 Methods

7.3.1 Calf chondrocytes

Chondrocytes were isolated from calf articular cartilage (a generous gift from Professor Charles Archer, Cardiff School of Bioscience, University of Cardiff). Cells were maintained in DMEM supplemented with calf serum (10 v/v%), gentamycin (0.1v/v%), HEPES (2.4%) and 50μg/ml ascorbate. Cells were stored in sterile conditions at 37ºC and 5% CO_2 and the media was refreshed every three days.

7.3.2 Other methods

All other techniques are described previously in appropriate sections. They are: the cell encapsulation method (section 4.3.3), PLIUS treatment (section 5.3.6), cell content study (section 4.3.5), hydroxyproline assay method B (section 5.3.7), glycosaminoglycan (GAG) assay (section 5.3.9), Alcian blue staining (section 5.3.10) and sample preparation for SEM (section 5.3.11).

7.4 Statistical analyses

The analyses same as described in section 5.4 and 6.4.

7.5 Results

The viability/proliferation of chondrocytes in the 3D alginate culture for both groups (control and PLIUS) shows a fluctuating pattern with the total cell number in the range 17 - 39 $\times 10^5$

cells/ml for the control group and 27 - 47 $\times 10^5$ cells/ml for PLIUS group (Figure 7.1). At no time period was there a significant different between treated and control groups.

The hydropxyproline assay (Figure 7.2) shows that PLIUS may have a tendency to enhance collagen production in the treated group. It appears to be significant on day 4. However, there is no evidence for PLIUS consistently enhancing collagen production for encapsulated calf chondrocytes.

GaG content was quantified using the DMB assay. With this assay, PLIUS treatment for 10 days in culture did not represent any significant effect in increasing GaG production (Figure 7.3). The graph itself shows a decreasing amount of GaG with time.

Alcian blue staining was done to confirm GaG accumulation around the encapsulated chondrocytes. After 8 days, as shown in Figure 7.4, the stained sample showed halos of Alcian blue around the cells indicating GaG production. Alcian blue staining revealed the formation of GaG for the whole experimental period. However, the results represent only qualitative detection; they showed no difference in GaG production for control and treated groups.

Figure 7.1 shows that PLIUS did not have any effect on chondrocyte viability and proliferation. When observed in SEM micrographs, the cells showed no differences when compared to the control group. However, the chondrocytes encapsulated in alginate had a rounded chondrocyte-like appearance (Figure 7.5). This was supported by previous findings that also show that chondrocytes maintain their rounded morphology in 3D culture

[Bittencourt *et al.,* 2009, Loty *et al.,* 1998, McConell *et al.,* 2004] but have a spread, fibroblast-like, shape in 2D culture (i.e., in a monolayer) [Bittencourt *et al.,* 2009, Loty *et al.,* 1998, McConell *et al.,* 2004]. It appears that chondrocytes maintain a rounded morphology when encapsulated in alginate while fibroblasts do not maintain their fibroblast-like shape when encapsulated in alginate gels (section 5.5.4 and section 6.4.4).

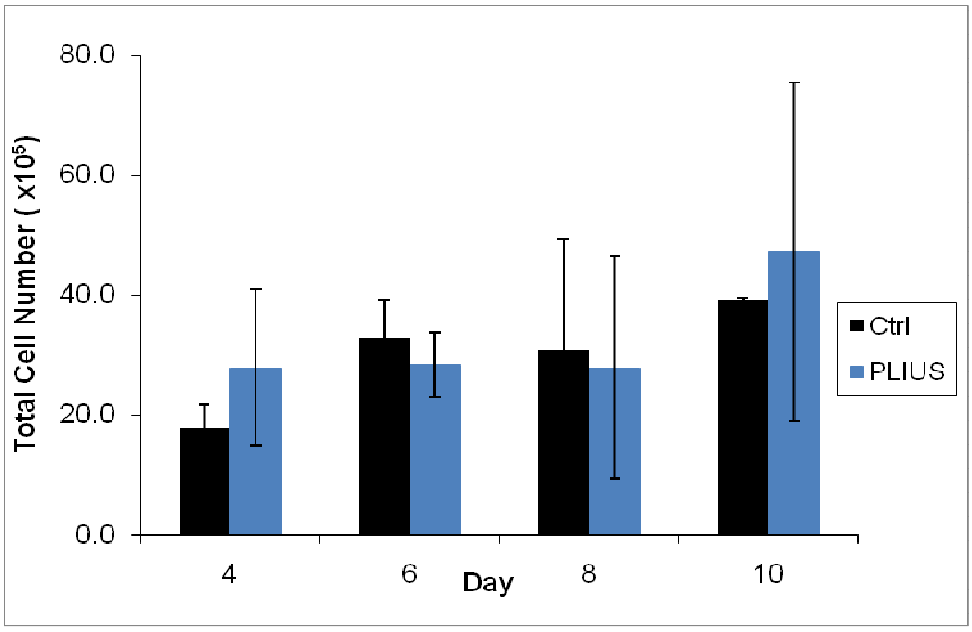


Figure 7.1: Cell proliferation study with high seeding density (2.0×10^6 cells/ml) in control (Ctrl) and treated group (PLIUS) after exposure with ultrasound in culture. Each result is the mean of three observations. Error bars represent standard deviations.

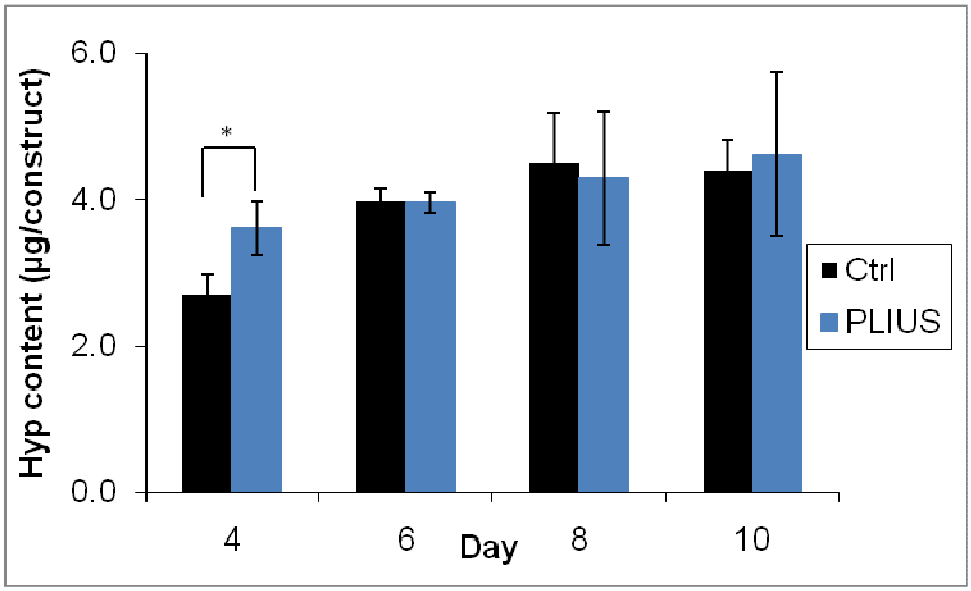


Figure 7.2: Hydroxyproline (Hyp) content with high seeding density (2.0×10^6 cells/ml) in control (Ctrl) and treated group (PLIUS) after exposure with ultrasound in culture. Each result is the mean of three observations. Error bars represent standard deviations**.** Results that are significantly different ($p < 0.05$) are marked with an asterisk.

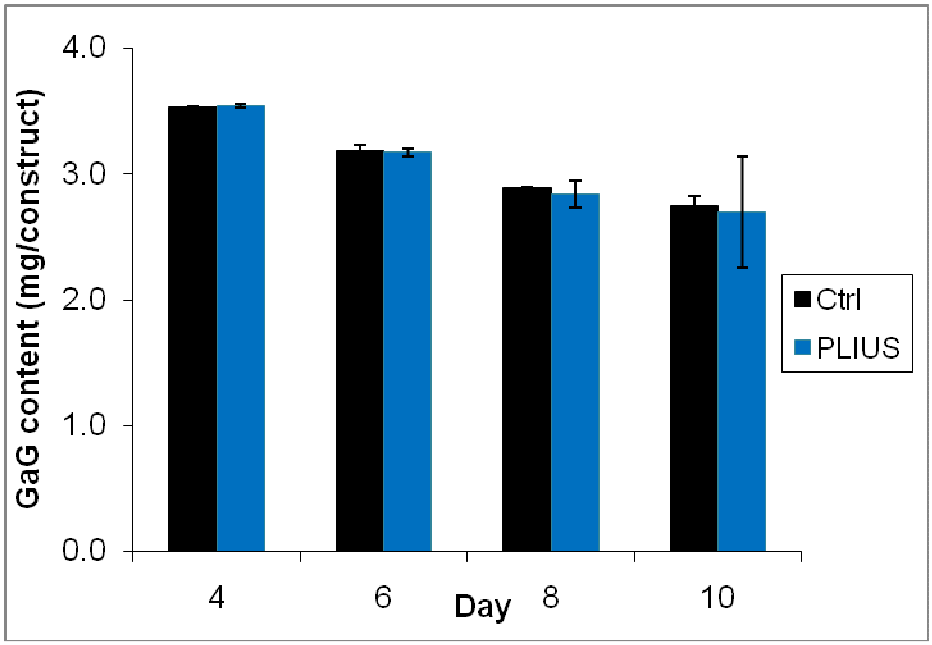


Figure 7.3: GaG content with high seeding density (2.0×10^6 cells/ml) in control (Ctrl) and treated group (PLIUS) after exposure with ultrasound in culture. Each result is the mean of three observations. Error bars represent standard deviations.

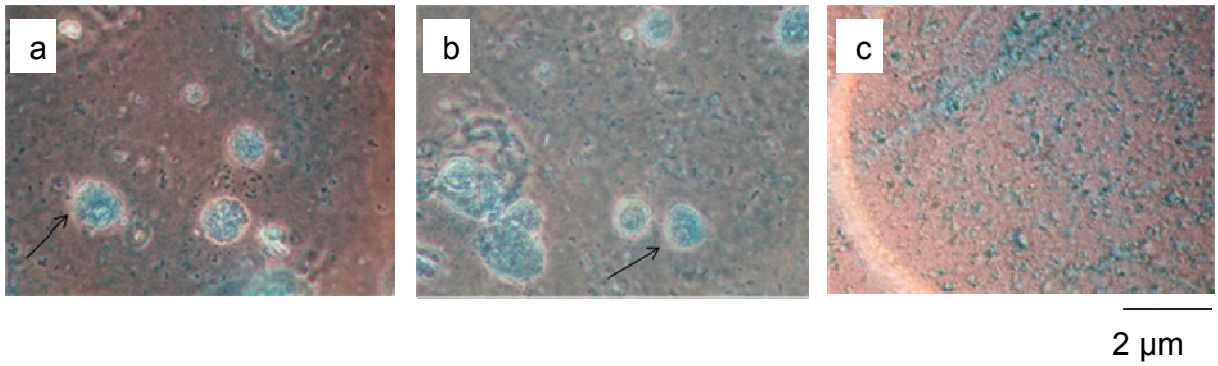


Figure 7.4: Alcian blue staining of calf chondrocytes encapsulated in alginate after 8 days in culture. The blue dark surrounding the cells shows detection of GaG (arrow). (a and b): control and treated group with seeding density 2.0×10^6 cells/ml, (c): alginate disc only. All micrographs are at the same magnification.

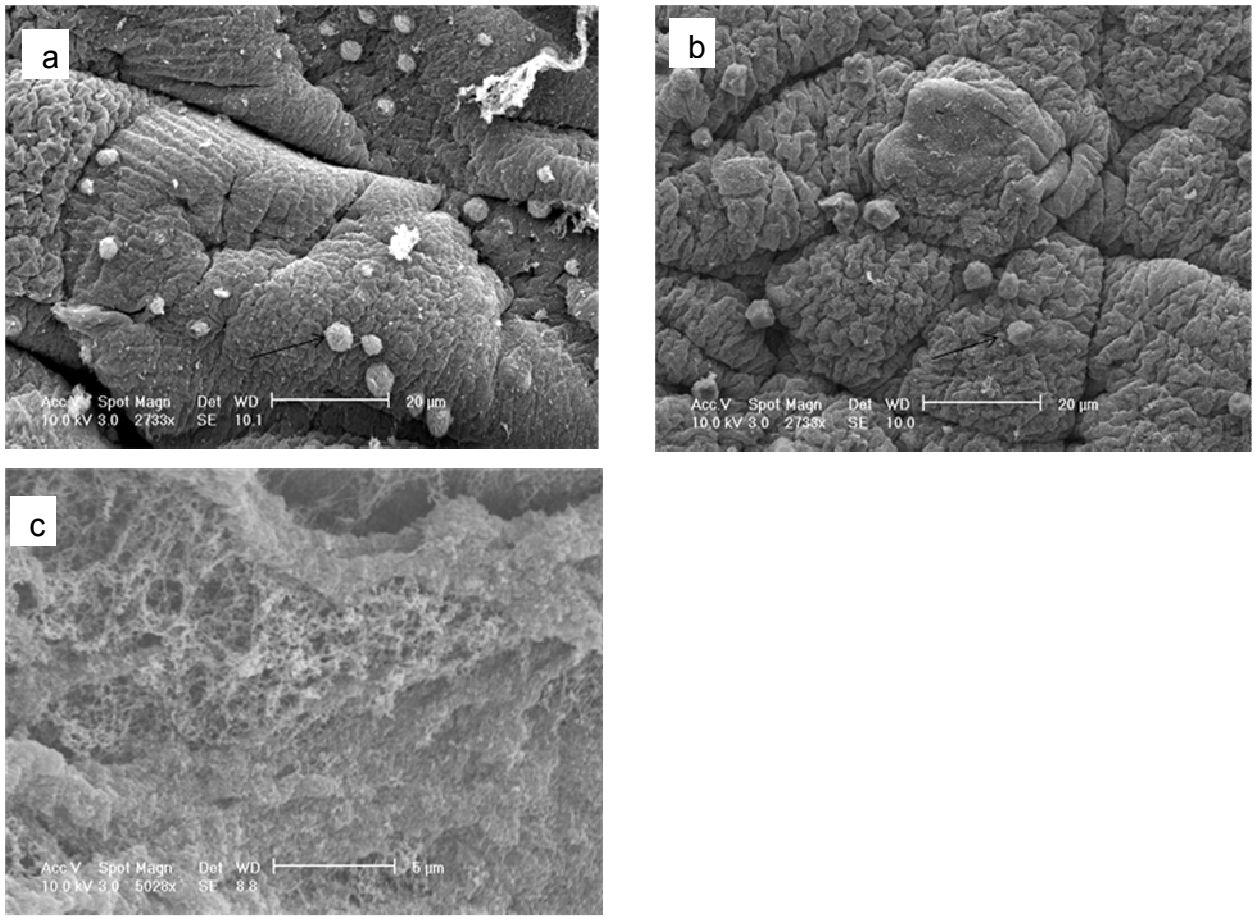


Figure 7.5: Scanning electron micrographs of a cross section of an alginate disc showing encapsulated calf chondrocytes after 10 days in culture. Viable cells can be seen on the surface of a disc (arrowed): (a) control group, (b) treated group (both at a seeding density $2.0\text{x}10^6$ cells/ml) and (c) alginate disc only.

7.6 Discussion

The aim of this study was to determine whether the PLIUS conditions used in this thesis can increase cell proliferation, enhance collagen production and stimulate GaG production. Previous studies have reported that PLIUS promotes cell proliferation [Hsu *et al.,* 2006, Zhang *et al.,* 2003] and matrix deposition by chondrocytes in 3D culture systems [Hsu *et al.,* 2006, Parvizi *et al.,* 1999, Zhang *et al.,* 2003].

Here it is shown that the PLIUS conditions used in this study did not promote chondrocyte proliferation in culture. The total cell numbers appear to fluctuate during the 10 days of the study. Previous studies showed no proliferation of chondrocytes when cultured in an alginate matrix [Chia *et al.,* 2005] and Parvizi *et a*l. (1999) reported no proliferation of chondrocytes when treated with PLIUS in a 2D culture system. There is no previous study of chondrocytes cultured in alginate that have been exposed to PLIUS, with the same dose used in the present study. From the results presented here, it can be concluded that PLIUS exposure has no effect in stimulating chondrocyte proliferation when cultured in alginate matrix.

A possible explanation, for this result, is that the alginate matrix is limiting the mass transfer of essential nutrients through the gel [McConell *et al.,* 2004]. As a result, the supply of nutrients becomes low and the cell population stabilizes (i.e., maintenance of viability without proliferation). However, a previous study reported that chondrocyte cell numbers increase when they are seeded in alginate with a low seeding cell density (1×10^4 cells/ml) [Gagne *et al.,* 2000]. This might be another reason why the chondrocytes in the present study did not show any proliferation in culture. Furthermore, Zhang *et al.* (2003) stated that PLIUS influenced chondrocyte proliferation in an alginate matrix in an intensity-dependent manner,

which means there is a possibility that the PLIUS intensity used in present study was not suitable to promote chondrocyte proliferation. A previous study also reported that the buffer system for culturing chondrocytes in an alginate matrix also plays an important role [Xu *et al.,* 2007]. According to their observations, supplemented DMEM with HEPES and bicarbonate buffer improves chondrocytes growth and matrix production in 3D alginate beads. In the present study, DMEM was supplemented only with HEPES buffer and this may be another reason for low cell proliferation and low collagen and GaG production.

Collagen production only showed a significant difference at 4 days of PLIUS treatment. Since that it only appear once for the whole experimental period, it can be considered PLIUS has no effect in stimulating collagen production.

GaG detection also shows no difference between control and PLIUS-treated groups for the whole experimental period. As discussed in the previous chapters (section 5.5.3 and section 6.4.3), there was a decrease in the amount of GaG produced, with time in culture, for the other cell types investigated in this thesis. Also, comparing the control and the PLIUS-treated group, there was no significance difference in GaG accumulation. Alcian blue staining showed qualitatively that GaGs accumulated around the cells [Chia *et al.,* 2005] in alginate discs.

SEM micrographs show a spherical morphology [Hsu *et al.,* 2006, Noriega *et al.,* 2007] for chondrocytes when cultured in alginate discs and no damage to the cells after exposure to PLIUS when compared to the control group. The cells did not proliferate in alginate when treated with PLIUS and there was no defect/injury to the cells seen in the SEM micrographs.

In the literature, TGF-β1 was reported to be able to stimulate collagen and GaG biosynthesis by chondrocytes in 2D culture [Redini *et al.,* 1988]. However, in this study there was only PLIUS exposure (physical stimulation) and no growth factor/biochemical stimulation involved. Another study showed that TGF-β1 did not stimulate collagen and GaG synthesis in 3D culture in agarose [Skantze *et al.,* 1985].

As mentioned in section 2.6.2, chondrocytes are reported to require a 3D scaffold in order to maintain their morphology [Bittencourt *et al.,* 2009] and that, when they are cultured in alginate hydrogel, they increase in number and viability as well as showing increased GaG synthesis [Bittencourt *et al.,* 2009, Chia *et al.,* 2005]. In the present study, there was no significant chondrocyte proliferation (Figure 7.1), collagen production (Figure 7.2) and GaG production (Figure 7.3) in alginate when exposed to PLIUS. A possible reason for this behaviour is that the kind of alginate [Domm *et al.,* 2004] used might be different in different studies but the exact reasons for the conflicting findings is still unclear.

8. OVERALL CONCLUSIONS

This thesis is aimed at investigating the effect of PLIUS on the viability/proliferation of different types of cells (3T3, HDF and calf chondrocytes) and their ability to produce ECM. As mentioned in Chapter 1, the original work is covered in five different chapters. The study begins by investigating the different concentrations of alginate solution (0.5, 1, 2, and 5%) in Chapter 3. Chapter 4 is about the effect of different alginate concentrations with different seeding densities on the viability/proliferation of 3T3 cells. After deciding on a suitable cell seeding density and the matching alginate concentration, the study continues with PLIUS treatment of encapsulated 3T3 cells in Chapter 5, HDF cells in Chapter 6 and, in Chapter 7, the study was extended to calf chondrocytes since many previous studies reported that treatment with therapeutic ultrasound caused chondrocytes to produce more collagen in culture [Hsu *et al.,* 2006, Parvizi *et al.,* 1999, Zhang *et al.,* 2003].

Chapter 3 describes the alginate properties. This chapter focuses on the viscosity, ageing and degradation of alginate solutions with different concentrations. In this chapter, it is shown that alginate solutions with higher concentrations had a high viscosity and consequently this would increase the shear stress applied to cells by the solution during mixing. An ageing process for the alginate solution was also clearly apparent for the higher concentration alginate (5%) but less so for the lower concentration alginate solutions. The degradation study in this chapter showed that when the alginate concentrations increased, the degradation process occurred slowly and took longer than at the lower alginate concentrations. From this it

can be concluded that 5% and 0.5% concentration alginate is not a suitable concentration as a medium to encapsulate the cells.

Chapter 4 focused on the viability and proliferation of 3T3 cells when encapsulated in different concentrations of alginate solution. Sections 2.4 and 2.5 explain, in detail, why alginate was chosen as a scaffold material. In summary, it is easily gelled when seeded with cells, it is biocompatible and it resembles the GaG gel that occurs in ECM [Rowley *et al.,* 1999, Wang *et al.,* 2003a]. As described in Chapter 4, the viability of 3T3 cells decreased when higher concentration alginate solutions were used; this could be explained by concentrated alginate solutions exerting a high shear stress at the cell surface, when the cells were dispersed before gelation, which could have damaged the cells (see section 3.5.1). Also in this chapter, spheroid formation occurred during cells proliferation. This can be seen clearly at lower concentration of alginate solutions and may arise because alginate lacks a cell attachment site, unlike ECM.

In Chapter 5, PLIUS was applied to 3T3 encapsulated cells to stimulate them to produce more collagen in culture. PLIUS alone stimulated collagen production by 3T3 cells. PLIUS with growth factor supplements showed more collagen production by the encapsulated cells at early experimental periods. GaG detection shows no difference between the treated and control groups. SEM micrographs show no damaged to encapsulated cells when treated with PLIUS. From this, it can be concluded that PLIUS treatment of 3T3 cells did not harm the cells and can stimulate collagen production alone or in conjunction with growth factor supplements.

Since positive effects were seen when the 3T3 cells were treated with PLIUS, it was decided to extend the study to include another type of fibroblast (HDF) in Chapter 6. For the proliferation study of HDF cells encapsulated in alginate, it appeared that PLIUS alone can stimulate cell proliferation. In the collagen production study, PLIUS alone stimulated collagen production of HDF encapsulated cells more than PLIUS treatment with growth factor supplements. PLIUS alone or with growth factor supplements showed no effect on GaG accumulation when compared to the control group. SEM images also did not show any damaged or injury to the HDF encapsulated cells after being treated with PLIUS alone or with growth factor supplements. It is concluded that fibroblasts from different sources can show different results when exposed to PLIUS. Furthermore, PLIUS alone was able to stimulate collagen and cell proliferation on HDF cells. This study suggests that PLIUS could be used as a physical stimulation tool for treating skin problems by tissue engineering.

In the previous chapters (Chapter 5 and Chapter 6), PLIUS showed a positive effect on 3T3 and HDF cells, when applied at the frequency and intensity chosen for this study. In Chapter 7, PLIUS treatment of calf chondrocytes was investigated since previous studies have reported that PLIUS has a positive effect on cartilage at the cellular level [Hsu *et al.,* 2006, Parvizi *et al.,* 1999, Zhang *et al.,* 2003]. However, in this study, PLIUS did not show any stimulation effect in the viability/proliferation study and in collagen and GaG production when cultured in alginate. As reported before, chondrocytes need to be cultured in 3D, to make them maintain their rounded morphology, in order to produce ECM and regenerate cartilage *in vitro* [Choi *et al.,* 2006, Hsu *et al.,* 2006, Langer and Vacanti 1993]. Consequently, it can be concluded that PLIUS , as used in this study, was not suitable for

increasing cell proliferation and stimulating ECM production, even though chondrocytes were cultured in 3D.

In conclusion, this thesis describes a series of studies on characterising alginate behaviour, cell viability study in different alginate solutions and PLIUS treatment on different cells type. The results from these tests have shown how different types of cells respond to PLIUS treatment. It should be possible to apply PLIUS (with the frequency, intensity and duty cycle used in the present study) to investigate further effects in tissue engineering applications.

Further studies can be done using different type of biomaterials (scaffolds) or a mixture of two or more different kind of materials to make the scaffold more durable in culture and at the same time support cell growth and encourage the cells to produce more ECM. When it is necessary, the scaffold can degrade as it is replaced by new tissue [Jones 2005, Wang *et al.,* 2003a].

In this study PLIUS treatment was only done at one frequency, intensity and duty cycle. Future studies may attempt to vary the frequency, intensity or duty cycle and this may produce different results than those presented in this study and could promote the cells to produce more ECM.

Finally, all of the cells that were used in this study are differentiated. Further studies could attempt to use mesenchymal stem cells. This is also another path for the application of PLIUS, to see if it can induce differentiation of stem cells to produce specific cells type in culture.

APPENDIX

A1 Viscosity measurements

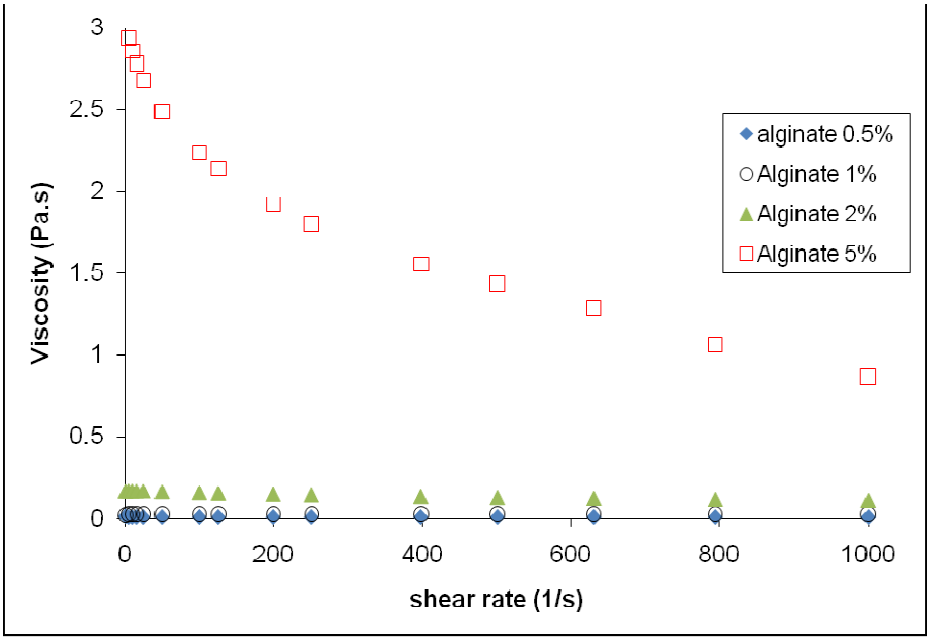


Figure A1.1: Viscosity variation on different alginate solutions at temperature 25°C.

A2 Ultrasound characterization

A2.1 Introduction

As mentioned in section 2.9, ultrasound is a mechanical vibration in form of sound wave with frequency > 20 kHz and frequencies in the range 0.8 to 3 MHz are usually used for therapeutic applications [Cambier *et al.,* 2001, Wu and Nyborg 2006]. The absorption of a sound wave results in molecular movement, i.e. heat that causes a temperature rise if it is not dissipated [Cambier *et al.,* 2001]. The phenomenon of cavitation can also contribute to the temperature rise; it happens when gas-filled bubbles in the tissue fluids expands and compress rapidly [Johns 2002, Speed 2001]. These rapid changes both in and around the cells may cause damage to them [Johns 2002, Speed 2001]. However, if the heat is dissipated, any effects of the ultrasound are expected to be non-thermal.

It is believed that non-thermal effects can occur at low intensities or with a pulsed output of ultrasonic energy [Cambier *et al.,* 2001]. It also proposed that non-thermal effects can stimulate fibroblast activity, increase protein synthesis, increase blood flow, increase tissue regeneration and stimulate bone healing [Speed 2001]. Indeed, a previous study reported that non-thermal effects of pulsed low-intensity ultrasound stimulated bone formation with no temperature rise at a power output of 0.1 W/cm^2 [Reher *et al.,* 1997].

The purpose of this study was to investigate the temperature rise with the setting that has been chosen (1MHz, 0.2 W/cm^2, 20% duty cycle (i.e. 20 pulses were emitted in 1 s, so that each

pulse had a duration of 0.05 s) for 5 min). This is important to understand how ultrasound affects encapsulated cells in culture.

A2.2 Methods

A2.2.1 Sonopuls 491 characterization

The same ultrasound source (Sonopuls 491, Enraf-Nonius, Rotterdam, Amsterdam) was used in all the experiments described in this thesis. The current experiment was done in conditions that resembled, as closely as possible, those used in the encapsulated cell studies. To investigate temperature rise, the ultrasound transducer was immersed in a water bath filled with deionized water maintained at 37°C [Doan *et al.,* 1999] that was treated with Sigma Clean Water Bath (50 ml, Sigma-Aldrich, Poole, Dorset, UK) to keep it free from bacterial and fungal growth. The water was changed every week. A beaker containing 5 ml water covered with parafilm, to minimise heat losses by convection, that had been kept overnight in an incubator at 37°C, was put on top of the transducer with the bottom of the beaker at a distance of approximately 1-2 cm from the transducer. A thermometer (Scientific Laboratory Supplies, Wilford, Nottingham, UK) with a range from -55.0°C to 148.8°C with a resolution 0.1°C was immersed in the beaker and the parafilm cover retained to reduce heat loss. The pulsed low-intensity ultrasound setting was applied for 5 min at 1 MHz, 0.2 W/cm^2 with a 20% duty cycle (i.e. 20 pulses were emitted in 1 s, so that each pulse had a duration of 0.05 s for 5 min). The experiment was done 3 times to measure the temperature increase.

A2.3 Statistical analyses

There was a weak but significant (R=0.97, p= 0.002) linear correlation (regression) between temperature (°C) and time (min) in which temperature, T, was related to time, t, by T=33.9

+0.0809t. This calculation was done using the Minitab Package (version 15, Minitab Ltd, Coventry, UK).

A2.4 Results

A2.4.1 PLIUS treatment

Figure 4.1 shows the temperature rise during the PLIUS exposure. As seen from the graph, the starting temperature of the experiment was at 33.9°C not 37°C. This probably arose from heat loss during inserting the thermometer probe in the beaker. However, the results show that the temperature increased only about 0.3°C for the whole 5 min exposure.

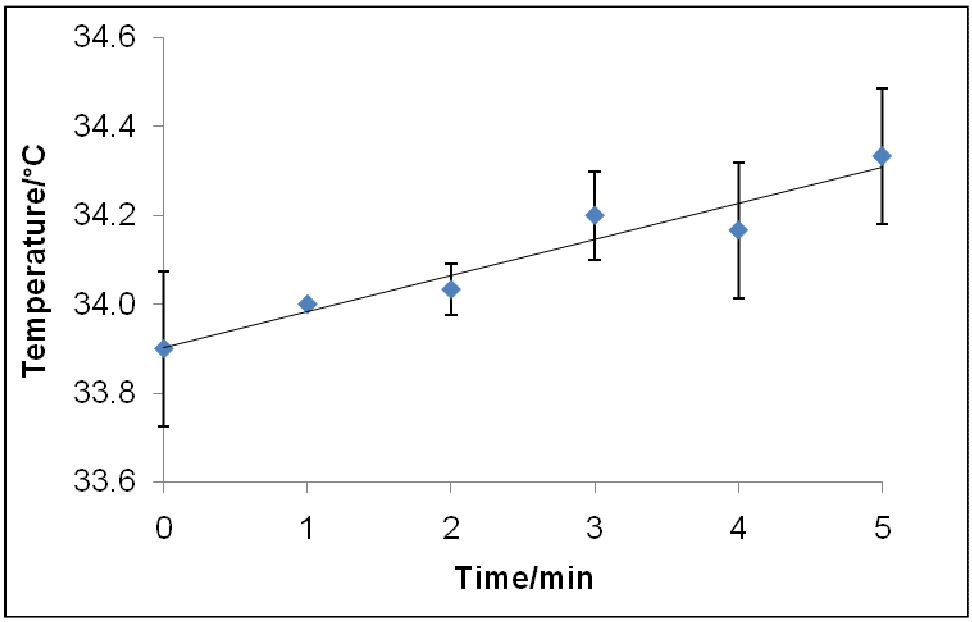


Figure A2: Temperature increase resulting from the ultrasound exposure as described in the text. Each result is the mean of three observations. Error bars represent standard deviations**.**

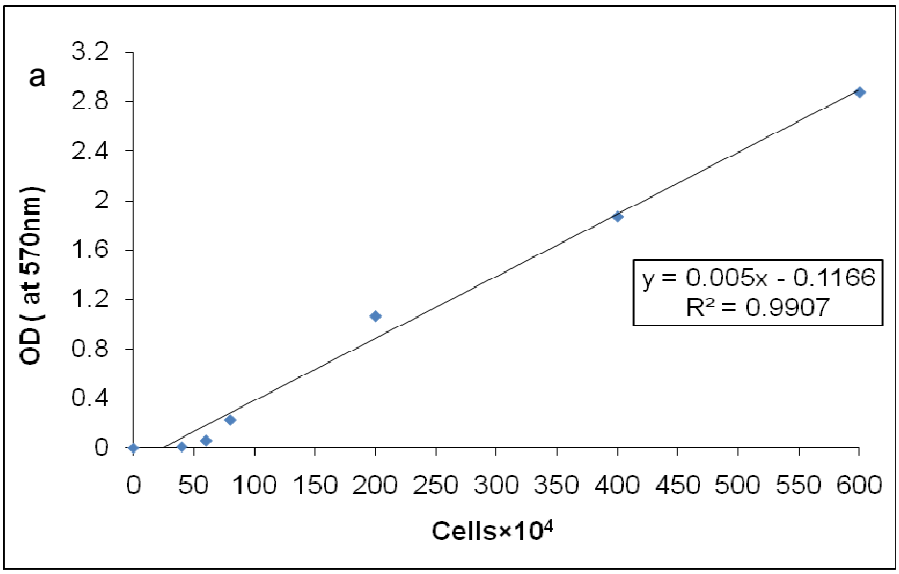
A2.5 Discussion

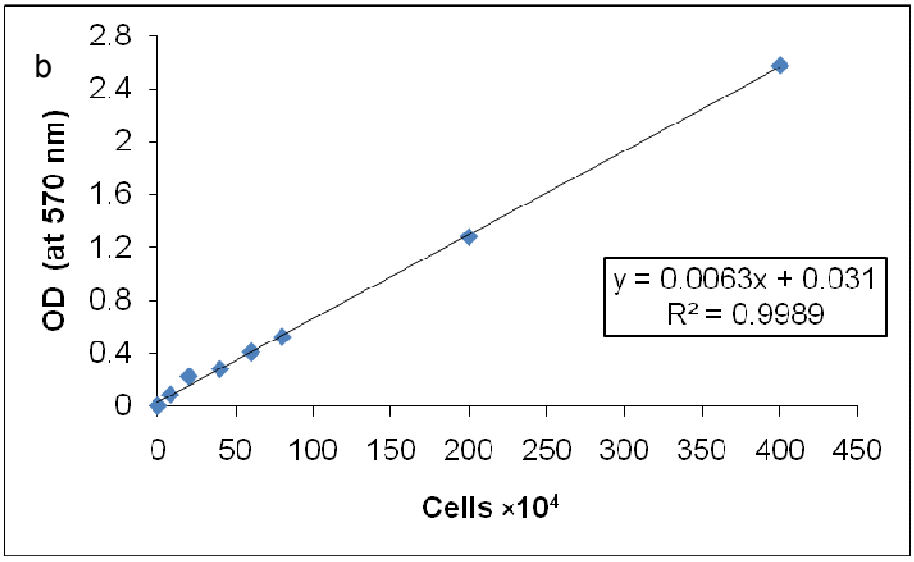
Cavitation could cause damage when living cells are treated with ultrasound; it involves the expansion of microscopic gas bubbles that lead to large temperature rises within and around

the cells. However, direct measurement on the surface or micro-environment of the cells are currently not possible [Johns 2002].

In the experiment described here, the temperature increased only 0.3°C over 5 min with the setting that been used to investigate the effect of ultrasound on cells (see Chapters 4, 5, 6). Previous studies by Johns (2002) and Reher *et al.*(1997) also demonstrated that temperature increases less than 0.5°C at the frequency 1 or 3 MHz. This study shows that ultrasound conditions that were used did not promote any dramatic changes in temperature that could have been harmful to the cells.

A3 Standard curve of MTT concentration against cell number





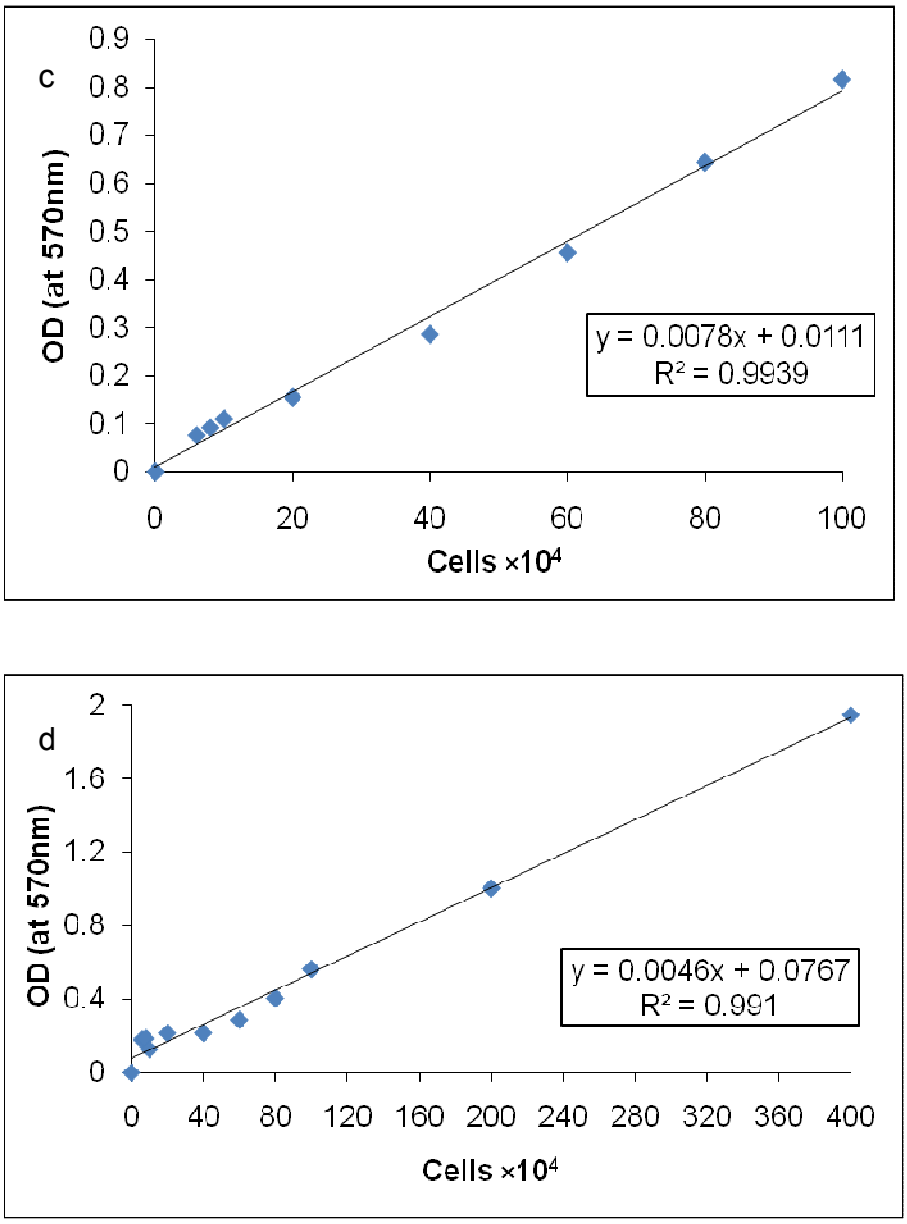


Figure: A3.1 Standard curve of MTT concentration measured at 570 nm against 3T3 cells encapsulated in (a): 0.5%, (b): 1% (c): 2% and (d): 5% concentrations alginate disc.

A4 Calibration curve of total cell number (Hoechst assay)

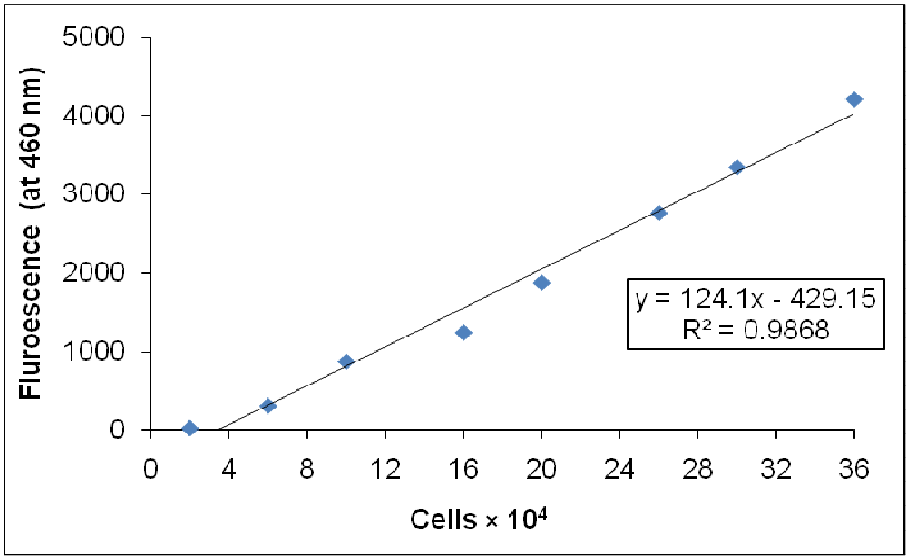


Figure A4.1: Standard curve of fluorescence intensity against 3T3 cells encapsulated in 2% concentration alginate disc.

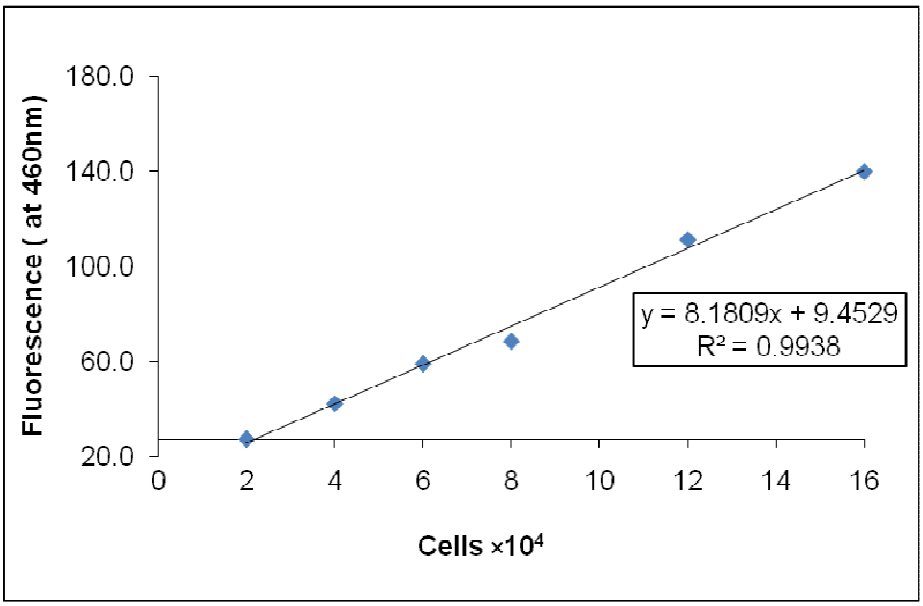


Figure A4.2: Standard curve of fluorescence intensity against HDF cells encapsulated in 2% concentration alginate disc.

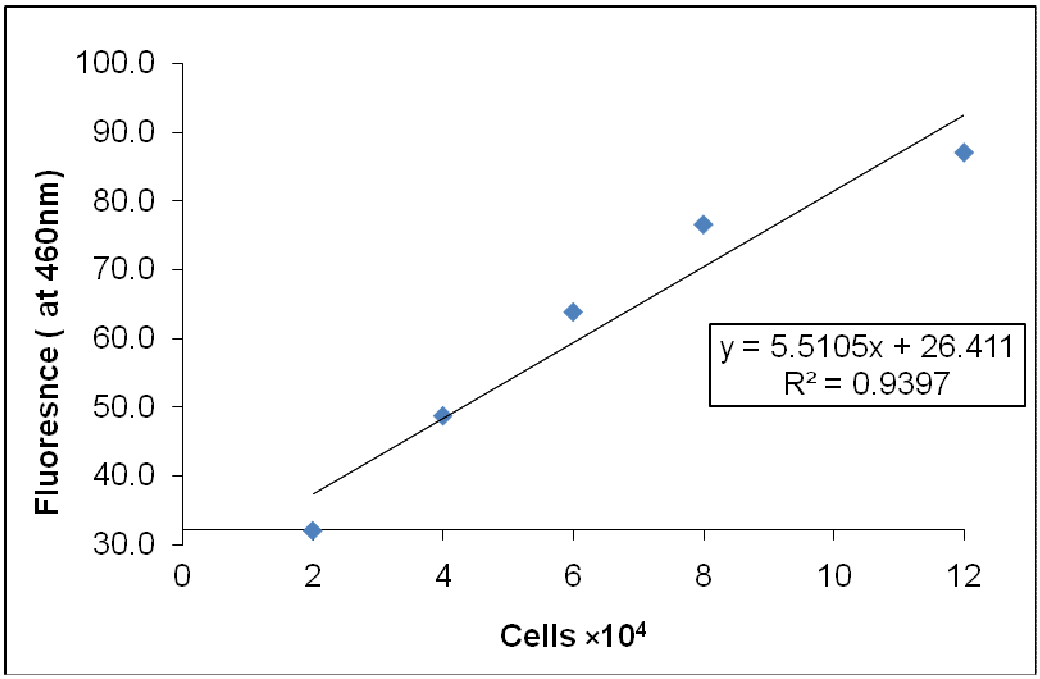
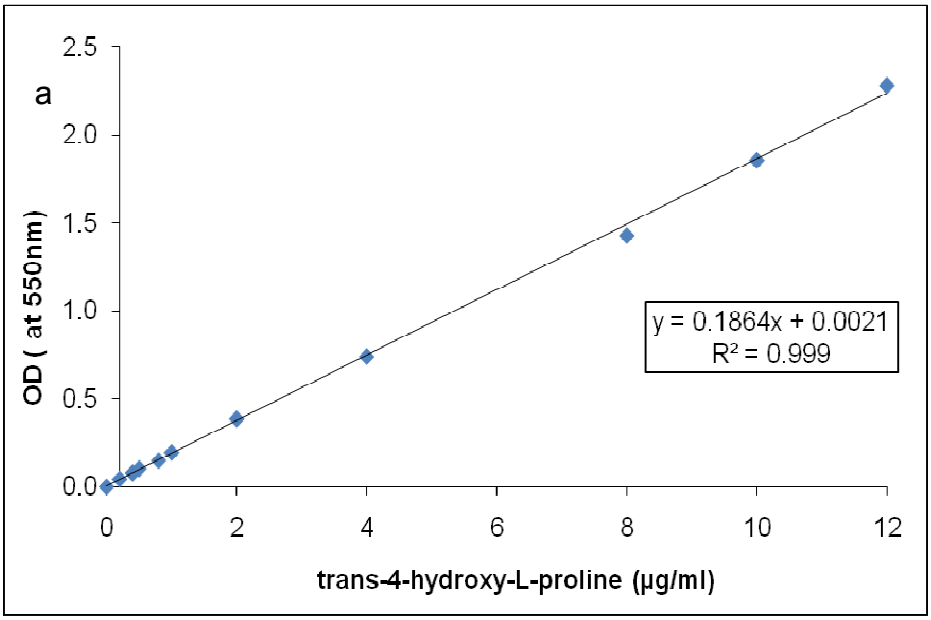


Figure A4.3: Standard curve of fluorescence intensity against chondrocytes encapsulated in 2% concentration alginate disc.

A5 Calibration curve of hydroxyproline content



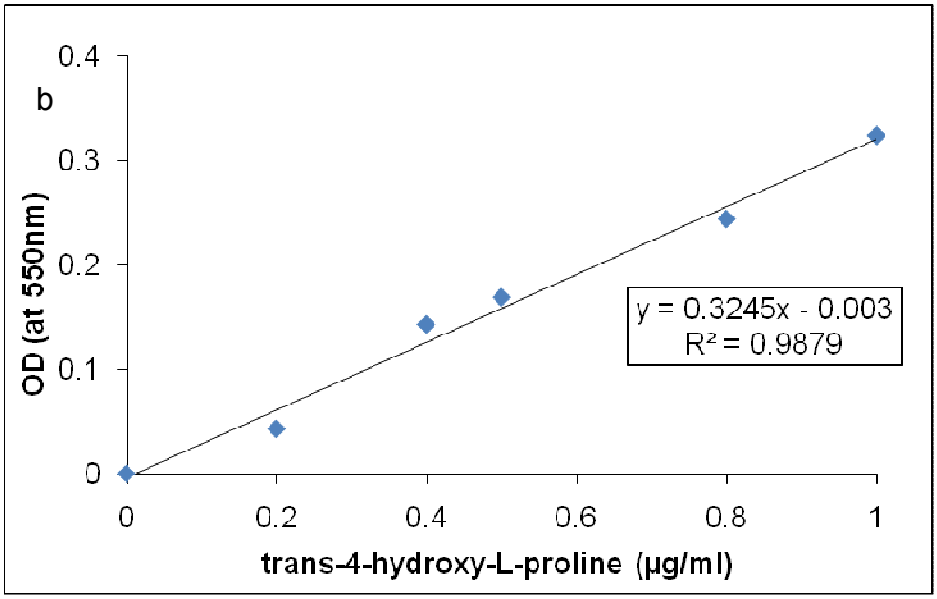


Figure A5.1: Standard curve for hydroxyproline assay (a): using method A and (b)**:** using method B

A6 Calibration curve for glycosaminoglycan (GaG) content

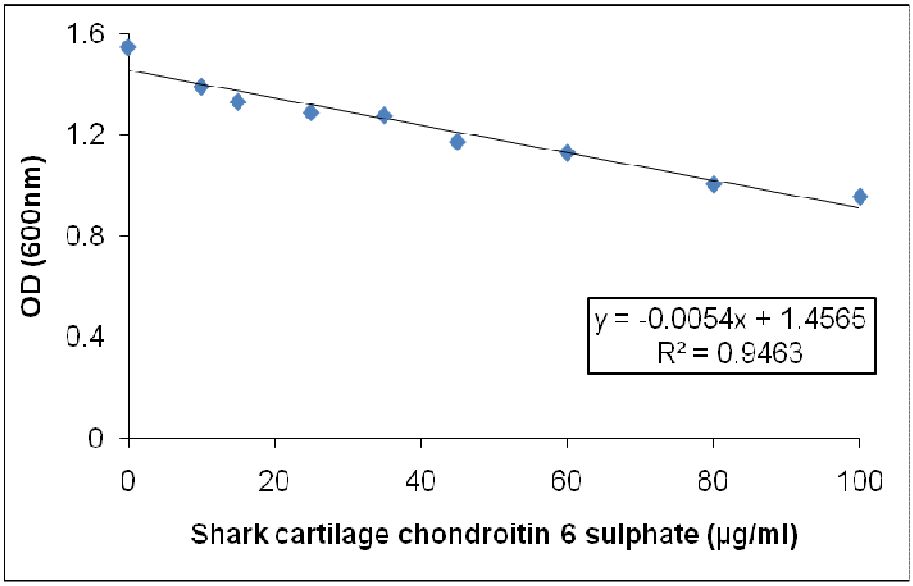


Figure A6.1: Standard curve of GaG content.

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