Use of Poly Vinyl Alcohol (PVA) Cryogelation for Tissue Engineering: Composites, Scaffold Formation and Cell Encapsulation

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Use of PVA Cryogelation for Tissue Engineering: Composites, Scaffold Formation and Cell Encapsulation

By

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Declaration

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Doctor of Philosophy (Ph.D.) is entirely my own work, that I have exercised reasonable care to ensure that the work is original, and does not to the best of my knowledge breach any law of copyright, and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

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6) N.E. Vrana, Y. Liu, P. Zorlutuna 2008. Controlling the vascular smooth muscle cell phenotype in photopolymerisable electrospun nanofibrous mats by microenvironmental mechanical changes, TERMIS-EU 2008, Porto, Portugal, June 2008.

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Awards

1) Finalist in Sir Bernard Crossland first year research awards (2007)

2) Finalist in TERMIS SYIS- Project proposal competition with two projects (2008)

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List of Abbreviations

BAEC	Bovine aortic endothelial cells
BASMC	Bovine aortic smooth muscle cells
BSA	Bovine serum albumin
CFDA	Carboxy-fluorescein diacetate succinimidyl ester
DAPI	4’-6-Diamidino-2-phenylindole
DMSO	Dimethlysulphoxide
DNA	Deoxyribonucleic acid
EC	Endothelial cells
EDTA	Ethlyenediamine Tetracetic Acid
eNOS	Endothelial Nitric Oxide Synthase
EtHD	Ethidium Homodimer-1
FACS	Fluorescent activated cell sorter
FBS	Foetal bovine serum
f-actin	Filamentous actin
FITC	Fluorescein Isothiocyanate

GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
h	Hour
KOH	Potassium Hydroxide
min	Minutes
NO	Nitric Oxide
PBS	Phosphate buffered saline
PI	Propidium iodide
P/S	Penicillin-streptomycin
PVA	Polyvinyl alcohol
RLT	Lysis buffer
rpm	Rotations per minute
RNA	Ribonucleic acid
RT-PCR	Real Time Polymerase Chain Reaction
SEM	Scanning electron microscope
SMC	Smooth muscle cells
TCPS	Tissue Culture Polystyrene

Nomenclature

a	Radius of rotation
ANOVA	Analysis of Variance
C_t	Threshold cycle
g	Gravitational acceleration
p	Density
n	Viscosity
f	Frequency
FI	Fluorescence Intensity
g	Gram
µl	Microlitre
M	Molar
µM	Micromolar
MW	Molecular Weight
cm	Centimetre
cm^2	Centimetre squared
^oC	Degrees Celcius

kg	Kilogram
kDa	Kilodaltons
σ	Stress
ε	Strain
τ	Shear Stress

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Glossary

Allograft: Organ or tissue transplantation from one individual of a species to another.

Xenograft: Organ or tissue transplantation from one individual of a species to another individual of another species.

Factorial experimental design: A series of experiments where n parameters were tested at x levels (values) to obtain all the possible combinations of the parameters for the selected range (x^n).

Extinction coefficient: A chemical index of a substance which denotes the absorbance capacity of the substance for a given wavelength

Phenotype: Expression of a particular set of genes by a cell that can be defined as a certain characteristic or trait.

Synthetic phenotype: Phenotype assumed by arterial smooth muscle cells upon injury. Involves high levels of migration, proliferation and ECM secretion.

Contractile phenotype: Phenotype of arterial smooth muscle cells under normal physiological conditions.

Inflammation: A non-specific immune response which can be triggered by infection, injury or irritation.

C_t: The cycle in Real time PCR, in which the increase in fluorescence becomes exponential.

Abstract

Title: Use of Poly Vinyl Alcohol (PVA) Cryogelation for Tissue Engineering: Composites, Scaffold Formation and Cell Encapsulation

PVA cryogelation is a physical hydrogel formation method, which yields cryogels with comparable mechanical properties to vascular tissue. However PVA cryogels are not suitable for cell attachment and proliferation alone. This can be overcome by the development of composite cryogels. Moreover, cryogelation provides a unique opportunity for encapsulation and storage of cells in one-step; if the correct composite structure and gelation conditions can be attained. In this study, PVA/Biomacromolecule composite cryogels were produced with a two step physical crosslinking (cryogelation and coagulation bath treatment) in the presence of different additives and a novel procedure to produce cell encapsulated PVA cryogels was developed for vascular tissue engineering. Also it was postulated that the disturbed shear stress could be used to facilitate endothelialisation of the PVA cryogel surface.

The results demonstrated that, the two step gel formation method was beneficial for degradation resistance and mechanical properties. All composites used supported cell attachment and proliferation, however PVA/Gelatin composites were superior compared to the others. Endothelialisation of PVA/Gelatin cryogels was achieved both under static and shear stress conditions with low levels of apoptosis and steady secretion of Nitric Oxide. It was shown that application of disturbed shear stress dramatically facilitated endothelialisation of the cryogel surface.

A general method of encapsulation via cryogelation was developed and robust cell-laden cryogels which promoted cell proliferation were obtained. Storage in frozen conditions did not affect the viability of the encapsulated cells, which suggests the prospect of safe long-term storage. Smooth muscle cell-laden cryogels were also able to support co-culture with endothelial cells. The results suggest that the novel encapsulation system developed is suitable for vascular and possibly other tissue engineering applications.

CHAPTER 1

1 Introduction

Tissue engineering is a promising technology that may provide solutions to a wide range of pervasive medical conditions. The tissue engineering research field represents an interdisciplinary effort to design, develop and manufacture artificial tissues and, in the long run, organs by combining engineering methodologies with knowledge stemming from the biological sciences (Mikos et al. 2006). Development of such artificial tissues would be a giant step forward in implant and transplantation technologies which are currently hampered by the long-term unreliability of synthetic implants and by donor scarcity, respectively. These products also promise to be useful experimental tools for researchers in both cell biology and pharmaceutical development by providing tissue-like structures that would enable *in vitro* testing of scientific or clinical hypotheses, involving therapies or drugs, on controlled 3D biological environments.

The need and urgency for development of tissue engineered products vary with respect to target organ or tissue. In some of the target tissues, such as cartilage and skin, tissue engineered products are mainly required for facilitation of the healing process and improving life quality. However, such as in the case of vascular tissue, tissue engineering products are desperately needed as a remedy for highly fatal conditions. It has been reported that each year cardiovascular diseases are responsible for 22% of premature deaths in Ireland (Irish Heart Foundation, 2009). Although the percentage of the cardiovascular disease related deaths has been

decreasing for the last 30 years, still 4 out of every 10 death are related to a cardiovascular condition. In a year, nearly 500,000 operations related to small diameter vessel replacement are conducted in the U.S.A. alone (Isenberg, Williams & Tranquillo 2006b). If tissue engineering can overcome the limitations of the current surgical procedures, cardiovascular disease related deaths can be further decreased.

As a way to imitate natural tissues, one philosophy of tissue engineering involves the presence of a biodegradable scaffold onto which cells from appropriate sources are seeded. After a suitable incubation time, tissue-like structures are formed (Freed et al. 2006). The scaling-up of this procedure would involve mass production of scaffolds followed by storage, with cell seeding and culture conducted immediately prior to implantation. Another option would be to develop storage methods for pre-manufactured engineered tissue, although this has not proven to be very feasible in practice. In this context, a system, where scaffold production, seeding and storage can be synchronized, with the possibility of a single batch of product being able to produce scaffolds with tunable functional properties, would be desirable.

Hydrogels have been among the most versatile tools in the arsenal of biomedical engineers (Brandl, Sommer & Goepferich 2007). They have been used as contact lenses, in drug delivery systems (Peppas et al. 2000), and for immunoisolation applications, and are now also the focus of much tissue engineering research (Hoffman 2002). Successful application of hydrogels in tissue engineering is due in part to the possibility of encapsulating cells without adversely affecting their

viability to a significant degree. Most of the connective tissue in the body is composed of tissue-specific cells encapsulated within a specific extracellular matrix (ECM) composition of their own secretion which has a complex function-oriented structure (Goh et al. 2007). The robustness of this structure is one of the signals that keep the cells in a certain phenotype and a change in structure, such as injury, triggers a wide range of cellular responses. Since connective tissue applications are an important part of the wider tissue engineering efforts, special attention should be given to this property. Entrapment of cells in a swollen hydrogel structure is a good imitation of cell-matrix interaction under *in-vivo* conditions. Therefore, as long as cell viability, function and the mechanical properties of the final hydrogel can be ensured, this is prospectively a viable approach for the production of tissue engineering constructs for connective tissues.

Tissue engineering investigators have long sought to incorporate the effects of physical strains and stresses on cell behaviour into their protocols (Barron et al. 2003). These stimuli might be used either to imitate the physiological environment or to replicate injury situations. The *in vitro* conditions under which an engineered tissue is produced and the physiological environment with which it will interact after implantation are different. Although this fact generates some technical problems, it can also be utilized to produce scaffolds which are more adaptable. One of the advantages of *in-vitro* scaffold culture is the ability to impose a wide range of possible stimuli. These stimuli can be at physiological levels, or, alternatively, sub- or supra-physiological stimuli levels can be selected when cellular response to such extreme conditions might have short term benefits.

Tissue engineering scaffolds have to be biocompatible, non-cytotoxic, and preferably biodegradable and should have the necessary mechanical properties to withstand the physiological stress and strain conditions of its target tissue (Suuronen et al. 2005). For thick tissues (above 500 µm in thickness) it is important to populate the whole scaffold structure with the cells (Malda, Klein & Upton 2007). To achieve this, two approaches are possible; either seeding cells evenly on to a porous scaffold which would enable cells to migrate and inhabit all available surfaces on and within the scaffold, or encapsulation of cells which are evenly distributed in a polymer solution which upon a certain treatment (such as photocrosslinking, physical crosslinking or non-cytotoxic chemical crosslinking) would turn into a hydrogel which contains cells within its 3 dimensional structure. The advantages of encapsulation include lesser dependence on cell migration and the immediate presentation of an extracellular matrix like environment to the cells from the start of the culture. In porous scaffolds, pores needs to be filled by the cells own secretion for the generation of a tissue like structure.

1.1Objectives of the Research Project

This study focuses on characterisation and utilization of PVA-based cryogels for vascular tissue engineering. The basis for this research is the proposition that combined hydrogels of PVA with appropriate macromolecules which would provide bio-functionality can act as scaffolds which exhibit surfaces conducive to endothelial cell attachment, display appropriate functional mechanical properties and degradation profiles for blood vessel applications, and successfully transmit

mechanical and biological cues which promote viability and functionality of encapsulated vascular smooth muscle cells.

It is anticipated that the preparation of such hydrogels by cryogelation of cell laden poly vinyl alcohol solutions, which involves freezing them to the sub-zero temperatures also necessary for cell storage, would ultimately allow the final functional properties of any given scaffold to be fine-tuned by control of the thawing process immediately prior to use.

Since a physical crosslinking method is proposed for a composite system, an additional physical crosslinking might be necessary to ensure the retention of the additives within the cryogels. For this end, coagulation bath treatment, another physical crosslinking method that works through precipitation of polymers via solvent/non-solvent interactions can be utilized. This method has not previously been considered as a reinforcement technique; thus it needs to be evaluated for this approach.

To test this proposition, the following key steps were taken:

1. The effect of the number of freeze-thaw cycles on specific PVA/Chitosan cryogel properties with respect to cellular behaviour was determined, to extend the previous body of work in this area (Mathews et al. 2008). In light of these results, the question of whether other biomacromolecular additives could also be used was answered via assessment of a set of three candidate natural macromolecular additives (Chitosan, Starch and Gelatin) for use in combined hydrogels with PVA. Moreover, coagulation

bath treatment as a reinforcement method was quantified, and its effect on composite cryogel properties such as mechanical, degradation, protein adsorption, and cell attachment was measured. On the basis of this experimental body of work, one PVA/biomacromolecule hydrogel composition was selected for further investigation.

2. A detailed investigation of the health of the endothelial lining and its behaviour under static and turbulent shear stress conditions, typical of flow conditions at anastomosis areas, on the selected PVA/biomacromolecule composite was performed. The possible positive effects of disturbed shear stress induced by oscillatory flow were assessed.
3. A novel cell encapsulation technique was developed, utilizing the cryogelation capability of PVA, to enable the storage and subsequent formation of cryogels with mechanical properties open to fine-tuning by adjusting the thawing rate and the gel components. The mechanical and biological performance of cell-laden hydrogel scaffolds was experimentally evaluated under cyclic strain conditions.
4. Finally, co-culture of encapsulated smooth muscle cells and endothelial cells was performed and the optimal conditions for commencing endothelialisation of the cell laden cryogels were determined.

The first step started with PVA/Chitosan cryogels and initially the effect of utilization of the coagulation bath on these properties was examined. Then the effect of increasing the number of freeze-thaw cycles on physical properties of the

cryogels was evaluated. The next step involved production of PVA-based cryogels using 3 different biomacromolecular additives (Chitosan, Starch and Gelatin), and then characterizing the mechanical properties, degradation, water content, surface hydrophilicity, protein adsorption capability and surface morphology (by phase contrast microscopy and scanning electron microscopy). These cryogels were then compared with respect to their ability to support endothelial cell culture on their surface and also their ability to encapsulate smooth muscle cells. One of the candidates was selected due to its overwhelming superiority in several items listed above.

The second step involved the formation of an endothelial lining on the selected hydrogel under both static and dynamic conditions. For this purpose, the characterisation focused on metabolic activity, proliferative capacity, apoptosis and expression of cell-cell contact proteins which are important either for activation or support of the endothelial lining (Resnick et al. 2003). The shear stresses created under oscillatory flow conditions are known to induce a higher endothelial cell proliferation rate (Chien 2008a); in the current study this effect was investigated as a possible route to facilitate endothelial lining formation. The analyses used were (i) Alamar Blue proliferation assay, (ii) Flow assisted cell sorting (FACS) system for proliferation and apoptosis analysis, (ii) Real-time RT-PCR for determination of the selected target protein expression, (iv) Griess method for determination of the Nitric Oxide release by the endothelial cells and Fluorescence microscopy and SEM.

The use of cryogelation for smooth muscle cell encapsulation is new, and necessitates other additives to ensure long term cell viability. The encapsulation

system was optimized for a wide range of parameters such as thawing rate, PVA viscosity and concentration of serum and cryoprotectants by utilization of factorial and Box-Behnken experimental designs which make it possible to see the interactions between different parameters. These allow the development of response models. Also, the effect of cyclic strain and long-term storage on encapsulated cells were evaluated. Cell viability was assessed by Live/Dead cell viability assay and Alamar Blue. Cell morphology and hydrogel structure were observed with SEM, phase-contrast microscopy and histology. The mechanical properties of the gels in the presence and absence of the smooth muscle cells were determined by uniaxial tensile tests. Finally, to ensure that the cryogels with encapsulated cells would support endothelial lining formation, co-culture experiments are undertaken.

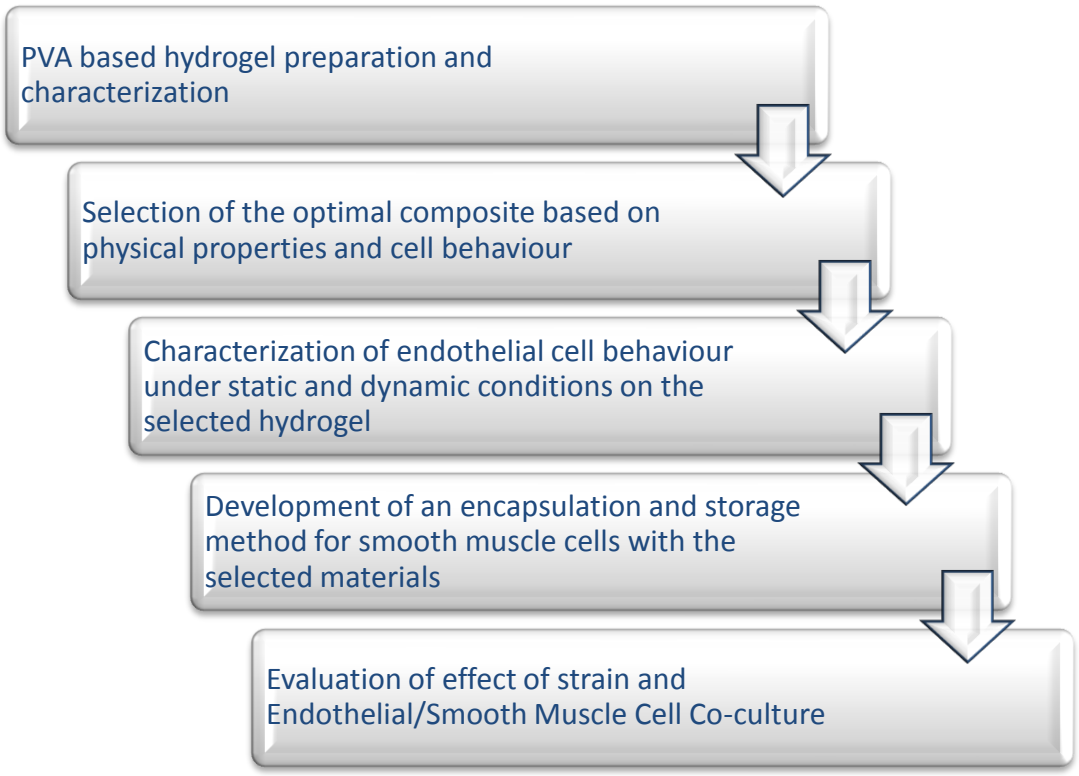

Figure 1.1 The scheme of experimental procedure followed in this study

CHAPTER 2

2 Literature Review

The following literature review deals with the reasons behind the necessity for and widespread use of vascular grafts, their shortcomings and the challenges these pose to scientists, engineers and clinicians. The structure of vascular tissue is briefly explained to understand the structures that need to be imitated with the artificial tissue constructs. Then, tissue engineering and specifically vascular tissue engineering is defined and the achievements and methods in the field are briefly discussed. After reviewing the available vascular tissue engineering scaffold structures, hydrogels are taken into focus and their physical properties, their capability to encapsulate cells and their feasibility as vascular tissue engineering scaffolds is introduced. Finally, mechanotransduction and its specific importance to vascular tissue (pulsatile blood flow induced shear stress and cyclic strain) and its possible uses for vascular tissue engineering are reviewed. This sets the background for why smooth muscle cell encapsulation and endothelialisation of the hydrogels are important, what kind of physical advantages are inherent to the hydrogels and how these can be exploited for vascular tissue engineering.

2.1 Cardiovascular Diseases and Current Remedies

Cardiovascular diseases are the leading cause of death in the world, especially in Western countries. Worldwide deaths related to cardiovascular diseases were estimated by World Health Organisation (WHO) as 17.5 million in 2005, which comprises nearly 30% of all recorded deaths (Figure 2.1). Specifically when Ireland

is considered, WHO statistics showed that 123,000 out of 312,000 deaths (nearly 40%) in 2002 occurred from cardiovascular diseases. For 2007, the Irish Heart Foundation reported similar percentages (36%). Aside from fatal conditions, cardiovascular system related surgeries are one of the most frequent type conducted. Because of this, together with the frequency of secondary surgeries, vascular health has become one of the most investigated areas in medical and biomedical sciences (Birla et al. 2004). Occlusion of the vessel lumen due to the accumulation of fat deposits (atherosclerosis) or multi-vessel dysfunctions, such as intimal hyperplasia, render the vasculature dysfunctional. Surgical intervention then becomes essential, especially in conditions such as myocardial infarction or critical limb ischemia (Brewster et al. 2007).

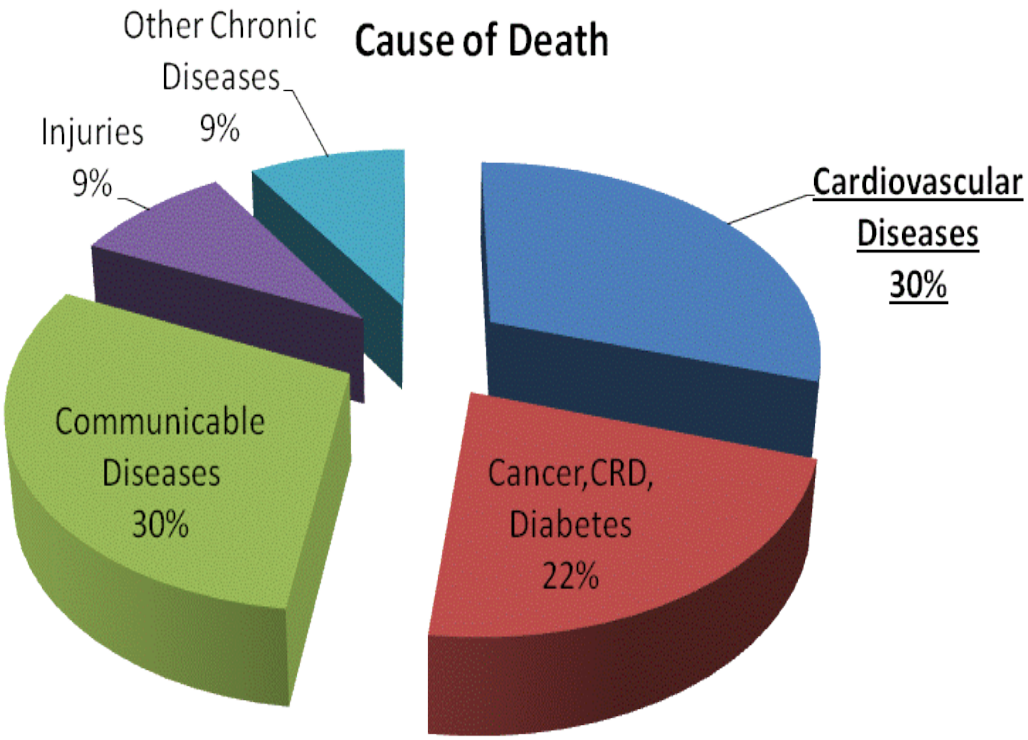

Figure 2.1. Cause of death statistics in 2005. 30% of all deaths worldwide were due to cardiovascular diseases. The graph was obtained from World Health Organization website.

The number of by-pass surgeries is already in the several millions around the world and the need is constantly increasing. For a significant group of patients, no autologous arteries or veins are available (Campbell, Campbell 2007). This incredible level of demand hinders, in some cases, the capabilities of surgeons in procedures such as coronary artery by-pass, since the availability of donor tissues is scarce (Figure 2.2). Normally, autografts are used as the golden therapy method, and with considerable success. There are some available donor sites in the human body (mainly saphenous vein, radial and internal mammary arteries), but in elderly patients these vessels might be unsuitable due to trauma, previous surgeries or vascular diseases such as varicose. Moreover, even after successful surgery, a graft may not function properly, due to compliancy problems, in which the mismatch in the extensibility of the natural vasculature and the synthetic graft material causes severe flow disturbance which in turn can lead to significant cellular response (Sarkar et al. 2007), such as intimal hyperplasia formation or thrombosis. In fact, vein grafts have only 50% patency within 10 years of implantation (Kannan et al. 2005). Allografts (vessels retrieved from other humans for implantation) and xenografts (vessel obtained from other species such as pig) face similar problems, and complications related to them are compounded by the risk of disease transmission and chronic inflammatory response, which might necessitate further surgeries. An attractive option is the development of artificial vascular grafts that would replace current grafts and more importantly would provide surgeons with readily available implants, which can be tailor-made and would be without the problem of scarcity.

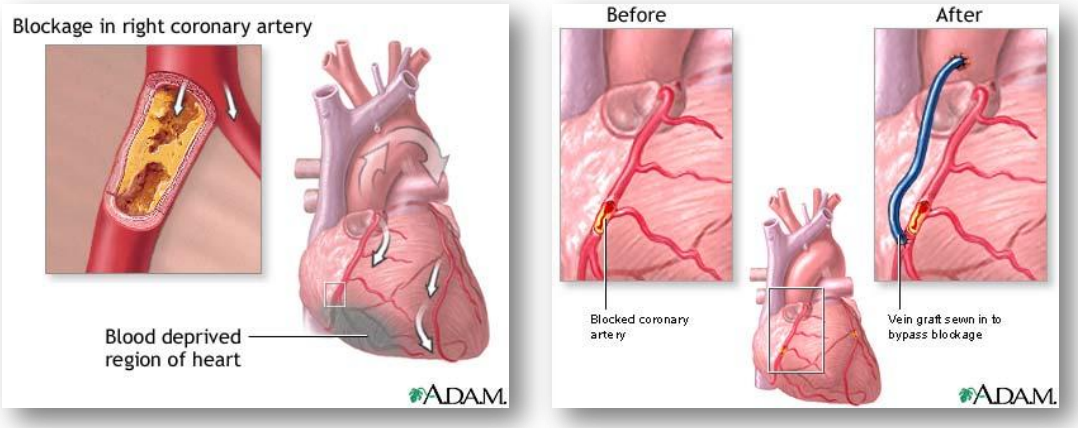

Figure 2.2 Arterial by-pass procedure a) Blockage of coronary artery causes nutrient and oxygen deprivation of the heart muscle, which causes necrosis and dysfunction. b) By-pass procedure to solve coronary artery blockage. An autologous graft (Saphenous vein in this case) is used to literally by-pass the blockage and re-direct the blood flow and re-establish the nutrient and oxygen flow to the heart muscle. Although highly successful, this procedure mostly depends on the availability of the autologous graft, which is not available in a big portion of cases. Images obtained from USA National Institute of Health Website, Medical encyclopedia (www.nih.com).

Artificial vessels without any biological components (such as Dacron, ePTFE (Expanded polytetrafluoroethylene), Polypropylene or Polyurethane based vessels) have been used as vascular grafts with limited success. Although they can be rendered relatively compliant, the biggest problem with these artificial vessels is thrombogenicity and neointimal hyperplasia formation, both of which can, in the long run, cause occlusion of the vessels due to thrombus formation and may

necessitate further surgical interventions (Clarke et al. 2001). Thrombogenicity, which can be defined as the proclivity of a material to induce blood clot (thrombus) formation when coming into contact with blood can be decreased for synthetic materials by surface coatings or use of smoother surfaces, but only to a certain extent. The surface would be still thrombogenic and this imposes a risk on long term viability of the graft, especially for small diameter grafts where occlusion is much easier. The main reasons behind surface thrombogenicity are serum protein adsorption, surface affinity to cells (such as platelets) and surface roughness. Attachment of thrombogenic proteins and subsequent platelet attachment has a positive feedback loop and subsequently the accumulation grows exponentially. When the graft surface is big enough (for wider arteries) this accumulation may not reach to a degree where the blood flow can be hindered. Thus this may be the reason why even though these artificial vessels are successful as large diameter substitutes, their utilization for small diameter vessel replacement (≤6mm) has low patency (Sarkar et al. 2007). These facts, together with parameters such as flow rate , the flow regime of the target artery environment compounded with the patient's own blood individual specific tendency to form clots, make it really hard to assess the feasibility of the artificial grafts for a given implant condition (Lopez-Soler et al. 2007). These problems were the driving force behind development of less thrombogenic vascular graft surfaces to obtain long term patency. Recently, it was shown that biologically derived graft materials, such as umbilical cords, suffer from similar thrombogenicity issues and neointimal hyperplasia formation, even though they are better for cell recruitment and integration, i.e. formation of a neo-endothelial lining on these grafts has a higher probability (Scharn et al. 2006). To

improve integration ability, synthetic grafts are implemented with several features such as a double velour knitted surface design to provide the necessary area for cell migration, hence improving integration with the surrounding vascular tissue (Figure2.3).

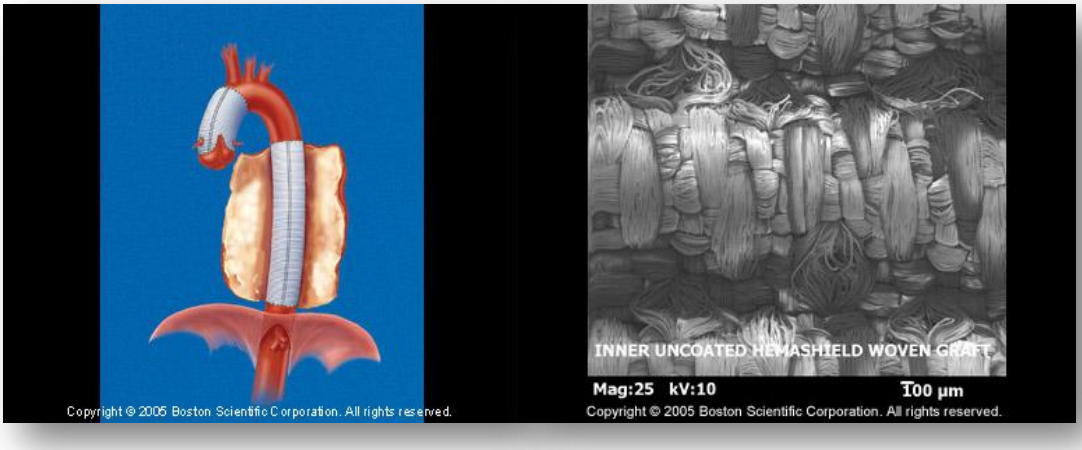

Figure 2.3 Arterial graft design. Hemashield Platinum Dacron based double velour graft for arterial surgeries produced by Boston Scientific Inc. A) Graphical representation of the grafts after implantation b) SEM micrograph of Surface structure (Double velour woven) for improved integration of the graft with the target vessel (Obtained from Boston Scientific Inc website www.bostonscientific.com).

One way to circumvent the thrombogenicity problem is the development of graft surfaces that are less thrombogenic and more conducive to endothelial cell migration and proliferation (Birchall, Lee & Ketharanathan 2001). In this way, the interaction of the graft surface with the blood can be kept minimal (Cuy et al. 2003). It has been suggested that this problem can be overcome by covering of the lumen surface of the artificial vessels with autologous endothelial cells. There was a significant increase in short-term and long term patency of the grafts via this route (Meinhart et al. 2001, Deutsch et al. 2008). However, problems such as the degree

of coverage and the strength of adhesion of the endothelial cells onto the artificial lumen surface still remain unsolved. *In-vitro* experiments showed that physiological level shear stress can cause detachment of endothelial cells from the polymer surfaces, thus exposing the graft surface to the blood flow. This can potentially be prevented by coating the lumen surface with cell adhesive proteins but no conclusive results have been obtained via this route (Chen, Barron & Ringeisen 2006). This lack of success is mainly due to the limited proliferative and migratory capacities of the endothelial cells and their mode of migration being two dimensional, which makes it nearly impossible to cover the whole area presented by an artificial graft. This can be overcome by the body by recruitment of circulating endothelial progenitor cells, which have high proliferation rates and can achieve fast lumen coverage (Melero-Martin et al. 2007), but the rate of this process versus the rate of platelet and fat deposition or intimal hyperplasia is difficult to control, unless with the inclusion of growth factor delivery systems (such as FGF) or gene delivery systems within the implant (Frerich, Kurtz-Hoffmann & Lindemann 2005). Moreover, these polymers are not very permissive to tissue in-growth and remodelling, and thus a total healing of the vasculature is impossible in their presence.

In short, what is expected of a functional synthetic vascular prosthesis could be impossible to achieve with a mere synthetic solution. They need to be non-thrombogenic, haemocompatible, fatigue and creep resistant yet compliant enough not to disturb the blood flow at the integration sites. It seems that the ideal solution for future generation of vascular prosthesis is to integrate cellular

components to better imitate the functionality. For this end, it is crucial to have an understanding of the structure and behaviour of the vascular tissue.

2.2 Structure and Composition of Vascular Tissue

Vascular tissue is composed of two main vessel types, arteries and veins which have different morphologies due to their different functions. Arteries are responsible from distribution of the oxygenated blood, whereas the veins function is the collection of the deoxygenated blood back from organs. These functional differences result in physiological differences too, such as higher burst strength for arteries and the presence of valves within veins for prevention of back-flow of the blood. The vascular health related problems are nearly always related to arterial dysfunction. There are two categories of arteries, elastic and muscular. Elastic arteries are the large diameter ones such as the aorta in which smooth muscle cells are accompanied by several elastic laminae. They are located proximal to the heart. Muscular arteries are smaller in diameter and their tunica media is mainly composed of smooth muscle cells and ECM. Their location in the vasculature is more peripheral. Arteries close to the heart are thick and more compliant whereas further along the circulatory system, they become stiffer and thinner. Arteries are composed of three layers, intima, tunica media and tunica adventitia (Figure 2.4). The thickness and the composition of each of these layers changes throughout the arterial tree due to the different pressure conditions present in the respective areas.

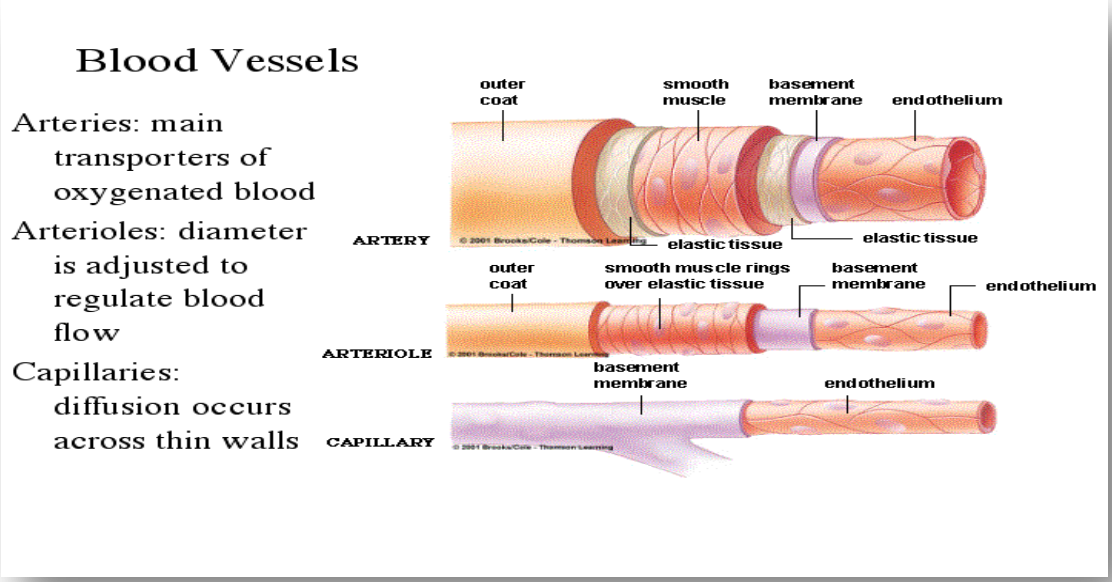

Figure 2.4 General structure of Arteries, arterioles and capillaries. The three layer structure of arteries, endothelium oriented in the direction of blood flow residing on a basement membrane and circumferentially oriented Smooth Muscle cells with interdispersed elastic tissue and the outer fibroblastic coating. For arterioles both elastic tissue and the outer coating(tunica adventitia) is less prominent due to less demanding loading conditions and for capillaries they are non-existent (Sherwood 2001).

The intima starts with a single layer of squamous endothelial cells which are elongated in the direction parallel to that of blood flow. They sit on a basal lamina which in turn lies on a sub-endothelial layer (this layer is more evident in highly elastic arteries). The tunica media is populated by smooth muscle cells. In the elastic arteries, it is formed of alternating elastic laminae and smooth muscle cells in a circular arrangement. The main components of its extracellular matrix (ECM) are collagen and elastin. The concentric arrangement of the fenestrated elastic laminae which are connected to each other with elastic fibrils is the reason behind the resilience and strength of elastic arteries. Smooth muscle cells within the framework are elongated cells with several extensions with a defined orientation.

The structure is held together by a fibrillar collagen network and mucopolysaccharides. This network structure provides the means to resist both longitudinal and lateral forces by the tunica media. The tunica adventitia is a collagen rich fibroelastic tissue. The nutrition of the vascular wall is supplied by vessels coming through the adventitia and it also contains innervations. The main function of adventitia is the stability and connection of the vessel to the surrounding tissue (Rhodin, 1980).

The media layer is rich in collagen type I and III, elastin and several proteoglycans. In this structure, the presence of the collagen fibrils contributes to the strength of the vessel and the elastin molecules render it elastic (Heydarkhan-Hagvall et al. 2006). This structure is important for the laminar flow of blood within the lumen and the control of blood vessel diameter as a response to hormonal or neural stimulations. Another role of the ECM is the sequestering of soluble biochemical signals (Noh et al. 2006, Heydarkhan-Hagvall et al. 2006). The load due to the pulsatile blood flow is mainly borne by the media layer. Vascular tissue is composed of nearly 70% water, and hence resists volumetric compression (Peattie, Fisher 2007).

2.2.1 Endothelial Cells

The endothelial lining is a single layer of cells covering the interior surface of the vessels, and is responsible for the antithrombogenicity of the inner surface, the response of blood vessels to injuries and the blood vessel activity control through secretions. The endothelial cells control blood homeostasis, regulate the transport of materials from and to blood, smooth muscle cell proliferation and fibrinolysis

(Table 2.1). Thus coverage of the inner vessel wall with a healthy, confluent endothelium is very important. The absence of an intact endothelium was shown to induce conversion to the synthetic phenotype for SMCs, which means that smooth muscle cells will proliferate more and secrete more ECM proteins due to lack of controlling signals by endothelium, and to play an important role in occlusion of the artificial grafts due to intimal hyperplasia (Deutsch et al. 2008). A healthy endothelium prevents initiation of the coagulation cascade. Their most distinguishing structure is their cobblestone like shape and their wide range of cell to cell adhesions. Under normal conditions endothelial cells secrete the anticoagulant thrombomodulin, but in case of an injury they start to express pro-platelet adhesive proteins such as selectins. They react to shear stress by increasing their surface area through spreading. Aside from shear stress it has been shown that the surface topography of the basement membrane plays an important role in the behaviour of the endothelial cells. The nanometre scale roughness of the inner surface of the vessels is said to be an essential aspect of the endothelial lining. This roughness is provided by the basement membrane secreted by the endothelial cells.

Table 2.1 Functions of Vascular Endothelial Cells(Davies 2009, She, McGuigan & Sefton 2007, McGuigan, Sefton 2007)

Function	***Mode of Action***	***Agents***
Vascular tone	Change in SMC morphology	Nitric Oxide, Endothelin, Angiotensin
Blood clotting, fibrinolysis	Platelet activation	Von Willebrand factor, Thrombin, Plasmin
Inflammation	Leukocyte and platelet activation	Complement system, Selectins
SMC activity	Proliferative, anti-proliferative	NO, prostoglandin
Transportation	Barrier function	Surface proteins
ECM secretion	Basement membrane	Collagen type IV, laminin, heparin sulphate

Endothelial cells are the first surface to be in contact with blood and they sit on a very thin basement membrane (40-120 nm). The basement membrane is composed mainly of collagen type IV, type V, laminin and heparin sulphate which are arranged as fibres. The lining also acts as a secretory tissue in the whole body with abilities to direct smooth muscle cell and blood cell responses and the onset of coagulation and inflammation. Some of the secretions that are important anti-thrombogenic or fibrinolytic factors include Nitric Oxide, prostocyclin and tissue plasminogen activator (Bachetti, Morbidelli 2000).

Endothelial cells act as a first line of response to the changes in shear stress due to the blood flow, which affect endothelial cells' gene expression profile which in turn causes changes in smooth muscle cell behaviour. Modulation of thrombosis is achieved through expression of several surface positive or negative feedback molecules (McGuigan, Sefton 2008). Endothelial cells express PECAM

(Platelet/Endothelial Cell Attachment Molecule) when they are in contact and are forming tight junctions. Thus PECAM expression is a good indicator of integrity of endothelial lining. Some other surface proteins such as ICAM (Intercellular Adhesion Molecule) and VCAM (Vascular Cell adhesion Molecule) which are mainly related to endothelium-leukocyte interactions can also be used to assess the health of endothelium, since they are expressed at a constant level under normal physiological conditions. Another protein that can be used to evaluate the endothelial lining is von Willebrand factor which is a protein that has an important role in coagulation cascade but is also expressed constitutively. Another important role of endothelial lining is the secretion of Nitric Oxide (NO) which has several functions such as vasodilatation, inhibition of vasoconstrictor signals and prevention of platelet adhesion and also anti-inflammatory and anti-proliferative functions. Antiproliferative functions are important for the prevention of intimal hyperplasia due to the overgrowth of smooth muscle cells. It was shown that endothelial cells respond to the underlying basement membrane or the coating of a tissue culture surface with cell attachment promoting molecules with a decrease in apoptosis and the maintenance of anti-thrombogenic phenotype. This is an important parameter that defines that the culture conditions have a strong effect on the behaviour of the endothelial cells (Prasad Chennazhy, Krishnan 2005).

Pro or anti-thrombogenic effects can be affected by the presence of shear stress but also by the substrate on which endothelial cells are grown; i.e. the strength of adhesion of endothelial cells and their ability to spread on the substrate surface. For a tissue engineered blood vessel, the endothelial lining should be in a

phenotypic state in which it prevents blood coagulation, so it is important to check signals and effects that trigger coagulation, complement activation and blood cell activation.

Endothelial cells have a cobblestone shape upon confluence in a tissue culture plate and their rate of division is much higher compared to that of *in-vivo* rates. Their presence is important for preventing platelet adhesion since platelets can easily attach to a denuded area of endothelium where they can interact with the underlying basal lamina. One of the active bio-signal that has an important role in homeostasis of the vasculature is Nitric Oxide (NO). It is a free radical with a wide range of functions in multicellular organisms; specifically in vasculature, upregulation of NO synthesis results in vasodilatation by directly affecting vascular smooth muscle cells. In the vasculature, NO synthesised by endothelial cells from arginine via the enzyme called e-NOS (endothelial nitric oxide synthase). NO can also act as an anti-proliferative signal for vascular smooth muscle cells during the remodelling process. NO also prevents platelet adhesion (Ibrahim, Ramamurthi 2008). NO synthesis can be affected by the presence of shear stress. Shear stress and cyclic strain are known to increase the expression of NO by upregulating eNOS.

2.2.2 Smooth Muscle Cells

The tunica media, populated by smooth muscle cells, is responsible for vascular tone/diameter and, in this sense; highly differentiated smooth muscle cells are responsible for the dilatation and constriction of vessels. This is how they regulate blood pressure and the distribution of blood flow. Smooth muscle cells appear in a helical pattern mainly in the circumferential direction. This orientation is important

for the vasoconstriction/dilation cycles and also for the mechanical strength of the vessels. They are also very sensitive to strain and dynamic culture experiments have shown that smooth muscle cells become more oriented under such conditions. Cell proliferation is also affected by the application of strain. They have fibroblast like highly orientated shapes which are closely related to their function (Figure 2.5). The vascular tissue is a stable tissue, which means that it can heal. This is possible due to the phenotypic plasticity of smooth muscle cells; which enables them to switch between a wide range of phenotypes with distinct characteristics; defined as contractile and synthetic phenotypes for the two extreme conditions (Owens, Kumar & Wamhoff 2004).

2.2.2.1 Contractile versus Synthetic Phenotype of Smooth Muscle Cells

Smooth muscle cells are mostly in the quiescent, contractile stage under in vivo conditions. However upon injury, they convert to a more synthetic phenotype resulting in cell proliferation, enzymatic degradation of ECM and remodelling via newly secreted extracellular matrix. When grown under *in vitro* conditions, SMC generally exhibit this conversion and proliferate. This is mainly related to the static culture conditions which are very different from the native vessel environment where there is constant stretching and radial distension due to the blood flow. These effects are sensed by SMCs through the interaction of their integrin mediated connections with the extracellular matrix (Owens 2007).

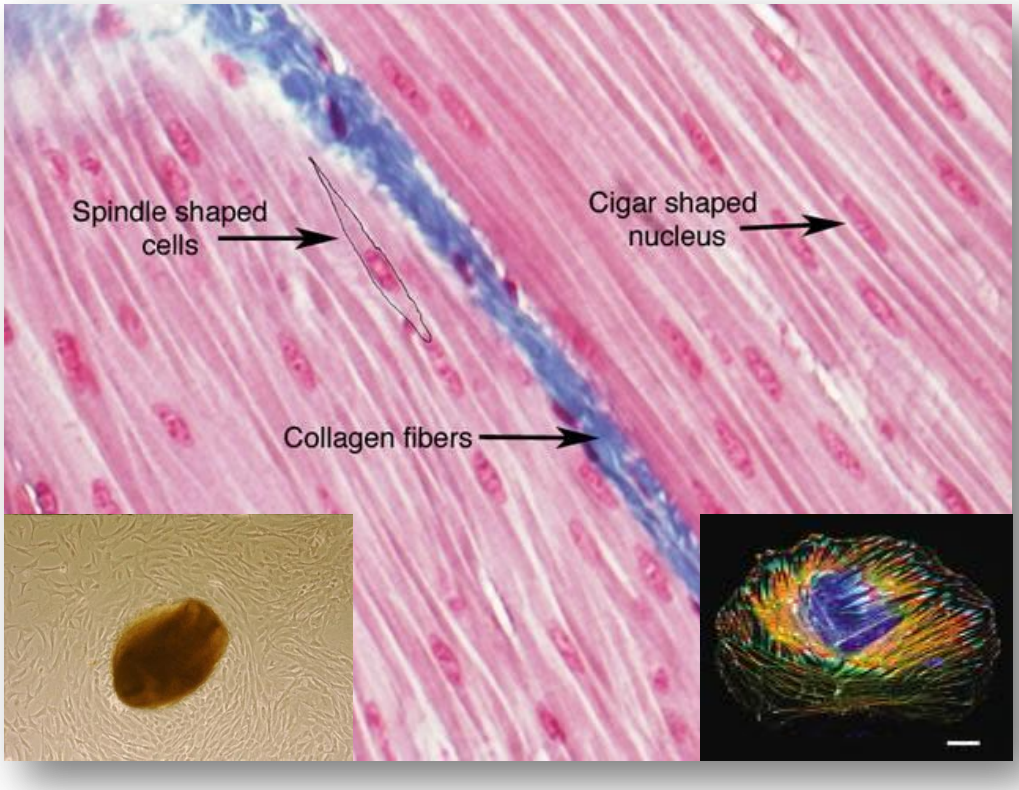

Figure 2.5 General smooth muscle cell**.** Spindle-like appearance of Smooth muscle cells with elongated cytoplasm in the direction of strain and also elongated nuclei. ECM secretion, collagen, follows the same pattern too (Obtained from **www.uscf.edu**, Histology Lecture Series, bladder). Left Inset) Appearance of vascular smooth muscle cells during explants culture (Obtained from www.strath.ac.uk) Right Inset) Single GFP transfected smooth muscle cell (Obtained from Nature Cell Biology, www.nature.com).

There are very notable differences between contractile and synthetic SMC phenotypes, such as increased smooth muscle actin expression in contractile cells and a pronounced increase in the ECM molecule synthesis in the synthetic phenotype. However, this phenotype conversion is not like a Boolean switch. It is more similar to a continuum of several phenotypes with differing degrees of contractile or synthetic properties. Mostly, maintenance of the contractile phenotype is dependent on the shear stress and pulsatile flow conditions and

biochemical signals, such as growth factors and/or the presence of ECM components (like GAGs). It has been shown that cyclic strain and shear stress are crucial for the well being of both endothelial and SMCs (Owens 2007). SMCs align themselves in the circumferential direction, in order to bear the stretching (Standley et al. 2002). This obviously has an effect on their phenotype and subsequent behaviour.

Another aspect of the SMC phenotype is the relation between the phenotype conversion and the ECM molecules. Several studies have been carried out in order to determine their effect. It has been shown that presence of collagen type I and fibronectin tend to trigger the synthetic phenotype, whereas components of the basement membrane, such as collagen type IV, laminin or elastin, suppressed the synthetic phenotype. However, such effects also show variation between 2D and 3D cultures. For example, even though collagen coatings increase the synthetic phenotype expressions, it was shown that cell proliferation was slower for cells encapsulated in a 3D collagen gel. Phenotype change from contractile to synthetic is important in atherosclerosis and restenosis. The main markers of the contractile smooth muscle phenotype are smooth muscle α actin, calponin, myosin heavy chain isoforms (Halka et al. 2006).

The modulation of smooth muscle cell phenotype is directly related to the tone of the underlying ECM. The mechanosensing capability of smooth muscle cells dictates the phenotype through sensing strains due to the pulsatile blood flow (Jeong et al. 2005).

The wide range of functions of both endothelial and smooth muscle cells indicates the reasons behind the shortcomings of purely synthetic grafts and a need to incorporate these cellular components in the future implant. Tissue engineering offers a method for achieving this goal.

2.3 Vascular Tissue Engineering

As a sub-field of tissue engineering, vascular tissue engineering deals with the production of bio-artificial implants that can replace arteries functionally and in the long run integrate with the host artery totally. This is planned to be achieved by successful culturing of arterial cells on biodegradable natural or synthetic scaffold materials which would endow the system with its mechanical properties and provide the arterial cells with a template to reproduce the 3 dimensional structure of arteries (Wang et al. 2007) . For this end porous matrices either produced via decellularisation of tissues or manufactured from synthetic polymers such as Poly Glycolic acid (PGA), Poly lactic acid (PLA) or from natural polymers such as collagen and fibrin have been used to promote smooth muscle growth and tunica media production (Cetrulo 2006, Thomas, Campbell & Campbell 2003). After maturation of the medial part, the inner surface is seeded with endothelial cells.

2.3.1 Aims and Requirements of Vascular Tissue Engineering

Vascular tissue engineering has three main aims. The first is the provision of a biological alternative to autografts and allografts used in current surgical procedures which is functional both structurally and biochemically, and is readily available or can be produced in a short time interval. The second goal is to develop *in vitro* systems that imitate the natural blood vessels with high fidelity, so some of

the research on cardiovascular diseases can be conducted on these systems and the final aim is to manufacture small diameter artificial vessels as complementary structures within the tissue engineering scaffolds for other target tissues in order to facilitate and improve revascularization. Although great achievements have been attained in the past, none of the stated goals have been fully realized yet.

The reasons behind the relatively low level of success, or rather low level of successful clinical outcomes, are the onerous requirements for a functional vascular tissue engineering product. Aside from the general prerequisites of biocompatibility and infection-resistance; as blood contacting materials, artificial arteries should be thromboresistant, with long term mechanical reliability (kink resistance, burst strength, compliance, suturability) and on top of that they should also act as a secretory tissues via the endothelial lining and be able to respond to mechanical and neural stimuli by being able to change its diameter (Thomas, Campbell & Campbell 2003).

There are some additional problems related to the engineering of the vascular tissue which are not encountered in other tissue engineering applications. The biggest issue is the necessity of the immediate functionality. Unlike other tissues such as bone, cornea or cartilage, the tissue engineered vessel does not have an opportunity to remodel prior to assuming full functionality following implantation. Since it is linked to the circulation, it is required to function as a circulatory element immediately. Moreover, integration of the artificial tissue with the host body happens under very dynamic conditions in the case of vascular tissue engineering, making design of such prostheses quite difficult. Moreover an unsuccessful attempt

can lead to several complications, such as formation of thrombus on the implant that may compromise the well-being of circulatory system. Thus, the design and production of an artificial vessel is a very sophisticated procedure. The mechanical demand on the scaffold is perpetual, and this demand might not be met by the secreted ECM especially if the application will utilise autologous cells from elderly patients, which are limited in capacity to grow and secrete.

2.3.2 Methods and Achievements of Vascular Tissue Engineering

There are three main approaches to vascular tissue engineering. The first is utilization of scaffolds which are populated with arterial smooth muscle cells. Population is achieved mainly through two routes; cell seeding at the beginning of the cell culture or encapsulation of the cells within the scaffold structure. As for scaffold material, the main choices are decellularised natural tissues (xenogenic arteries or small intestine submucosa) or manufactured synthetic or natural polymer based structures (Kakisis et al. 2005). As for scaffold forms the main choices are porous foams or hydrogels, mainly for encapsulation purposes. Generally, seeded scaffolds are conditioned in bioreactor systems under pulsatile flow to imitate the effect of blood flow (Isenberg, Williams & Tranquillo 2006, Buttafoco et al. 2006). The second approach is to use the patient's own body as a bioreactor for production of the artificial vessels and a third option is the scaffoldless systems which rely on the triggering of ECM secretion capabilities of the smooth muscle cells and fibroblasts (Baguneid et al. 2006). Below, the most prominent examples of these methods are discussed.

The first successful example of vascular tissue engineering has been demonstrated by Weinberg et al in 1986 (Weinberg, Bell 1986). They used bovine originated cells in collagen based hydrogels and after a long-term culture vessel like structures were obtained, albeit with low strength (Figure 2.6). Their model was quite strong, since it had all three cellular components (endothelium, SMC layer and the fibroblast layer). However a Dacron mesh was needed as a mechanical stabilizer. Other natural polymers, especially fibrin since it can be obtained from patient's own blood so would be less immunogenic, have also been successfully used in similar schemes (Helm, Zisch & Swartz 2007, Mol et al. 2005).

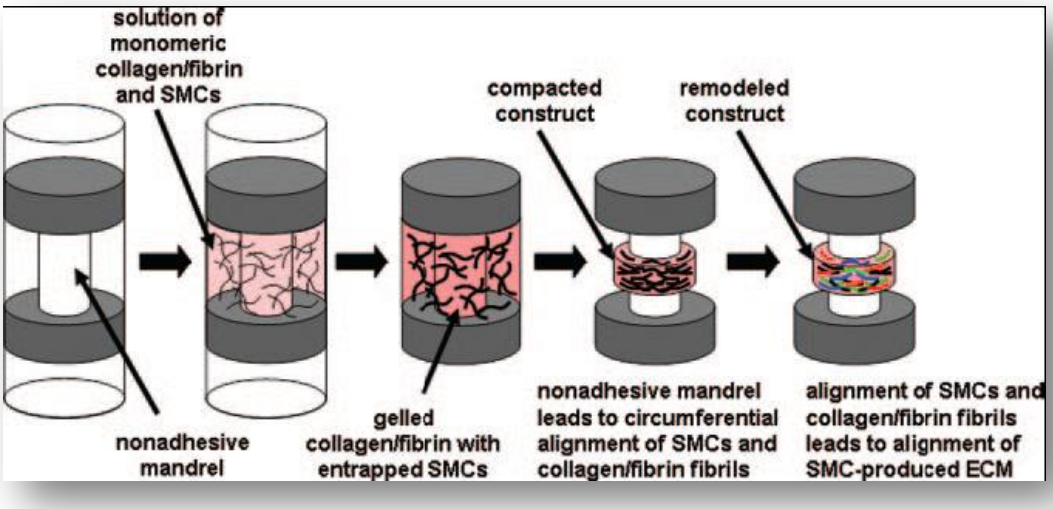

Figure 2.6 Step by step production of Weinberg Bell arterial model. Even though, it is quite beneficial for smooth muscle cell circumferential alignment, it suffers from lack of physical strength due to the weakness of the starting collagen gels (Isenberg 2006b)

An option to overcome the physical limitations of the natural scaffolds yet having natural components is to utilize decellularised vessels, which have been used with

limited success (Kerdjoudj et al. 2006, Martin et al. 2005). Decellularised scaffolds are advantageous in the sense that the orientation and distribution of the ECM molecules are very close to those of native structures. Also, decellularised scaffolds may retain entangled or fixed growth factors that can be further released after culture and remodelling. However, the generally harsh nature of the decellularisation process can result in batch to batch differences between different decellularised scaffolds. Moreover, decellularised systems, especially from other donors or species, also pose disease transmission problems.

For improving the mechanical properties of the conduits, several synthetic materials have been utilized as vascular tissue engineering scaffolds (Figure 2.7). Utilized polymers include PCL, PLGA, PGA. Although synthetic materials offer better mechanical properties and long term stability, their degradation rate and the effect of degradation products on the behaviour of the cells is of major concern. Niklason and co-workers used PGA based scaffolds and have successfully covered the surface of these scaffolds with endothelial cells (Niklason, Langer 1997, Gong, Niklason 2006). Polyurethane scaffolds were also used as a scaffold for SMCs under a laminar flow regime. They were populated by cells and cells synthesized ECM. Following endothelialisation, scaffolds were grafted to dog coronary artery and remained active for 1 month.

A very novel approach was carried out by Campbell et al, in which silicone tubes were inserted into rabbit peritoneal cavity and a vessel-like structure was obtained through the inflammatory response of the host body (Campbell et al. 2004, Chue et al. 2004). After silicone tubes were covered with cells they were removed and

grafted to the same animal, to serve as autologous grafts, with moderate success. Another quite innovative experiment conducted by L'Heureux et al, suggests production of a functional artificial vessel without any scaffold (L'Heureux et al. 2006). In this system, confluent layers of SMCs and fibroblast were wrapped around each other and after maturation of these layers; the inner lumen was seeded with endothelial cells. This structure stayed patent for at least one month, with a burst strength comparable to that of native blood vessels (Figure 2.8). However, the total manufacturing period is more than three months for each vessel and there was a high degree of interference with the culture system, which is very risky when the probability of contamination is considered.

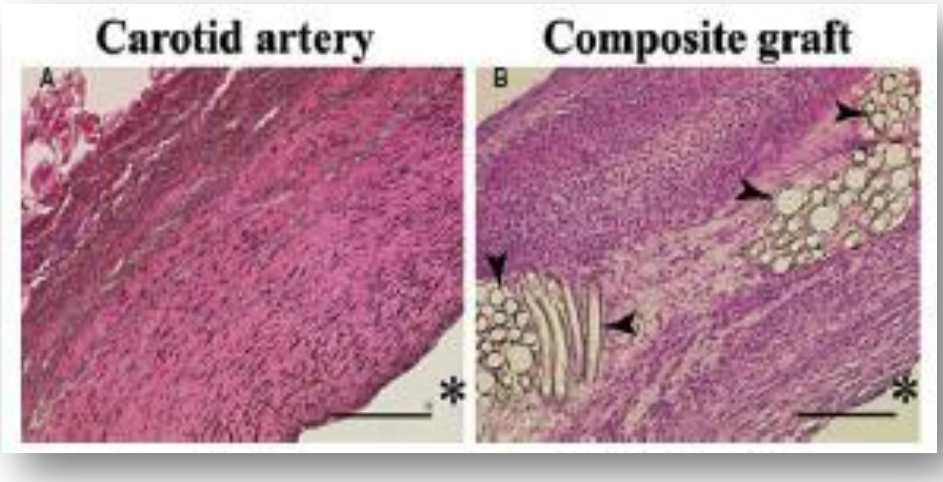

Figure 2.7. Cell seeded synthetic scaffold vs. carotid artery. Comparison of smooth muscle cell seeded synthetic composite graft (made of PVDF and fibrin) and natural carotid artery cross-sections after 2 weeks of cell culture. Similar cell numbers were obtained in the composite graft; the original polymer scaffold is still visible in places (Marked with arrows) (Tschoeke et al. 2008).

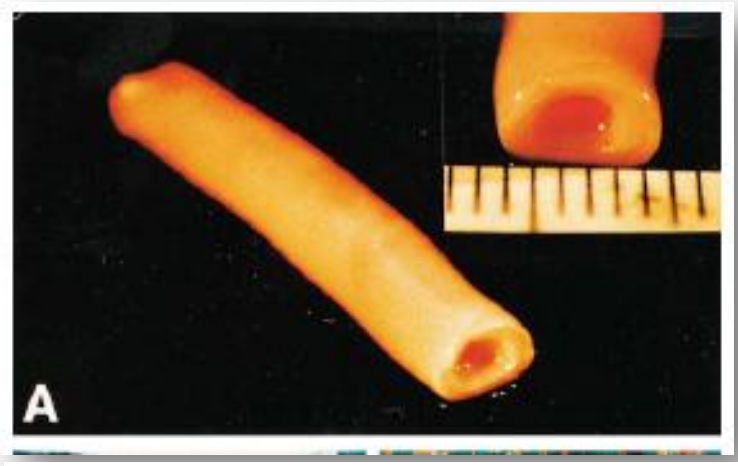

Figure 2.8 Scaffoldless vessel production**.** L'Heureux Model of scaffold-less artificial vessel after 9 weeks of maturation, a self-sustaining vessel like structure without using any scaffold material (L'Heureux et al. 1998)

2.3.3Endothelialisation

One of the most important aspects of vascular tissue engineering is the endothelialisation of the construct and the retention of the endothelial layer after exposure to laminar flow. Unfortunately, most of the current designs are not successful in this respect. Several approaches have been developed to overcome this problem, such as different seeding techniques, magnetic orientation of the cells, or coating of the lumen with cell adhesion promoting molecules and the preconditioning of the cells at lower shear stresses (Alobaid et al. 2006, Feugier et al. 2005, Turner et al. 2004). Even though there are problems with the endothelialisation, tissue engineering constructs are easier to endothelialise

compared to the standard vascular graft, due to the generally more suitable nature of the substrate for cell attachment (Ibrahim, Ramamurthi 2008, He et al. 2005) especially for the case of decellularised tissues and natural polymer based scaffolds. In addition to this, *in-vitro* shear conditioning of the endothelial layer is hugely beneficial (Inoguchi et al. 2007).

As can be seen from the examples, the main short term aim is the development of the first two layers of the vasculature; the intima and the tunica media. This composite structure actually is composed of two different tissue types, namely epithelium and smooth muscle tissue. Thus what is necessary is to successfully develop the basement membrane for the endothelium, so that there would be a strong attachment of the endothelial cells and the connection between the endothelium and the tunica media would be possible. The second challenge would be the development of the media with smooth muscle cells in a contractile phenotype so that upon implantation they could react to the nerve impulses and be able to change the diameter of the vessel. The main purpose of using smooth muscle cells is to achieve ECM secretion during the degradation of the scaffold, so that it can be replaced with the native ECM which is the product of smooth muscle cells. The removal process can be passive if the scaffold is mainly made of synthetic polymers or active if it contains natural polymers which can be degraded by the smooth muscle cells by enzymes, such as metalloproteinases. The level and rate of ECM secretion, composition of the final ECM structure and modulation of smooth muscle cell phenotype between contractile and synthetic phenotypes are the main parameters that need to be controlled during the process of artificial vessel

manufacture. Therefore, one of the most important parameters in vascular tissue engineering is the control over this conversion, since proliferation of smooth muscle cells is necessary in the beginning of the culture. However, after a certain density is reached, this proliferation must stop. Otherwise, overpopulation of the structure with SMC may occur, and this would result in something similar to intimal hyperplasia.

Another important aspect of vascular tissue engineering is the determination of the effects of stresses or strains present under in vivo conditions on different cell types and possible methods of exploiting these relationships. Physical properties of the scaffolds, such as porosity, surface roughness and stiffness can also have marked effects on smooth muscle cells. Also another important parameter is the organization and orientation of the smooth muscle cells which are oriented in a spiral-like structure in native vessels with respect to the direction of the load and this orientation has an effect on ECM secretion too (DeLong, Moon & West 2005/6). The stiffness and the density of the encapsulating scaffold are also important for cell behaviour, because the density of the scaffold would have an effect on the migration rate of the cells and the stiffness of the 3D structure will influence the attachment profile.

Mechanical properties have a high priority in vascular tissue engineering applications. Compliancy of the grafts is especially crucial. Thus, any material that is to be used as a vascular tissue engineering scaffold should exhibit viscoelastic properties similar to those of soft tissues and they should be resistant to fatigue

and creep due to cyclic stretching. For these purposes, hydrogels in general are viable candidates.

2.4 Hydrogels

Hydrogels are one of the most prominent forms of scaffold in tissue engineering owing to the similarity of their mechanical properties to soft tissues, their ease of fabrication, their comparable water content to that of tissue and their suitability for cell encapsulation processes either as pre-made structures or as injectable scaffolds (Kopecek 2007). Injectability is especially important for tissues where a complex architecture is necessary. There are a wide range of natural and synthetic polymers which can be used in the manufacture of hydrogels, such as alginate, collagen, fibrin, polyethylene glycol (PEG), polyvinyl alcohol (PVA), poly ethylene oxide (PEO), Polyacrylic acid (PAA), Polypromyl fumarate (PPF) (Hong et al. 2007, Drury, Mooney 2003). Hydrogels are hydrated networks of one or more polymers which are held together either by entanglements of the polymer chains or crosslinks between them (Figure 2.9). Several different methods can be used to trigger gelation such as temperature change, physical crosslinking, chemical crosslinking, photocrosslinking, or the utilization of radiation (Razzak et al. 1999).

Hydrogels have high water content and generally show viscoelastic behaviour. They have been used as contact lenses, artificial corneas and artificial cartilage scaffolds. They have been shown to be beneficial for three dimensional cell network formation, which facilitates the formation of a tissue-like structure. One of the most important properties of the hydrogels is their hydrophilicity which decreases the rate of protein adhesion onto hydrogels and thus increases their biocompatibility

(Nicodemus, Bryant 2008a). However their highly hydrophilic nature can sometimes be detrimental for some applications, since cells generally tend to adhere to surfaces with intermediate hydrophilicity. The reason behind this is that the cells adhere to a surface through the adhesion proteins absorbed onto that surface and the protein adsorption is quite low for highly hydrophilic or hydrophobic surfaces. This situation can be improved by addition of cationic building blocks, which are known to promote cell adhesion. Another plausible route would be the development of composite hydrogels (Lozinsky, Savina 2002). The hydration level of hydrogels is generally higher than 30% (Drury, Mooney 2003).

Synthetic hydrogels are advantageous since their chemical properties can be easily altered which in turn provides more control over essential hydrogel properties such as the degree of crosslinking, degradation, the dynamics of gel formation and mechanical properties. Another advantage is their high level of reproducibility. However, synthetic hydrogel structures have an inherent problem with cell attachment. Unlike collagen, most hydrogel forming molecules do not have cell attachment promoting domains. Thus cellular attachment depends on the absorption of adhesion proteins. On the other hand, natural polymers have properties quite similar to ECM which improve their biocompatibility and performance as tissue engineering scaffolds. Collagen, fibrin, and hyaluronic acid are all natural components of the ECM and thus they are favourable for cell attachment and spreading. The mesh size of the hydrogels has an effect on both degradation and biochemical signalling. The mesh size of the hydrogel can affect the diffusion of biomacromolecules which in turn may change the enzymatic

cleavage of the natural constituents of the hydrogel, but can also hinder growth factor penetration (Dikovsky, Bianco-Peled & Seliktar 2006).

It can be said that the ideal hydrogels for tissue engineering would be composites of natural and synthetic polymers which can then demonstrate the beneficial attributes of both groups. For soft tissue engineering applications one of the available synthetic polymers is Polyvinyl Alcohol.

2.4.1 PVA Hydrogels

PVA is a synthetic polymer with pendant –OH groups. It is formed through hydrolysis of acetate groups of poly vinyl acetate which itself, is polymerized from vinyl acetate monomers. The main properties that define PVA behaviour are the degree of hydrolysis and the degree of polymerization. It can be used to form films, foams and hydrogels in the tissue engineering area, but it also has several other functions, such as surfactant, sealant or cement filler. It is water soluble and its solubility depends on its molecular weight (Guo, Xu 2005).

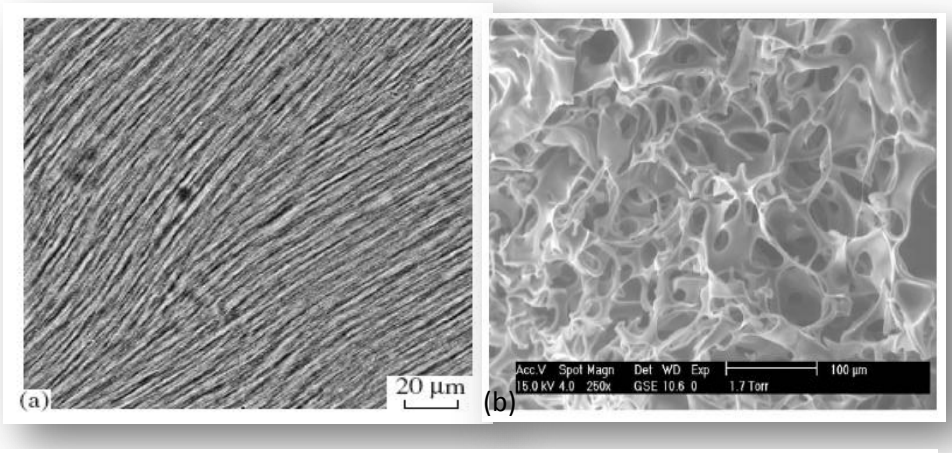

Figure 2.9 Typical structure of cryogels a)Polyvinyl alcohol (Lozinsky et al. 2007) which shows cryostructuring as can be observed by the presence of aligned PVA chains b) Polyacrylamide (Plieva et al. 2004) which shows a mesoporous structure

2.4.2 Network Formation

PVA can form hydrogels of high water content with several different methods such as chemical crosslinking (such as glutaraldehyde, succinyl chloride), freeze/thawing, gamma irradiation and photopolymerisation. PVA hydrogels have desirable mechanical, swelling and optical properties which make them suitable for tissue engineering (Mangiapia et al. 2007). The freeze/thaw or cryogelation method has an important advantage over chemical methods. Due to its purely physical nature there is no risk of remnant chemicals that might compromise the biocompatibility of the final hydrogel. Also, gels formed with this method are highly elastic and durable, which is quite important for soft tissue engineering. However, PVA based cryogels have some disadvantages such as the need for sterilization, which is not necessary in the case of the gamma-irradiation crosslinked hydrogels which are inherently sterile (Chowdhury et al. 2006). Moreover, in the presence of the natural macromolecular additives, such as chitosan, dextran and bacterial cellulose, the gelation process is different because generally these additives are not as prone to gelation as PVA chains. This causes heterogeneity in the structure and also faster loss of the additives than the PVA backbone (Mathews et al. 2008). PVA has been shown to be biocompatible, eliciting a minimal inflammatory response upon implantation (Stammen et al. 2001). One of the most promising properties of PVA is its ability to form physical hydrogels, described as cryogels, via repetitive freezing and thawing cycles.

2.4.3 Cryogels

Cryogelation is one of the methods of physical hydrogel formation. These gels are formed through processes which force formation of non-covalent bonds such as

hydrogen bonds, ionic bonds or by basic entanglement of the polymeric chains and crystallites after freezing and thawing cycles (Figure 2.10). Most of these gels are reversible gels due to these factors. The gels are very beneficial in the sense that there is no need for addition of any chemical crosslinker or application of UV light, which in some cases cause cytotoxicity and problems due to the remnant chemicals. Cryogels form under moderate freezing conditions in which frozen solvent causes phase separation and acts as a porogen, leading to a gel with high water content. Gelation can occur in each of the three steps of the freeze-thawing process; freezing, storage in frozen state or during thawing. For PVA the most important step is thawing, since this is where most of the gel formation occurs (Komarova et al. 2008). One of the main aspects of cryogelation is that not all of the solvent freezes under these conditions and there is always a portion of the solvent in the liquid phase (Lozinsky 2002). The surface tension between the thawed solvent and the gel phase causes round pores. The conversion between spongy and non-spongy cryogels depends on the freezing regime and the concentration and composition of solute. The physical gelation occurs through formation of a three dimensional non-covalent bond structure, either hydrogen bonds or hydrophobic interactions depending on the nature of the solutes. The degree of hydrolysis is important for PVA, since a high level of presence of acetyl groups on the chain causes inhibition of bond formation.

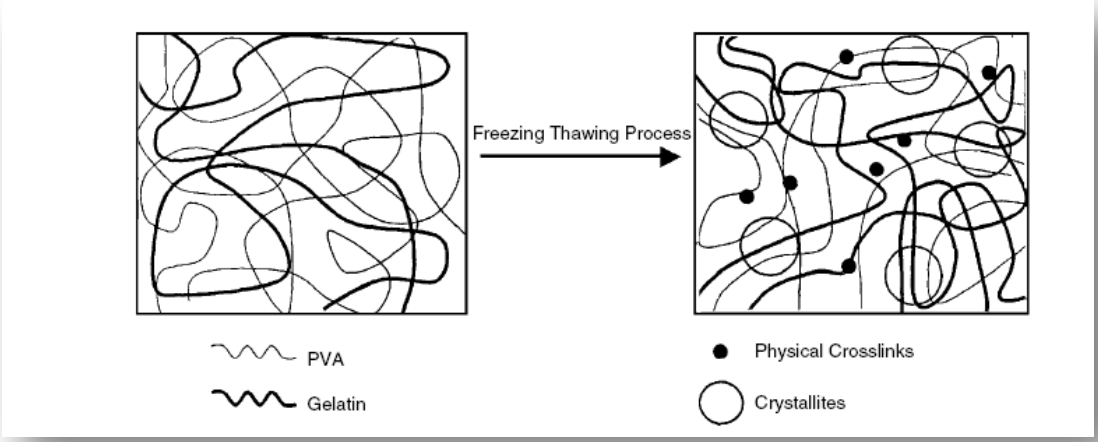

Figure 2.10. Cryogelation process, Gel formation via entanglement, hydrogen bonding and formation of crystallites. Reproduced from (Bajpai and Saini 2005) adapted (Peppas and Stauffer 1991) for Gelatin.

2.4.3 Additives and Coagulation Bath

PVA by itself is a very poor surface for cell attachment, basically due to its inability to adsorb proteins because of its hydrophilicity. This is a limiting property for its utilization in tissue engineering applications and this has been overcome mainly by two methods; tethering of cell adhesive molecules to the PVA backbone covalently (Nuttelman et al. 2001) or development of composite hydrogels. For this end a wide range of biomacromolecules, such as hyaluronic acid, egg albumin, chitosan and gelatine has been utilized for different applications (Sbarbati Del Guerra et al. 1994, Koyano et al. 1998, Koyano et al. 2007, Moscato et al. 2007, Bajpai, Saini 2006). Each of these additives has a different effect on the cryogelation and on the subsequent final physical properties of the resultant cryogel. Thus, utilization of several different additives to determine the optimum additive for a given application merits investigation.

Chitosan is a linear polysaccharide composed of (1–4)-linked d-glucosamine and N-acetyl-d-glucosamine which is obtained by deacetylation of chitin, which is extracted from the exoskeleton of arthropods. Deacetylation degree and molecular weight affect several characteristics of chitosan, such as its crystallinity. Chitosan is generally soluble in dilute acids due to its pendant amine group, which gets protonated in low pH, but it can be made water soluble. Chitosan breaks down in-vivo enzymatically, through the action of lysozymes. Chitosan can be used for various scaffold forms (Chen et al. 2007, Inanc et al. 2007) and it has anti-microbial properties (Shi et al. 2006).

Starch is a plant based storage polysaccharide with good biocompatibility. In plants, the end product of photosynthesis, glucose, is stored in the form of starch. There are two basic structures in starch: amylase which is a linear chain of glucose, and amylopectin which has a branched structure. The ratio of amylase to amylopectin differs from species to species. As a water soluble polysaccharide, starch has been used in several tissue engineering applications (Tudorachi et al. 2000).

Gelatin is formed by hydrolysis and denaturation of collagen which is an irreversible process. So gelatin lacks the fibrillar structure of collagen, but still contains cell attachment sequences and still demonstrates the properties of a protein. The ease of extraction and storage and being cost-friendly makes gelatin a widely used biomacromolecule in the biomedical field. The main source of collagen for gelatine production is either animal skin or tendons or bone (bovine skin is one of the most widely used sources). It is used in several different biomedical and food applications

(Fan et al. 2006, Young et al. 2005, Zhu et al. 2004). Gelatin can form physical hydrogels by hydrogen bond formation.

Utilization of these biomacromolecules provides the necessary surface or bulk properties for cell attachment, but in the meantime they also interfere with the cryogelation process and compromise the mechanical properties of the final hydrogel. Moreover, since these polymers do not gelate as strongly as PVA during freeze-thaw cycles, their loss during culture period through leeching is likely to be quite significant. To prevent this, additional crosslinking procedures might be necessary. As PVA cryogels are physical hydrogels, another physical crosslinking method would be beneficial to keep the positive attributes of physical crosslinking. Polymer coagulation, which is a fast and cost-efficient physical way to crosslink polymers, can be used in this manner. PVA hydrogels are known to have the ability to change volume and density when contacted by solutions containing solutes basically by eliminating water within the hydrogel network (Termonia 1995). This can be used for further crosslinking of the hydrogel when highly concentrated solutions are used. The so-called coagulation bath treatment results in co-precipitation of the two components of the composite hydrogel system together and produces a stronger hydrogel without modifying the chemical characteristics of the hydrogel structure. However, this might also change other physical characteristics of the hydrogel, and these need to be quantified.

In tissue engineering studies, cryogels have been mainly used as substrates for cellular growth, which is a suitable scenerio for endothelial cell culture. However smooth muscle cells would need a 3 dimensional environment to grow and

utilization of cryogelation for cell encapsulation is a less explored territory (Lozinsky et al. 2007). The next section discusses the advantages of the cell encapsulation route for 3 dimensional cell-laden tissue engineering constructs.

2.5 Cell Encapsulation and Storage

In most of the current scaffold schemes, and also in standard tissue culture substrates, cells have an empty space around them. This is quite unlike *in-vivo* conditions, where cells are surrounded by an ECM even during wound formation. The empty area for cell migration in the ECM *in-vivo* is not comparable to the area presented by pores or inter-fibrillar space in meshes which are produced as tissue engineering scaffolds. These open interconnected pores are necessary for cell repopulation, but the effect of this discrepancy on overall cell behaviour is not fully known. The need for a solid scaffold is a logical prerequisite due to the need (i) to keep a specific shape pertaining to the target organ, (ii) to be able to exert some sort of control over anchorage-dependent cell attachment and proliferation and (iii) to obtain the ability to load these constructs with factors that can direct cell behaviour. This is one of the main advantages of tissue engineering over cell therapy, as with a scaffold approach the spatial and temporal distribution of the cells can be controlled better. However there are some inherent problems too, such as vascularisation of the scaffold structure, especially beyond a certain thickness. Utilization of different cell types in the given orientation in a scaffold may be necessary, since they are generally isotropic and not cell selective. Achieving homogenous cell densities within the scaffold is also required, because population of the scaffold is inherently dependent on cell migration (Mironov et al. 2009).

Moreover, the porous nature of these scaffolds actually provides a 2-dimensional environment for the cells, since cells face an empty liquid filled area in their upper region, unless they are encapsulated. It is hypothesized that this might lead to a slow down on the rate of ECM deposition, since secreted ECM is prone to be washed out due to a lack of three dimensional restraining environment like in the tissues (Zimmermann et al. 2007).

An alternative to overcome this problem is encapsulating cells within the scaffolds, by which the physiological structure can be somewhat imitated. Cell encapsulation first started as a method to immunoisolate cells, such as pancreatic and hepatic cells (mainly xenogenic or allogenic cells) from the host without compromising their functionality (Canaple, Rehor & Hunkeler 2002). This treatment scheme can be used for a wide range of endocrine secretion related diseases and also some neurodegenerative syndromes (Uludag, De Vos & Tresco 2000). Another reason for encapsulating cells is to protect them from the detrimental effects of in-situ crosslinking of a complex, load bearing system, where cells also are desired to be functional (Payne et al. 2002). Where accurate control of cell differentiation is needed, encapsulation can be used as a diffusion barrier, acting as a rate controlling element for the diffusion of growth and differentiation factors and moreover as an easier method to distinguish and separate sub-cell populations (Maguire et al. 2006) (Figure 2.11). For vascular tissue engineering specifically, it will enable the separation of the smooth muscle cells within the hydrogel body and the endothelial cells on the surface, since SMCs will be encapsulated but ECs would be seeded afterwards.

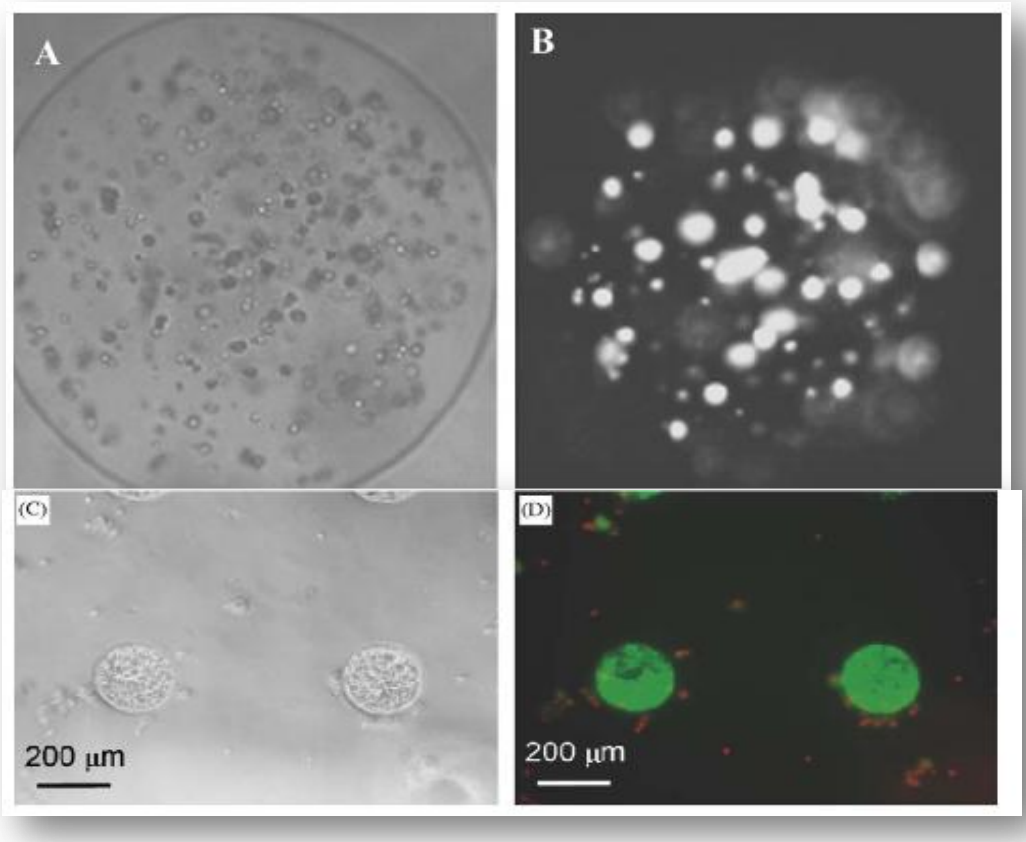

Figure 2.11 Micrographs of encapsulated cells, phase-contrast and live-dead assay results. Phase contrast microscopy (Left hand side images) can be used to locate the cell distribution, as cells can be seen within the hydrogel structure at different depths, individually or in groups. However to ensure viability, additional tests are necessary. Live/Dead assays (Right hand side images) are developed for these situations, where green areas denote viable cells, whereas red areas are dead cells (Maguire et al. 2006, Khademhosseini and Langer 2007).

Storage of tissues and tissue engineering products is an important obstacle for the commercialization of tissue engineering research outcomes. As a patient-specific technology, tissue engineering promises to deliver patient-specific products in a timely manner. However, the variation in physical properties of each tissue, even within the same patient's system, makes this promise unfeasible and storage

systems crucial. There is evidence that shows that cryopreserved tissues and tissue engineering products have markedly different characteristics, such as different mechanical properties and cellular response with respect to fresh samples (Liu et al. 2002). Encapsulated cells are also a good alternative for storage of the tissue engineered constructs or other cell based systems such as biosensors, since it has been shown that cell viability under cryopreservation conditions is higher when cells are encapsulated within a hydrogel structure (Itle, Pishko 2005).

This approach would also be beneficial in the sense that the tissue engineering constructs can be stored as a ready-made product which contains cells instead of needing a long culturing period prior to implantation. Encapsulation of cells has been tried for a long period of time for various applications (Jen, Wake & Mikos 1996). Gelation of the solution containing the cells can be achieved by several methods like photopolymerisation, ion addition (e.g. preparation of cell encapsulated alginate microspheres by divalent cation crosslinking) and physical gelation initiated by changes in pH or temperature. Gelation properties can be controlled by pH, temperature and ionic strength but the essential problem concerning cell encapsulation is the narrow range of durability of cells for all these parameters. Thus, optimization of the final structure is severely hindered by the limitations imposed by the presence of the cells. Such limitations were the case for silk fibroin hydrogel which under physiological conditions take hours up to days to set. This can be prevented by utilization of low pH high temperature conditions which are obviously not suitable for cell encapsulation (Wang et al. 2008). In the same study, after optimization of gelation, it was observed that cells survived much

better in low concentration hydrogels (4%) than in high concentration hydrogels (8 and 12%). Similar effects of concentration and density of the final gel on cell behaviour have been seen with other encapsulation systems too (Burdick, Anseth 2002). However, generally, the concentration of the gelation solution is dictated by mechanical requirements. Cell proliferation is highly restricted in an encapsulation system due to the absence of the necessary surface area and volume for the dividing cells. Thus the final cell number is reached relatively early during culturing. Then further growth is dependent on the degradation rate and it is further restricted by cells' own ECM secretion. Different encapsulation techniques impose different sets of threats to cell viability. For example encapsulation via freeze-thawing is impeded by the ice crystal formation and rupture of the cells which can be partly solved by addition of cryoprotectants such as DMSO (Qi et al. 2004). A secondary limitation is the size of the hydrogels, since encapsulated cells require a steady diffusion gradient for oxygen and nutrients and also for the removal of the waste products, which obviously becomes untenable for thicker hydrogels. Diffusion of the nutrients will also be affected by their propensity to be absorbed on the hydrogel network, which would cause sequestering of certain elements in the outer parts of the hydrogel while depriving the inner core. However, hydrogels are still better options for thick products; as evidenced by the promising results obtained by the 8 mm thick PEO hydrogels with photoencapsulated chondrocytes (Bryant, Anseth 2001). This is mainly due to the high water content of hydrogel structures in general, which improves permeability properties of hydrogel structure and enables better diffusion of the nutrients. Such studies have not established the possible differences between different areas within the hydrogel, due to the

uneven exposure to light (Baroli 2006). Moreover, the encapsulation process itself would impose abrupt changes to the cellular microenvironment which could result in phenotypic responses by the cells.

Encapsulation based therapies can also be utilized to form bottom-up tissue engineering scaffolds which would contain different cell types to produce complex tissues in a tailor-made manner from small micro-moulded units (Yeh et al. 2006). Utilization of photolithography, micromoulding and microfluidics techniques has resulted in precise control over cell-laden hydrogel properties (Khademhosseini, Langer 2007). This would create the opportunity to be able to separate different cell types present in a given tissue while keeping the biochemical communication between them intact through the hydrogel network. Cryogelation is a promising candidate to be used in this manner too.

The relationship of the scaffold with cells can be divided into three categories for tissue engineering applications. Namely, these are migration of the cells of the host *in-vivo* (recruitment), movements of the seeded cells and their subsequent distribution *in-vitro* and encapsulation of the cells within the scaffold structure *in-vitro*. All these three conditions have different effects on the cells and the decision of which one would be best suited for a given application is very crucial. Interactions of the cells with the scaffold material are very different for all these situations. For example, the mode of differentiation of mesenchymal stem cells was very different under 2D culture in a differentiation induction medium and in encapsulated form in hydroxyapatite containing microspheres, mainly due to the interaction and the morphology of the cells within such a structure (Inanc et al.

2007). Another aspect of encapsulation is the control over the stiffness of the environment faced by the cells in a 3D manner; this way, cellular activities such as ECM secretion might be altered as a response to the surroundings. Also, control over how cells would attach within the hydrogel is of great import. One of the possible methods to control this is chemical addition of cell adhesive protein sequences to polymer chains to improve the adhesiveness of the inner space of the hydrogels.

Cell encapsulation has been predominantly achieved by using natural polymers, due to their superior conduciveness to cell attachment. Unlike the attachment and migration mode of events in cell-seeded on to the scaffolds, in cell entrapment there is not enough time between interaction of the medium and the polymer for adsorption of proteins for cell attachment and the following initial cell migration. Following the gelation, cells are trapped in a restricted area and if this area is not permissive for attachment, the result would be apoptosis or necrosis. The most widely used encapsulation materials are collagen, gelatine, alginate, chitosan and fibrin (Elisseeff et al. 2006, Guaccio et al. 2008), which are known to be highly permissive to cell attachment. Another possible advantage of using natural polymers is the possible effect of degradation products on cell behaviour, since most of them are recyclable within the hydrogel system as base materials by the cells. However, some synthetic polymers are very suitable for encapsulation due to their physical properties and ease of polymerization, even though they generally need to be modified for improving cell behaviour within them. Some options are

polyethylene glycol (PEG), polyvinyl alcohol (PVA), polypropylene fumarate (PPF), poly ethylene oxide (PEO) (Elisseeff et al. 2006, Wu et al. 2008).

Additional delivery systems to control the inner biochemical composition of the structure is a viable option, but encapsulation of cells in a polymeric system is a highly closed system in which all the parameters are greatly dependent on each other, which makes the optimization of the process much harder than normal tissue engineering methodologies. Restrictions on the system are quite different in semi-permeable membrane encapsulation, which is mainly used for metabolic product engineering, and the case of connective tissue engineering where mechanical properties and ECM secretion also needed to be considered. This necessitates utilization of complex statistical models for multiple-variable optimization of these parameters for both the start and end point of the construct maturation (Villani et al. 2008). For example, for photopolymerisation reactions it is essential to determine an optimum photoinitiator concentration that would result in enough polymerization while compromising the cell viability minimally (Nuttelman et al. 2008). However, this is not a very clear-cut procedure, because the cytotoxicity is highly dependent on cell type and it was found that proliferative capacity decreases the tolerance of a cell line to photoinitiators (Williams et al. 2005). This would hint that the condition of the cells in culture just before encapsulation is an important determinant of their behaviour. Thus, the confluency level of the cells before trypsinisation and seeding becomes an important parameter. Also the degree of polymerization would have an effect on the mesh size of the hydrogel network, which in turn will define the rate of diffusion to and

from entrapped cells. The initial density of the cells, final desired cell density and attainable cell density are in this sense very important factors during the design of the scaffold. The viscosity of the initial polymer solution imposes an essential limitation on several aspects of encapsulation, such as distribution of the cells within the final structure, porosity or water content of the structure, mechanical properties, roughness, permeability and the amount of area available to cells for ECM deposition (Zimmermann et al. 2007).

In this study, one of the main aims is to develop an encapsulation system for vascular smooth muscle cells by utilizing freeze/thaw based physical crosslinking of PVA chains. The advantage of this system is that it also acts as a storage medium for the cells and the scaffold. An important aspect is the protective capabilities of the polymer chains on the cells by mainly keeping the cell membrane intact. However, cryopreservation of encapsulated cells by using other encapsulation procedures can be problematic and may need high levels of cryoprotectants (Chin Heng, Yu & Chye Ng 2004). An encapsulation system utilizing freeze/thawing may need to incorporate several items to minimize the damage incurred due to freezing, such as addition of cryoprotectants and sugar solutions. Another important parameter is the cooling rate, which directly effects the ice crystal formation. To prevent ice crystal formation vitrification can be utilized; but it has mainly been successfully utilized for encapsulation of single cells (Demirci, Montesano 2007). Our aim is to employ cryogelation first for encapsulation of the cells and then develop a procedure from the data obtained from the short and long term response of the cells to the encapsulation conditions.

Encapsulation and the nature of the encapsulating material may have other indirect effects on tissue properties, such as the mechanosensing ability of the cells and their subsequent response. This is one of the main reasons that necessitate (Nicodemus, Bryant 2008b) the modelling of cell behaviour in 3D encapsulation schemes under different physical conditions and determination of the underlying material-independent mechanisms. Currently, characterisation of a wide range of encapsulation systems has given widely scattered and occasionally contradictory results. This is mainly due to the early stage effects of loading and resulting ECM secretion, changing the interaction of the cells with their surroundings (Nicodemus, Bryant 2008b). Moreover, the main method to prepare hydrogels with different mechanical properties is to change the initial solution's concentration, which also has a direct effect on the degradation properties of the hydrogels (Bryant et al. 2004). Hence analysis of the interaction of these two effects on mechanosensing capabilities of the hydrogels overlaps. Moreover, the aforementioned reverse gelation event causes a very abrupt change in the microenvironment of the cells and even though it does not cause a failure, it might have more subtle effects on cell behaviour. For example, as the secretion of the collagen and GAGs takes place, their diffusion within the hydrogel system is dependent on the mesh size. Thus at lower mesh sizes there would be an accumulation of collagen around the cells due to this diffusion restriction. This might change the behaviour of the cells by itself, since the nearby microenvironment of the cells would become distinctly different from that of the whole hydrogel structure. Thus, a mesh size that would permit the movement of molecules as big as collagen fibrils should be attained. Encapsulation systems are also need to mimic the mechanical properties of their target tissue,

which is predominantly a soft tissue. The next section discusses the mechanical properties of soft tissues and the similarities of hydrogel mechanical behaviour to that of soft tissues.

2.6 Mechanical Properties of Soft tissues and Hydrogels

Soft tissues, such as ligaments, cartilage and blood vessels, are highly elastic tissue which might need to go through huge deformations as a part of their function. Soft tissues are highly complex fibre reinforced composites with interdispersed cells, which is the reason for their non-linear viscoelastic behaviour. Mechanical behaviour of the soft tissue is directly related to the arrangement and relative abundance of these components. The main constituents present in the ECM of soft tissues are several types of collagen, elastin and proteoglycans. Collagen is the main load carrying element. In blood vessels the most abundant collagen types are collagen type I and type III. Collagen is generally found in the form of fibrils in a certain orientation which renders soft tissues anisotropic. Elastin is another protein and as its name implies it is highly elastic. Three dimensional networks of elastin endows the vessels their elasticity. The main mechanism behind the elasticity of this network is its ability to get oriented with the increasing stress and thus store potential energy.

The main characteristics of the mechanical properties of the soft tissues are their anisotropic response to stress and strain, non-linear stiffening and dependence of the final strength on strain rate. They can go through large deformations and exhibit viscoelastic properties, which are mainly related to the activities of the gel-like proteoglycans. The response of the soft tissues can be divided into three

phases. The first is a nearly isotropic, linear response mainly due to the stretching of elastin fibre. The second phase is a nonlinear phase as the collagen fibres straighten and start to resist the stress. In the third phase the collagen fibres are fully elongated and the material becomes stiffer as the stress increases (Holzapfel, 2001). In arteries, the arrangement of the smooth muscle cells also contributes to the mechanical properties. Arteries contract about 40% when isolated, proving that they are under tension under physiological conditions and stress induced by the blood flow corresponds to 10-20% strain (Silver, 2003)

For arteries the most important mechanical parameter is the burst strength; which varies with respect to thickness and diameter. An artificial vessel should at least match the burst strength of the target area. Another important parameter is compliancy. Any compliancy mismatch will result in neointimal hyperplasia formation. The elastic vessel wall is highly extensible and has a great capacity to recoil; which is crucial for the dilation and constriction of the vessels by smooth muscle cells; in the absence of this elasticity artificial vessels can dilate irreversibly and can be weakened by creep (Lillie, Gosline 2007).

Hydrogels are viable options to imitate these behaviours, since they demonstrate similar general viscoelastic characteristics. The only difference is the determining factors behind the behaviour, such as the density of the crosslinks, degree of swelling of the hydrogel (Anseth, Bowman & Brannon-Peppas 1996) or the concentration of the initial solution (Anseth 1996). Moreover, the mode of gelation, crosslinking, polymerization or physical gel formation, also has an effect on the final properties. Hydrogels owe their deformability to the ability of the polymer chains to

rearrange themselves under stress conditions; just like the reorientation of elastin and collagen chains in native tissues. Depending on the density of the crosslinks and the degree of entanglements of the polymer chains, this reorientation will have different time-dependency which gives rise to the different viscoelastic properties of different hydrogels. Specifically for PVA cryogels, the similarity in the mode of non-linear viscoelastic behaviour with the vascular tissue is quite good. Generally the results of mechanical tests fit quite well with that of animal tissue sample tests (Wan et al. 2002). The response hierarchy of the natural tissues, the low stiffness region response by elastin and high stiffness region response by collagen, is replaced in the case of PVA cryogels by the amorphous and crystalline parts respectively (Millon, Mohammadi & Wan 2006). Due to these properties, PVA cryogels have been used as MR phantoms for more than a decade (Chu, Rutt 1997) and this similar response of PVA cryogels to arterial tissue under stress and strain conditions is beneficial for their ability to convey mechanical signals to the encapsulated or seeded cells for tissue engineering applications, since the cellular response will be affected by the response of the substrate to the mechanical forces.

2.7 Mechanotransduction

2.7.1 Bioreactor systems

Requirements for an animal cell culture bioreactor are based on the properties of animal cells. Bioreactors are systems that are designed to amplify cells rapidly while providing them with necessary conditions for their viability, without the labour intensive manual fed-batch methodologies. Since most of the animal cells are anchorage dependent, bioreactor designs for animal cells generally include a solid

support matrix. Problems faced in an animal cell based bioreactor system are, sensitivity of the cells to high mechanical stimulations, a stringent oxygen and nutrient requirement and high risk of contamination. The need for bioreactors for animal cells is due to two main needs; production of some important biochemical molecules such as antibodies or growth factors and simulation of physiological flow and stress/strain conditions (Scragg 1991). In the area of vascular tissue engineering the second need is crucial and there have been lots of efforts in developing custom-made vascular tissue engineering bioreactors that would provide pulsatile flow conditions similar to that of blood flow. These pulsatile flow bioreactors, which are designed to imitate the pulsatile nature of blood flow due to the beating of the heart, are actually derivatives of more general fluidized bed bioreactor designs. The effects obtained range from better nutrient transport, improved extracellular matrix formation, enhanced mechanical stability and phenotypic changes depending on the target tissue (Flanagan et al. 2007, Jeong et al. 2007, Abilez et al. 2006). There are also commercialized bioreactors for vascular cells such as CellMax Capillary System (Figure 2.12) which is used for endothelial/smooth muscle cell co-culture systems under pulsatile flow conditions. The stress/strain environment for tissue engineering bioreactors is dependent on the target tissue and the responsiveness of the cell type used. Whether it is compression, tension or shear; all these dynamic loading conditions trigger a wide spectrum of cellular responses depending on the cells in question, which can be defined as mechanotransduction in general.

Figure 2.12 CellMax Hollow Fibre Bioreactor for co-culture of Smooth Muscle cells and endothelial cells on hollow fibres, which provide the physical segregation of the cell types. Pulsatile flow is obtained by a pump system and 4 bioreactors can be run simultaneously (Image is obtained from Spectrum Labs Inc. website, www.spectrumlabs.com).

Mechanotransduction is the general term used for the biochemical or physical responses of cells to a mechanical stimulus. In different parts of the body the intensity and the mode of response change and also different parts of the body are under different mechanical environments. For cartilage and bone, compressive forces due to movement and the structural support of the organism are important; whereas for the tendons the dominant stimuli are tensile strains. In the case of blood vessels, two dominant stress/strains are very important. These are the shear strain that endothelial cells are exposed to due to blood flow, and cyclic strain (due

to the circumferential stress created by the blood pulses) stemming from the pulsatile nature of the blood flow that mainly affects smooth muscle cells (Figure 2.13).

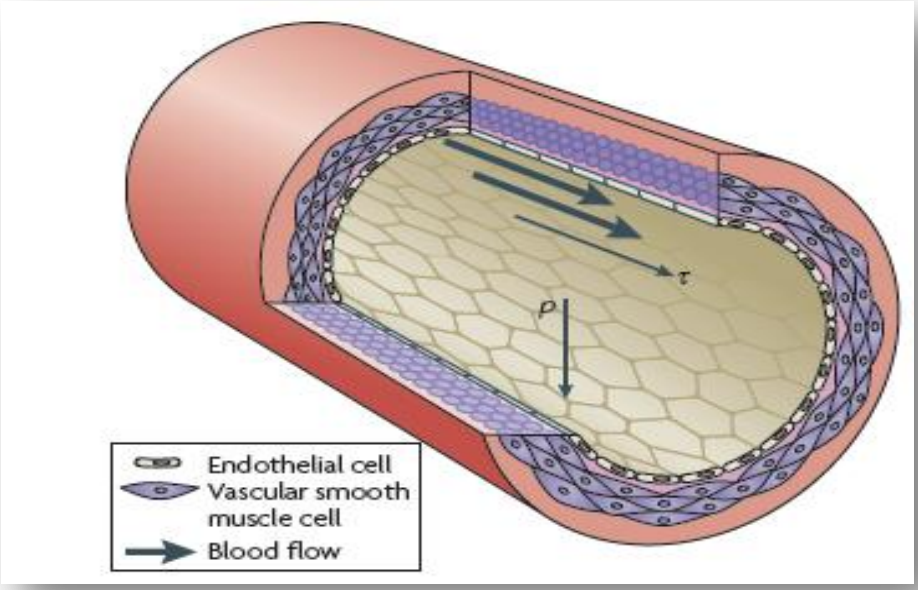

Figure 2.13 Blood flow induced mechanical stimuli. Mechanical stimuli which affect endothelial cells and smooth muscle cells respectively, shear stress and cyclic strain (Hahn 2009)

2.7.2.1 Shear Stress

The wall shear stress component, due to the lateral frictional force, of the effect of blood flow on vascular cells is mainly felt by endothelial cells, unless the lumen surface is denuded. Under normal conditions, the laminar fluid flow related shear stress is the main signal for the endothelial cells to maintain quiescence and release the signals that would ensure quiescence by the smooth muscle cells also (Kudo et al. 2005). In this sense laminar flow induced shear stress limits cell proliferation and

prolongs the turnover rate, i.e. decreases apoptosis. Moreover, it promotes NO secretion and also inhibits smooth muscle cell activation and proliferation (Ballermann et al. 1998). For these reasons, laminar flow is defined as atheroprotective. On the other hand, disturbed shear stress, where the shear stress direction and distribution is not even or the blood flow is turbulent, is defined as atherogenic since it triggers high EC cell turnover, increased proliferation, poor alignment, expression of inflammation related genes and high permeability (Chien 2008b). For example, disturbed shear (oscillatory) increases expression of ICAM (Dardik et al. 2005). But this is also evident under physiological conditions in disturbed flow areas such as carotid bifurcation. Under laminar shear, *in-vitro* activation of several genes occur in the beginning, like prostacyclin and eNOS but then they return back to their baseline levels and cell proliferation is inhibited (Chatzizisis et al. 2007). Also the Akt pathway was suggested as one of the reasons behind the difference between laminar and turbulent flow, since it gets phosphorylated under laminar flow and thus inhibits apoptosis under laminar flow condition, but not under turbulent flow (Dardik et al. 2005). This is the general atheroprotective effect of physiological level of laminar shear. Even a low level of laminar flow has an atherogenic effect. The atherogenic effect depends on the disturbed flow, and resulting turbulent flow which increases proliferation.

There is a proven correlation between the mode of flow and arterial thickening. In arterial branch where the flow is unidirectional and laminar there is no intimal thickening; however when the flow slows down and becomes turbulent arterial thickening can be observed (Davies 2009).The effect of the shear stress exerted by

the flow starts with its effect on endothelial cell proliferation and morphology (Johnson, Barabino & Nerem 2006). Moreover, attachment of the cells should be strong enough so that the endothelial layer would not be removed under shear stress due to the blood flow (Feugier et al. 2005). This is especially crucial in the anastomosis sites, since turbulence also contributes to the stress sensed by endothelial cells and the removal of them is easier. Thus, in general, a strong endothelial lining which will act as a non-thrombogenic surface and at the same time biochemical activities that would direct smooth muscle cells is a necessity either for synthetic grafts or tissue engineered products (Inoguchi et al. 2007).

2.7.2.2 Effects of Cyclic strain on Smooth Muscle Cell Activity

The second component of the mechanical effect of blood flow is mainly effective on tunica media and the cells within this layer, smooth muscle cells. Cyclic strain of a an average of 8-10% acts on the smooth muscle cells and it has been shown that cyclic strain can trigger a variety of responses from smooth muscle cells, especially under *in-vitro* conditions whereas the response of endothelial cells was comparatively insignificant. Under *in-vivo* conditions normal levels of cyclic strain keep smooth muscle cells in contractile phenotype and regulate the expression of ECM molecules such as elastin and collagen and also ECM degrading enzymes, metalloproteinases (MMPs)(Moore et al. 2001) . An elevation or depression in blood pressure triggers vascular remodeling by smooth muscle cells, which is called the myogenic effect, and can cause elevation in proliferation, secretion of ECM and also degradation (Stegemann, Nerem 2003b). This response can be either due to pathological causes or as a result of injury. For example, atherosclerosis evolution

causes “out of phase” cyclic strain and shear stress and it is known to have a further atherogenic effect on the induced area (Hahn, Schwartz 2009). Although there is a consensus on the general importance of the cyclic strain on smooth muscle cell phenotype; the literature available on the subject is largely contradictory, with regard to cell proliferation, apoptosis and secretion and the effects are not as clear-cut as in the case of endothelial cells and shear stress (Kim et al. 1999, Standley et al. 2002, Stegemann, Nerem 2003a). The effects are highly dependent on amplitude and the frequency of the strain as well as the initial phenotype and origin of the smooth muscle cells used (Nerem 2003). However, from a tissue engineering point of view, cyclic strain is a valuable asset to modulate smooth muscle cell phenotype (Butcher, Barrett & Nerem 2006, Stegemann, Hong & Nerem 2005) and there are several attempts to utilize this property, either by custom-made systems or by commercial units, such as the Flexercell System (Figure 2.14). Either systems such as these or pulsatile flow bioreactors can be used to modulate cell behaviour in vascular tissue engineering (Jeong et al. 2005, Engelmayr et al. 2006, Kurpinski et al. 2006).

For vascular tissue engineering one of the most suitable scaffold form is hydrogels, due to their similarities to the soft tissue mechanical properties. However, the most readily available hydrogel options such as collagen and fibrin based hydrogels are substantially weak mechanically. As a model, such as a vessel phantom, PVA cryogels has shown great promise to mimic mechanical properties of the vessels. So if this mechanical superiority can be matched by good cell behaviour PVA cryogels can be viable options for vascular tissue engineering. The current study focuses on

applying the cryogelation property of PVA as a fast and cost-friendly scaffold producing method.

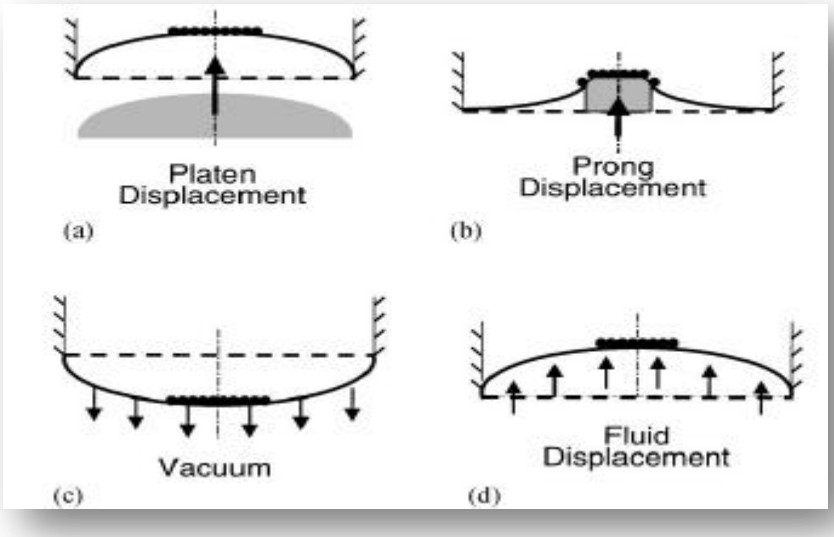

Figure 2.14 Different modes of applying strain to cells in-vitro. Flexible membranes can be displaced by several means such as platens or vacuum. Flexercell system used in this study uses the third mode of action. Different modalities result in different strain distribution on the circular elastomeric tissue culture plate surfaces (Brown 2000)

2.8 Summary

Cardiovascular diseases will continue to be a major health care problem in the near future and, as the population grows, the need for artificial blood vessels will become more urgent. Despite the presence of some well established procedures, especially in the area of small diameter vessels (≤6mm) there is a need for new methods, as the standard grafts have major patency problems. It is hoped that tissue engineered blood vessels will provide the answer to this need as the successful attempts outlined in this review demonstrate their potential. However,

the methods have not been perfected yet and novel systems are required for improvement of the currently available scaffolds and models. PVA cryogels with their viscoelastic properties similar to vessel structures and their ability to support cell encapsulation could be promising candidates in this respect.

Arteries are complex structures which are composed of several different cell types. The extremely dynamic environment of the arteries due to the blood flow has a huge effect on the cell behaviour. Moreover, the constant stress and strain conditions present necessitate mechanical robustness, which has been achieved through evolution with fibril and cell alignment and with the special composition of the ECM of the blood vessels. So any vascular tissue engineering procedure should implement these stress-strain conditions for conditioning of the scaffold and the cells within.

The premises of the current study were drawn with respect to these parameters together with the physical characterisation of PVA based cryogels as scaffolds.

CHAPTER 3

3 Materials, Methods and Research Equipment

3.1 Materials

All materials were of the highest purity commercially available and included: two types of PVA, Molecular Weight (MW) = 78400, Vassar Brothers, USA and Sigma-Aldrich, MW= 89000-98000. The following items were purchased from Sigma-Aldrich Inc.; Starch from potato, Gelatin Type B from bovine skin, Collagen, Potassium Hydroxide (KOH), Sodium Sulfate (NA_2SO_4), FITC Labelled Phalloidin, PBS (Phosphate buffered saline), RPMI 1640 cell culture medium, DMSO (Dimethyl Sulfoxide), FBS (Foetal Bovine Serum),Triton-X, BSA (Bovine Serum Albumin), α-smooth muscle actin antibody, 99.6% Ethanol, quartz cuvette, Trypsin, Penicillin/Streptomycin, SYBR Green, Trizma base, elastin staining kit, trypan Blue.

The list of the other agents used and the supplier companies are given below: Chitosan (85% deacetylation, Jinan Haidebi Ltd, China), Alamar blue (ABDSerotec), CFDA cell proliferation assay, Live/Dead cell viability assay, Vybrant apoptosis assay, AlexiFluor 546 labelled Anti Mouse IgG antibody (Invitrogen, CA, USA), Anti-eNOS type III (endothelial Nitric Oxide Synthase) antibody, FACS flow, FACS clean, FACS rinse (BD Biosciences, USA), 0.2 µm filters, sterile pipettes, 6 and 24 well cell culture plates, 96 well Elisa reader plates, rings, 25, 75, 175 cm^2 cell culture flasks, autoclave tapes, centrifuge tubes, frosted slides (Sarstedt, Germany), iscript cDNA synthesis kit (Promega, Germany), BCA protein determination assay, BSA standards

(BioSciences, UK) DNase, RNeasy RNA isolation kit , (Qiagen, USA), Real-time RT-PCR plates and tubes (Applied Biosystems, USA).

3.2 Methods

This study was conducted in 3 main parts. In the first part; three parameters were dealt with to elucidate the cryogel formation and the properties of the final cryogel structure. As a secondary physical crosslinking method, coagulation bath treatment, was evaluated as a reinforcement and additive retaining system. Then, the effect of the number of freeze-thawing cycles on cryogel properties and cell attachment to cryogels was examined using PVA/Chitosan cryogels. Furthermore, 3 different additives (Chitosan, Gelatin and Starch) were used to assess cryogel properties and to determine whether any of them were better for endothelial cell attachment when compared to the pure PVA cryogel surface. Moreover, secondly, after establishment of the most promising gel type, endothelial cell behaviour on this gel under static and shear stress conditions was evaluated and the functionality of the endothelial lining was demonstrated. The utilization of shear stress induced by turbulent flow was also assessed for facilitation of endothelial cell adherence and growth.

Finally, an encapsulation technique with the selected composite was developed and the final cell laden gel was assessed for cell attachment and incorporation under static and cyclic stress conditions (Figure 3.1).

The experimental procedure for the first part is presented below in a scheme where experiments pertaining to a given property are listed in columns as there are overlapping parts in these experiments (Figure 3.2) and the methods description is

organized accordingly by stating the relevant experiments to each individual part of the first experimental group. The lists of materials and the equipment used are also listed in the corresponding subsections.

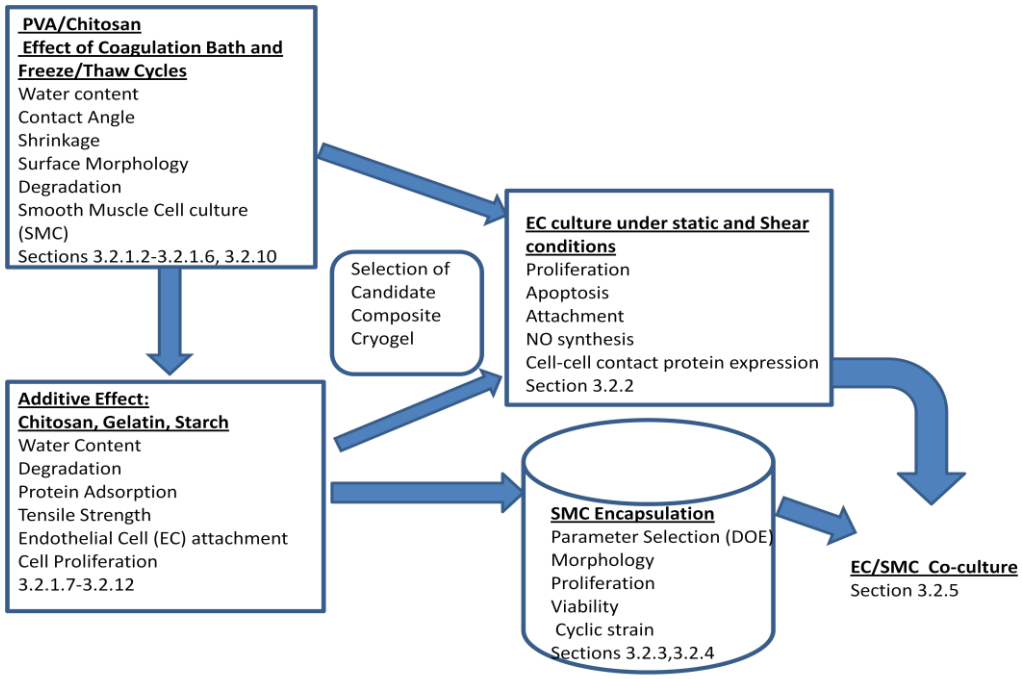

Figure 3.1 The general scheme of the experimental route. The results of first section of experiments provided the most suitable candidate for sections 2 and 3, whereas results of sections 2 and 3 provided the necessary information to develop the co-culture system.

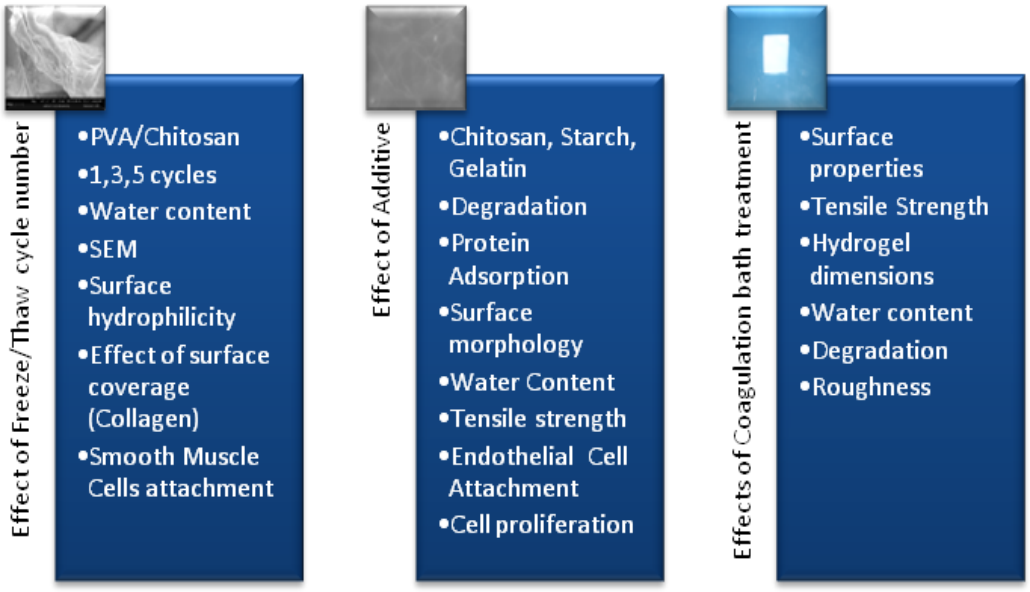

Figure 3.2 Experimental procedures for 1st step of the study. The effects of increasing freeze-thaw cycle number on cryogel properties and smooth muscle cell attachment and proliferation was studied using PVA/Chitosan cryogels, while the apparent physical effects of coagulation bath treatment was carried out simultaneously. These experiments were repeated for the case of different additives and the propensity of the surfaces to cell attachment was determined with endothelial cells

3.2.1 Determination of Additive, Freeze/Thaw Cycles and Coagulation Bath Effects

3.2.1.1 Cryogel Preparation:

10% (w: w) PVA (Molecular Weight (MW) = 78400, Vassar Brothers, USA) and water soluble chitosan, solutions were prepared in distilled water and mixed at a w: w ratio of 9:1. For even distribution of the additives within PVA solution, the mixture was stirred at 60 ºC for 2h. The mixture was then poured into custom-made

persplex templates (Appendix A) with a thickness of 500 µm and frozen at -20 ºC overnight. Hydrogels were obtained by thawing the frozen solutions at room temperature for an additional 12 hours (1st cycle).

After the 1st freeze-thaw cycle, two routes were investigated: (i) the effect of cycle number on gel properties and cell behaviour with PVA/Chitosan hydrogels and (ii) the possible improvement of gel properties by using different additives; namely starch and gelatin. PVA/Starch and PVA/Gelatin cryogels were prepared with the same procedure described above (Figure 3.3).

To determine the effect of number of freeze/thaw cycles, the procedure was repeated 2 or 4 more times (3rd cycle, 5th cycle). Hydrogels were further crosslinked by immersion into a coagulation bath composed of 1M KOH and 1 M Na_2SO_4 for 1 hour under constant agitation at room temperature. After the coagulation bath treatment the hydrogels were washed with dH_2O several times and stored at 4 ºC until use.

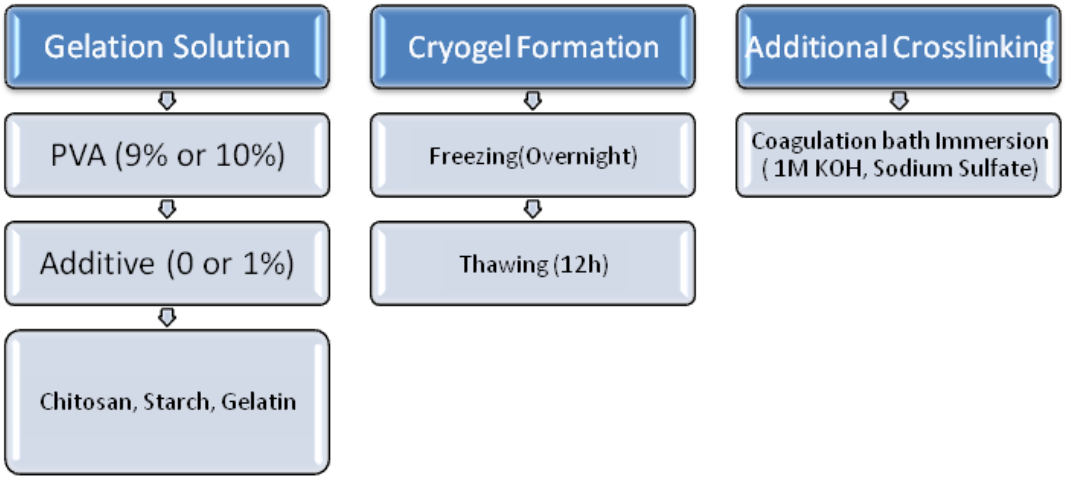

Figure 3.3 Cryogel Formation procedure. First either pure PVA or 9:1 w:w PVA/Additive solutions were prepared, then gel formation was induced by successive freeze-thawing cycles and the resultant gels were further crosslinked with the coagulation bath treatment.

Properties Affected by Coagulation Bath Treatment

The initial experiments prove the validity of the claim that coagulation bath can help retaining the additives within the cryogel structure while improving mechanical properties. However, as it was noticed that coagulation bath treatment also causing significant physical changes in cryogels, tests 3.2.1.2- 3.2.1.6 were carried out to quantify these effects. Then tests 3.2.1.7-3.2.1.9 was done to evaluate the effect of coagulation bath with different additives. These experiments provided the basis for comparison of different additives and different freeze-thaw cycle number with PVA/Chitosan specifically prior to cell culture experiments.

3.2.1.2 Determination of Water content

To assess the effect of the coagulation bath treatment on water content of the hydrogels, hydrogels were cut into 1x1 cm^2 pieces weighed after blotted dry and air-dried for 24 hours. The water content was determined by the measurement of the difference between wet and dry weight of the hydrogel samples before and after coagulation bath treatment ($n \geq 6$). Results were given as % Water content.

3.2.1.3 Surface Hydrophilicity

Surface hydrophilicity is an important determinant of protein adsorption and cell attachment. Surface hydrophilicity of the hydrogels was measured by determination of the water contact angle (the Sessile drop method) using a goniometer on hydrogel surfaces which have been blotted dry (Figure 3.3). Flat hydrogel surfaces were positioned under the syringe tip and the angle measurements made automatically after the contact of the drop with the hydrogel

surface. Measurements were done for before and after coagulation bath for 1st, 3rd and 5th cycle PVA/Chitosan hydrogel samples (n≥4). To better assess the wettability of the hydrogels, continuous measurement of contact angle was performed for 20 secs with PVA/Gelatin hydrogels (20 measurements, n≥ 3).

Figure 3.4 Contact angle measurement. Representative measurement of contact angle on the hydrogels. (PVA/Chitosan 5th cycle hydrogel)

3.2.1.4 Shrinkage due to the crosslinking

Cylindrical Hydrogels with a thickness of 1 mm and a diameter of 1.6 cm were produced and their dimensions verified by 6 measurements per sample with a digital micrometer for 1st cycle cryogels. The changes in hydrogel dimensions (diameter and thickness) were measured by a micrometer and recorded as percentage change (n≥3) after coagulation bath treatment of the specimens.

3.2.1.5 Surface Roughness

Surface roughness was determined by Mitutoyo SurfTest 402 Profilometer for 1st cycle cryogels. The profilometer was first calibrated with a surface with known roughness (1 μm), before the flat samples were placed in contact with the tip of the profilometer and the measurements were made on random areas on the cryogel surface with a precision of 0.1 micrometer over a length of 5 mm (n≥6).

3.2.1.6 Surface Morphology

Surface morphology of the hydrogels in both the dry and wet state was assessed by microscopy. For the wet state, hydrogels were observed before coagulation bath under an inverted phase-contrast microscope to assess the change in surface morphology due to freeze thaw cycle number and also the type of additive. For higher magnification observation, samples before and after the coagulation bath were freeze-dried for at least 6 hours and then sputter coated with gold for 80 seconds before being observed using a Scanning Electron Microscope under high vacuum conditions.

Effect of Freeze-Thaw Cycle number and the Additives

The main areas where the effects of freeze-thaw cycles on PVA/Chitosan cryogels were assessed were the mechanical properties, degradation, protein adsorption and smooth muscle cell attachment and proliferation as listed through 3.2.1.7-3.2.1.13. The same experiments with endothelial cells instead of smooth muscle cells were carried out in the presence of all three different additives. The physical characterisation experiments outlined between 3.2.1.1-3.2.1.7 were utilized whenever they were beneficial for explanation of the results.

3.2.1.7 In-situ Degradation

In order to assess the effect of the coagulation bath on hydrogel degradation, pre-weighed swollen PVA based hydrogels, before and after treatment, were cut to 1x1 cm squares and transferred to 24 well plates with sterile forceps. The hydrogels were sterilized with 70% ethyl alcohol for 2 hours and then alcohol was washed with sterile PBS several times before the hydrogels were suspended in 2 ml sterile PBS (10 mM, pH 7.4) and incubated under standard tissue culture conditions (37 °C and %5 CO_2). The degradation medium was changed weekly. After 2 weeks of incubation, samples were removed, washed with copious amounts of distilled water and subsequently blotted dry before they were weighed (n≥ 3). Degradation was reported as % weight loss. The effect of degradation was also observed with SEM imaging.

3.2.1.8 Uniaxial Tensile Strength Test

To assess the tensile strength and elasticity of the hydrogels, uniaxial tensile strength tests were carried out with dog bone samples that were cut from hydrogel slabs with a custom made cutter (length x width: 25 mm x 3mm). Tensile strength tests were conducted by a Zwick-Roell Z005 Universal Mechanical tester with custom made grips for tissue and polymer samples (Zwick-Roell, Germany) at a strain rate of 60%/min. Initially, the hydrogels were put through a preconditioning cycle (1 cycle) at the same loading rate up to 60% strain (n≥6). All types of hydrogels were tested both before and after coagulation bath treatment and after 1st and 3rd freeze/thaw cycles. Samples were tested up to failure.

3.2.1.9 Protein Adsorption

Protein adsorption onto the hydrogels was quantified by Bicinchoninic acid assay (BCA) (Pierce, USA). Two standard proteins 1 mg/mL bovine serum albumin (BSA), a globular protein with an average Molecular weight of 65 kDa and 1 mg/ml rat tail Collagen type I, a fibrillar protein with an average MW of 285 kDa were applied onto the hydrogels at room temperature. The protein adsorption was allowed to proceed overnight. The desorption of the proteins was performed by incubating the hydrogels in a 3M Urea/5% sodium dodecyl sulfate (SDS) solution overnight (n≥6). The amount of protein inside the desorption solution was determined by incubating 10 µL of the solution in 200 µL of BCA reagent solution at 37 ºC for 30 minutes and measuring the absorbance at 562 nm with a microplate reader (Biotek ELx800). Absorbance values were converted to concentration with respect to a standard curve of known BSA and collagen concentrations (Appendix B).

3.2.1.10 Cell Culture conditions

For determination of surface feasibility for cell attachment; bovine thoracic aortic smooth muscle cells (BASMC) and bovine thoracic arterial endothelial cells (BAEC) (Coriell Institute, NJ, USA) from the same animal were cultured under standard tissue culture conditions (37 ºC, %5 CO_2) in RPMI 1640 medium (Sigma-Aldrich, UK) supplemented with %10 Foetal Bovine serum and 1 % Penicillin/Streptomycin. All experiments were conducted with cells between passage 10-18 for smooth muscle cells and passage 8-12 for endothelial cells. Smooth muscle cells were initially used for determination of the effect of freeze-thaw cycle number on cell behaviour on PVA/Chitosan cryogels. The initial use of endothelial cells was to determine the PVA/biomacromolecule composite cryogel that supported the best cell attachment

and proliferation. Cells were either seeded separately or successively for co-culture experiments.

All hydrogels, except hydrogels used for smooth muscle cell encapsulation, were sterilized by immersion into 70% Ethyl alcohol for 2 hours and following washing steps with sterile PBS, stored in culture medium in a CO_2 incubator overnight to promote protein adsorption. Alternatively, sterilized PVA/Chitosan cryogels were coated with sterile filtered rat tail Collagen type I, which is a constituent of the native ECM environment of SMCs (Sigma-Aldrich, UK) at a concentration of 100 $\mu g/cm^2$. The adsorption was achieved by overnight incubation at 37 °C before the hydrogels were washed with PBS several times. This was only done for smooth muscle cell experiments on PVA/Chitosan cryogels for different freeze thaw cycle numbers.

The standard culture on PVA composite cryogels were carried out after incubation of cryogels in serum containing medium overnight following sterilization. The cells were trypsinised and the cell number was quantified by trypan blue before they were seeded onto the hydrogels, at a concentration $5x10^4$ cells/cm^2 in 20 µL of medium. After an initial attachment period (15-30 min), the medium was completed to 500 µL.

3.2.1.11 Fluorescence Microscopy

To assess cell attachment, proliferation and morphology, hydrogel samples were stained with DAPI (a DNA binding dye that stains cell nuclei) and FITC-labelled Phalloidin (a fungal toxin with specific affinity to f-actin fibrils; that visualizes the cell cytoskeleton). Hydrogel samples were fixed with 3.7% formaldehyde for 5 min

and then rinsed with PBS. Afterwards cells were permeabilised by treatment with 0.1% Triton X solution. After removal of Triton-X, samples were incubated in 1% PBS-BSA solution at 37 °C for 30 minutes to decrease non-specific absorption of the dyes. 1:1000 dilution of DAPI and 1:200 dilution of FITC-Phalloidin were applied to the samples before the samples were incubated in the dark for 15 minutes. After washing with PBS several times, the samples were observed under an epifluourescence microscope under single or multiple fluorescence modes (Olympus, Japan).

3.2.1.12 Alamar Blue Cell proliferation assay

Cell attachment and proliferation was quantified by Alamar Blue Cell proliferation assay (AbBiotech, USA) at 24 h and 7 d post seeding for BASMC and day 2, 5, 10, and 15 for BAEC. Alamar Blue solution (10% in serum free RPMI 1640 medium) was applied onto the hydrogels and absorbance of the dye at 562 and 595 nm for initial BASMC tests and 570 nm and 600 nm for the rest of the tests, was determined after one hour of incubation in culture. Absorbance readings were converted to dye reduction % as per instructions of the manufacturer (Appendix C). Calibration curves for cell numbers determined by trypan blue assay was drawn for both cell types. Dye reduction (%) is indicative of cellular metabolic activity, i.e. higher reduction signifies higher cell number for a given cell type, as the metabolic activity differs between cell types.

Summary of the Structure of First Experimental Part

The determination of cryogel properties was initiated with the determination of the physical effects of secondary crosslinking step (coagulation bath), followed by the

determination of effects of different freeze-thaw cycle numbers and additives in a staggered series of experiments to obtain the data on the possible effects of physical characteristics on cell behaviour outcomes

3.2.2 Endothelial Cell behaviour on PVA based hydrogels

After determination of the best candidate for the additive to PVA; a more elaborate examination of the endothelial cell adhesion and proliferation was carried out on the selected additive (PVA/Gelatin). To this end, endothelial cell proliferation and apoptosis were determined via FACS analysis and expression of several cell-cell contact molecules was determined with Quantitative Real-time RT-PCR tests. As a marker of endothelial functionality, the expression and activity of eNOS synthesis of Nitric Oxide (NO) was determined. All these tests were also conducted under shear stress conditions produced by direct or ramped turbulent flow produced by an orbital shaker.

3.2.2.1 CFDA Cell Proliferation Assay

CFDA is a non-toxic dye which can penetrate the cells and change in its distribution over time within a cell population can be used as direct evidence of mitosis. For labelling with CFDA, cells were seeded onto the hydrogels and allowed to recover overnight. Assay solution was prepared by adding 90μl DMSO to the CFDA SE solution and mixing well by pipetting gently (stock solution of 10mM). A 1:2000 dilution of the stock CFDA SE was prepared in sterile PBS, resulting in a working concentration of 5μM for the dye. The samples were removed from the incubator and washed with PBS. After removal of PBS, PBS-CFDA SE solution was added to each sample and the samples were placed into the incubator for further 30 min.

PBS-CFDA SE was removed and replaced with complete media and the samples were incubated in a CO_2 incubator. At days 2, 5, 10 and 20, the cells were harvested by trypsinisation (2x) and placed in 1.5 ml eppendorf tubes on ice. The tubes were centrifuged at 1000-1500 rpm for 5 mins @ 4^{O}C. The supernatant was removed without disturbing the cell pellet and 500µl of 1 X PBS containing 0.1% BSA was added into each eppendorf tube and the centrifugation was repeated. After removal of PBS-BSA the pellets were gently resuspended in 200µl of 1x PBS-0.1% BSA and placed into FACS tubes for analysis. Analysis of Fluorescence of CFDA per cell was done for at least a cell population of 10000 for each sample (n≥3) with the FACScalibur flow cytometer system (BD Sciences, USA). Decrease of CFDA fluorescence intensity per cell is an indicator of active cell division (Figure 3.5). For comparison purposes the average fluorescence intensity (FI) was determined and 1/FI was used for quantification of proliferative activity.

3.2.2.2Vybrant Apoptosis Assay

To determine the health of the cells on the hydrogel surface and the strength of the initial attachment, the level of apoptosis and necrosis were determined with Vybrant Apoptosis assay. This assay is based on labelling of Annexin V, which is a marker of apoptosis that binds to phophatidylserine (PS) which is normally located in the cytoplasmic side of the membrane and which translocates to the outer layer in apoptotic cells, and Propidium Iodide (PI) which is impermeant to viable and apoptotic cells and can penetrate only damaged or necrotic cells. Unlike TUNEL or caspase based assays single cell apoptotic condition can be determined, thus this assay is more precise for determination of the percentage apoptotic cells. Cells were seeded onto the hydrogels at the density required and allowed to recover overnight. The next day, cells were removed from the incubator and washed once with 0.2% BSA media (1ml per well) to remove the serum. The BSA wash was rinsed

and replaced with 0.2% BSA the media for each well before the cells were allowed to quiesce overnight. At day 2, 5 and 10 and 20, cells were washed with 1ml of PBS and then the cells were trypsinised with 2x Trypsin-EDTA solution for 5 min. After trypsinisation, the cells were placed in 1.5 ml eppendorf tubes and placed on ice. Cells were kept on ice up until addition of the reagents.

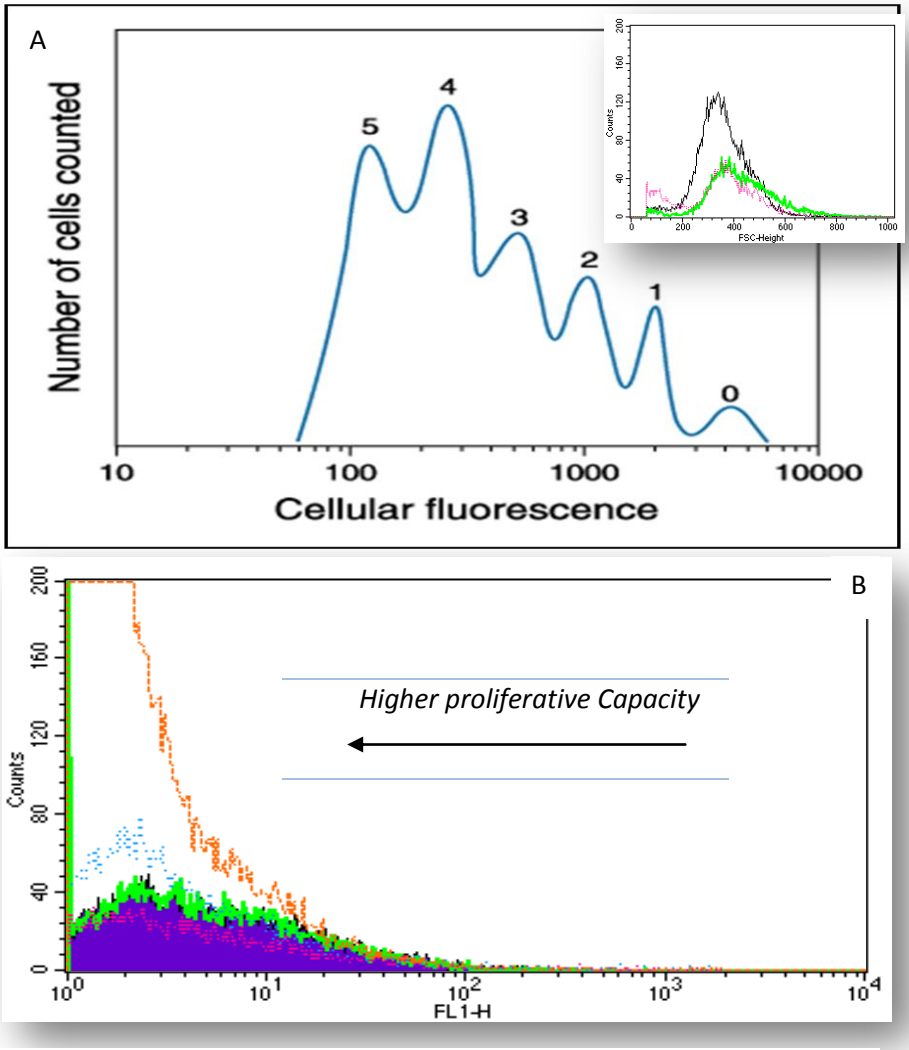

Figure 3.5 Representative CFDA read-outs from FACS analysis a) A generic evolution of the fluorescence readings over culture interval; from 0 to 5 the decrease in fluorescence intensity denotes cellular division(adapted from Vybrant CFDA proliferation assay manual) Inset) Number of cells vs. FSC (cell size), which can be used to differentiate between different cell types or to validate the uniformity of the cell shape after different treatments b) CFDA Fluorescence reading after day 15 of culture 4 read-outs from PVA/Gelatin hydrogel samples vs. one TCPS control (Red line). The proximity of the readings to y axis is an indication of proliferation.

The Apoptosis assay solutions were prepared as follows:

An appropriate volume of 5 X ABB is diluted in PBS-0.1% BSA (to 1 X) to allow addition of 200 µl to each sample. A 100µg/ml working solution of Propidium Iodide (PI) was prepared by diluting 5µl of the 1mg/ml stock in 45µl of 1 X Annexin Binding Buffer (ABB). Meanwhile, the eppendorf tubes containing the cells were placed into the centrifuge and the cells were pelleted by centrifugation at 1000-1500 rpm for 5 mins @ 4^{o}C. Cells were placed on ice and the media was removed from each tube without disturbing the cell pellet. A 500µl solution of 1 X PBS containing 0.1% BSA was added into each eppendorf tube and the centrifugation was repeated. PBS-BSA solution was removed without disturbing the pellet and the cells were gently resuspended in 100µl of 1 X ABB. Then, 1µl of Annexin V and 0.4µl of PI was added to each 100µl volume of cell suspension and the preparation was incubated at room temperature for 15 mins. Following the incubation, a further 100µl of 1 X ABB was added to the cells, the cell suspensions were placed into FACS tubes, and return the samples to ice. Annexin V and Propidium Iodide fluorescence was analysed for at least a cell population of 10000 for each sample ($n \geq 3$) with the FACScalibur flow cytometer system (BD Sciences, USA). Annexin V is an apoptosis marker and PI is a nuclei stain; the ratio of their fluorescence intensity is an indicator of the cell condition; viable, necrotic, early apoptotic or late apoptotic. Cells that shows low level of PI and Annexin V fluorescence are viable cells where as cells which only show high fluorescence in Annexin V are early apoptotic cells, high fluorescence in both channels denotes late apoptotic cells, whereas only high fluorescence in PI filter marks necrotic cells (Figure 3.6).

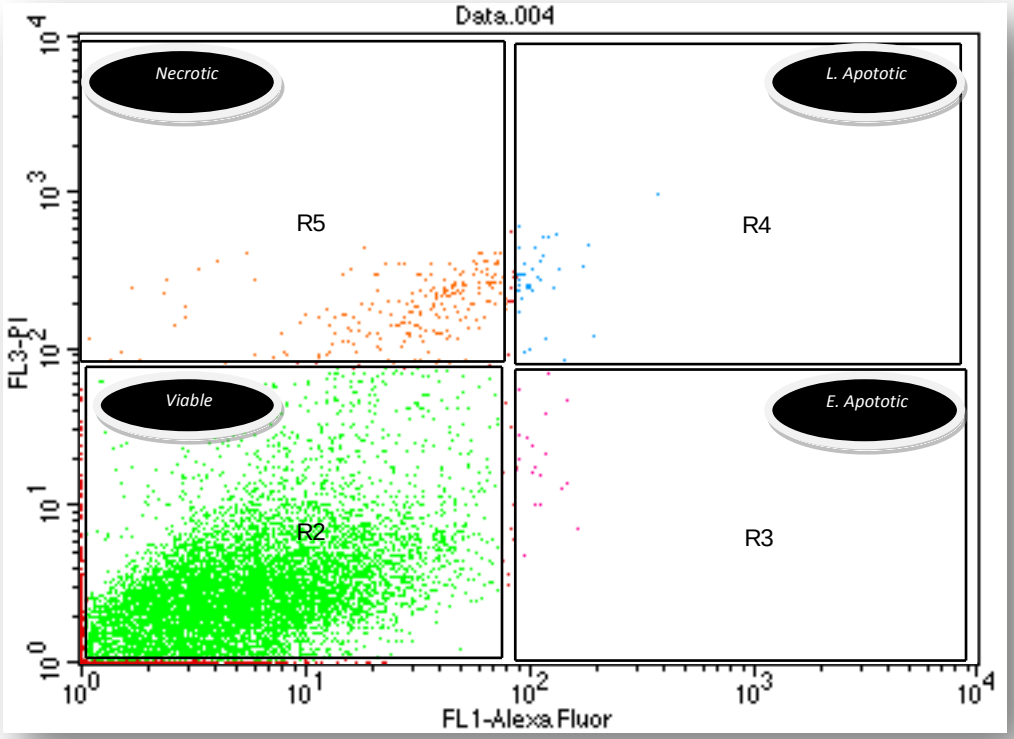

Figure 3.6 Determination of the apoptotic cells by FACs using Vybrant Apoptosis assay. Regions are divided as: R2) viable cells R3) Early apoptotic cells R4) Late apoptotic cells R5) Necrotic cells.

3.2.2.3 RNA Isolation and Quantification of gene expression (Real time-RT PCR):

Cell-cell contact is one important part of inflammatory response and also impacts on the formation of an endothelial barrier. To this end, three cell-cell contact adhesion molecules were selected for quantitative analysis of their expression by RT-PCR. Endothelial cells (BAEC) were seeded on both PVA-Gelatin hydrogels and TCPS (Tissue Culture Polystyrene) at a density of 5 x 10^4 cells/cm^2, and the total RNA was isolated by RNeasy RNA isolation kit (Qiagen, USA) after day 2, 5 and 10. The cells were trypsinised using 0.25% Trypsin-EDTA solution for 5 minutes and were then collected into RNase-free centrifuge tubes and centrifuged for 5 mins at 300g.

Afterwards, removal of supernatant cells was disrupted by RLT (Lysing buffer) buffer and the lysate was homogenized using a 20 gauge sterile needle (BD Sciences) by aspirating cells at least 5 times. An equal volume of 70% Ethanol (prepared with RNase free water (GIBCO, USA)) was added and the sample was transferred to Rneasy spin column and centrifuged at 8000g for 15 s. Flow through was discarded and the same volume of RW1 buffer was added and the column was centrifuged again at the same speed. The column was washed by RPE buffer two times by the centrifugation at the same speed for 15s and 2 minutes, respectively. The spin column was placed inside a collection tube and 40 µl of RNase free water was added and the column was centrifuged again for a minute. Isolated RNA was quantified by diluting the RNA sample 50 times in Tris-HCL (pH 7.0) buffer and measuring the absorbance at 260 nm; theoretically an absorbance of 1 corresponds to 44 µg/ml of RNA. Ratio of absorbance at 260/280 was used for the purity measurement. Measurements were initially done standard UV-Vis spectrometer with a quartz cuvette and later verified with Nanodrop spectrophotometer for RNA samples.

cDNA synthesis was performed by using iScript cDNA synthesis kit. The volume corresponding to 1 µg of RNA for each sample was added to 4 µl of 5x iScript reaction mix, 1 µl of Reverse Transcriptase and the necessary amount of water to complete the reaction mix to 20 µl. The reaction was carried out at the following conditions; 5 minutes at 25 °C, 30 minutes at 42 °C and 5 minutes at 85 °C. The amount of cDNA and its purity is again quantified by Nanodrop spectrophotometer (Figure 3.7).

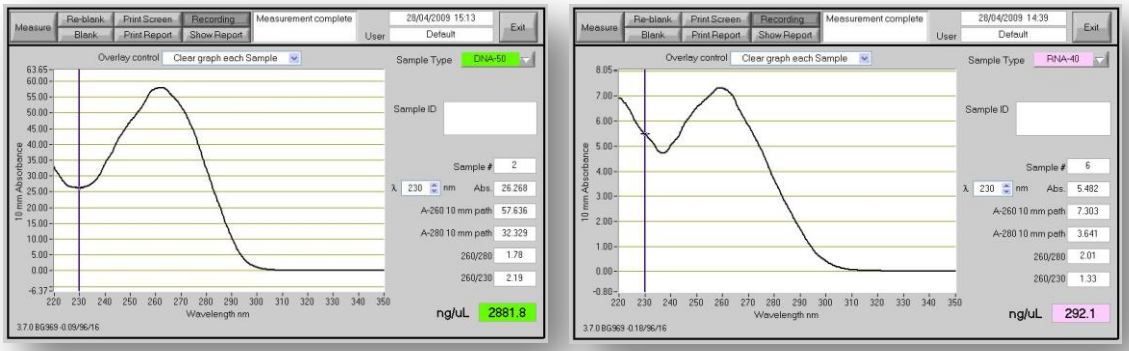

Figure 3.7 Representative mRNA and cDNA amount measurements

Finally cDNA was analysed by using SYBR Green Jumpstart Taq ReadyMix for 3 endothelial phenotype markers (ICAM, Selectin P, and PECAM) and one house keeping gene (GADPH). Primers for these genes were designed by using Primer 3 Software by using mRNA sequences obtained from Entrez Nucleotide Database (Appendix D). Reaction was carried out by using 25 µl of SYBR Green with 0.2 µM of forward and reverse primers 10 ng of cDNA and the necessary amount of water to complete reaction mix to 50 µl. Then the PCR reaction was carried out for 40 cycles with the following cycling conditions: Initial denaturation (94 °C): 2 min, then 40 cycles of denaturation (94 °C, 15 s), annealing (60 °C, 1 min), extension (72 °C, 1min). The efficiency of the primers were checked with calibration curves obtained with serial dilutions of target cDNA and then the results were normalized against housekeeping gene and negative controls (Figure 3.8). The primer sequences and details can be found in Appendix D. Final results are reported by $2^{-\Delta\Delta Ct}$ method.

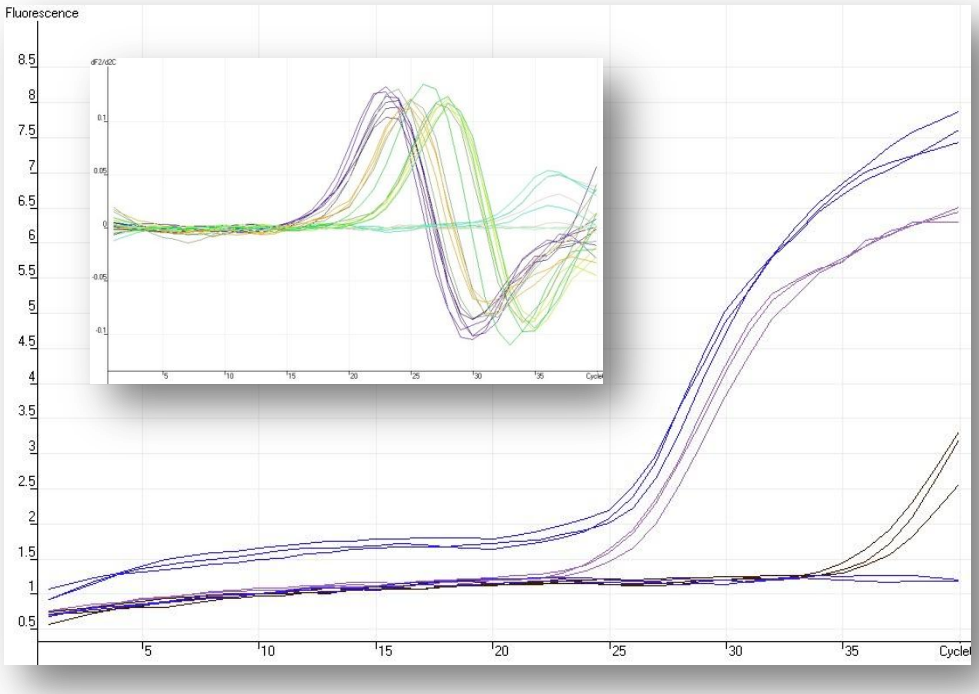

Figure 3.8 Representative readings for Real-time RT-PCR amplification curve. Dark Blue Line (GADPH, Housekeeping gene), Light blue (PECAM), Black (Selectin-P) and horizontal lines are negative control. Threshold values (C_t) were determined by an algorithm available within the analysis program R 2.2 Inset) Melting curves, to assess the specificity of the reaction

3.2.2.4 Nitric Oxide Synthesis

Nitric Oxide (NO) synthesis, which is an important indicator of endothelial cell function, was determined by the Griess method. Levels of NO secreted by endothelial cells were evaluated by measuring nitrate, nitrite metabolite levels by mixing 500 µl of culture medium obtained from the samples with the equal amount of Griess reagent and measuring the absorption of the final solution after 15 minutes of incubation at 540 nm with a microplate reader. The absorbance values were converted to micromolar through a calibration curve made from known concentrations of Sodium Nitrite ($NaNO_2^-$).

3.2.2.5 Effect of shear stress

To determine the effect of shear stress on endothelial cell behaviour was applied to the hydrogels fixed under a nylon O-ring by mounting and securing the culture plates on an orbital shaker unit. The shaker unit is placed inside a water-jacketed CO_2 incubator. The shear stress produced as a result of the orbital motion (Stuart Scientific Mini orbital shaker) can be determined by the following formula, which is a validated empirical fit (Ley et al. 1989):

$$\tau = \alpha\sqrt{\rho n(2\pi f)^3}$$

where:

α is the radius of rotation = 0.02 m, ρ is the density of full culture medium (with serum and antibiotic addition, measured in house as 987.1 kg/m^3), n is the viscosity of the full culture medium (measured in-house by a cone and plate viscometer with the cone angle of 2 degrees as 9.1 x 10^{-4} Pa.s), f is the number of rotations per second (rps). So from the equation the necessary revolutions per minute were calculated for each of the following shear values: 2.5 dyn/cm^2, 5 dyn/cm^2, 10 dyn/cm^2. Shear stress was applied either by direct application of physiological stress levels (10 dyn/cm^2) or ramping of the shear rate slowly (referred to as ramping) in 3 steps from 0 dyn/cm^2 to 10 dyn/cm^2 (6 hours at 2.5 dyn/cm^2, 6 hours at 5 dyn/cm^2 and 12 hours at 10 dyn/cm^2) (Figure 3.9). Ramping effect was selected with respect to the previous work in our lab that showed that endothelial cells could withstand immediate application of shear under these conditions up to 4 dyn/cm^2 , so for acclimatisation the first step was kept below this limit, upper limit was decided on the literature values (Inoguchi et al. 2007, Dangaria, Butler 2007).

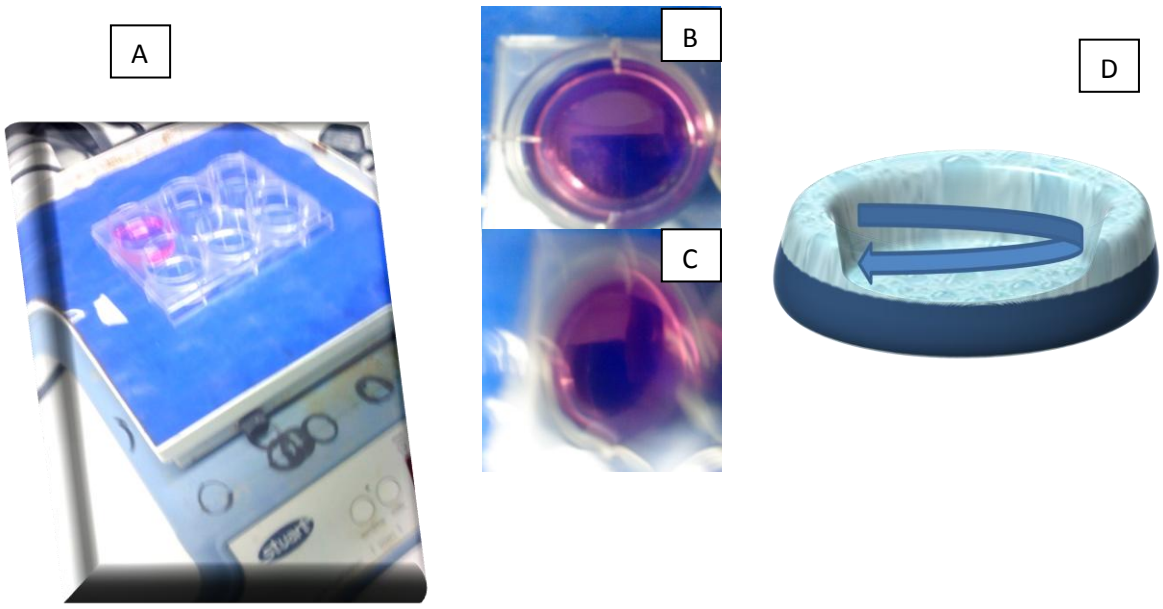

Figure 3.9 Orbital shaker setup. a) Tissue well plates are secured to the holder area of the orbital shaker. b-c) Effect of the orbital movement, b) before start of the motion c) During 100 rpm rotation. d) Movement of the culture medium within the tissue culture wells.

Cell proliferation and apoptosis were determined by Alamar Blue, CFDA and Vybrant Apoptosis assays as described above. Cell morphology was observed with DAPI/Phalloidin staining. Gene expression both under direct and ramping shear was also determined by Real Time RT-PCR as described above. The Griess method was used for determination of the NO synthesis levels. Additionally, endothelium formation was observed with SEM, and cell removal due to direct application of 10 dyn/cm^2 shear stress and its effect on surface roughness was characterized by obtaining 3D SEM images and subsequent roughness measurements using Alicona Max 3D Image production software. Briefly, 3 images of the same surface were taken by three tilting positions (0, 10°, 20°) and then these images were matched in

the program and 3D constructs were produced. Surface roughness was estimated by line and area measurements (n≥ 3).

3.2.3 Smooth Muscle Encapsulation and Storage via Cryogelation

The third part of the study was to develop an encapsulation system via cryogelation to produce a cell-laden scaffold and a method to cryopreserve the product. For this purpose, statistical experimental design methodologies were utilized to assess the interaction and effect of several parameters on gelation and cell viability in order to reach an optimum design. The design of experiments is an efficient way to determine the effect of several parameters on a systems behavior while reducing the number of experiments necessary. 2^n factorial designs are also beneficial for determination of interaction between several parameters changed between two levels. Box-Behnken design is good for observation and derivation of higher level models around a centralised experimental design.

3.2.3.1 Determination of Pre-encapsulation parameters:

pH of the solution mixtures before and after buffering and in the presence and absence of the several additives was determined to evaluate its effect on the outcome. The viscosity of the different PVA solutions and composite solutions were quantified by cone and plate type viscometer analysis (n≥3).

3.2.3.2 Smooth Muscle Cell encapsulation in Hydrogel structures

For encapsulation of the smooth muscle cells in a 500 μm thickness hydrogel, several factorial experimental design schemes were developed using DesignExpert 8.0 software (StatEase, USA) to determine the optimum encapsulation conditions concerning multiple variables. Variables dealt with were:

1) Solution viscosity
2) Presence of cryoprotectants and their type (DMSO, Optifreeze cryopreservant media),
3) Concentration of the nutrients within the encapsulation system,
4) Application of further crosslinking with a coagulation bath.

Two levels of each factor was taken (for example, presence of DMSO and Serum, 10%, absence 0%) for the experiments and all the possible combination for these two levels (Hence 2^4 factorial design) were checked in 3 separate randomized blocks of experiment for determination of the most significant factors effecting the cell viability and their interactions. Cryoprotectant and coagulation bath sterility was ensured by sterilization, sterile filtration. Response curves were drawn in light of cell survival following encapsulation procedures. The initial screening was done by a general factorial experimental design. After evaluation of the results of these tests, further determination of the effect of DMSO and serum concentration was performed by a separate Box-Behnken experimental design (with cryoprotectant type as the third factor) with three replicate blocks. Box-Behnken designs are suitable for deriving quadratic interactions between the factors observed, by doing 3 level combinations of the factors around a central design point. Repetition of the central design point solidifies the reliability of the trends (Goodness of fit) set by the experiments.

After initial viability results, with the optimal cryogel formulation, cell proliferation was assessed by Alamar Blue assay up to 14 days. Cell distribution was assessed by DAPI staining and phase contrast microscopy. Hydrogel formation was observed and the stability test was carried out in sterile PBS for 72 hour periods.

3.2.3.3 Histology

To observe the distribution of the cells within the hydrogels, the hydrogel samples were fixed with 3.7% Formalin overnight on days 1 and 7 of the culture period. The samples were washed and dehydrated via a series of ethanol treatments (%70 up to pure ethanol) and finally after treatment with pure xylene before the samples were embedded into paraffin wax and cut with a microtome to obtain 4 µm sections. Sections were fixed onto frosted glass slides and stained with standard Haemotxylin& Eosin stain. Sections were observed with a standard light microscope and the images taken by a digital camera fitted to the eyepiece.

3.2.3.4 Effect of Thawing Rate, Storage Time and Cell Type

Since one of the aims of the system is to store viable cells and the thawing rate is an important determinant of the final gel mechanical properties, the storage and thawing rate effects were examined for the encapsulation. Storage was done for the periods of 1, 2 and 4 weeks under -70 °C. Immediately after thawing, cell viability was checked with Alamar Blue assay and after 3 days of culture cell presence and viability was tested by Live/Dead Cell viability assay (described in 3.2.3.5). The thawing rate was in between 0-30 minutes prior to addition of pre-heated culture medium. To show that the encapsulation procedure can be applied for different cell types, rat arterial smooth muscle cells and bovine thoracic endothelial cells were also encapsulated. To prove the protective properties of the hydrogel encapsulation, confluent monolayers of bovine smooth muscle cells were frozen and thawed with conventional slow-cooling cell storage method and observed 2 h after thawing to assess the level of viability. Hydrogel structure and the cell distribution were further evaluated by SEM.

3.2.3.5 Long Term Cell Viability (Live/Dead Viability assay)

In order to prove that the cells are sustained throughout the hydrogel and they were viable, live and dead viability assays were conducted on samples which were freshly removed from culture. The working principle of the kit is that the fluorescence of Calcein, which is initially present in the kit solution as Calcein-AM, is cell permeant but non-fluorescent. The conversion of Calcein-AM to calcein is only possible via intracellular esterase activity and as a result viable cells which catalyze this reaction would fluoresce green. The second component, Ethidium homodimer (EtHD1) is non-cell membrane permeant and it has ability to bind DNA or RNA; thus its fluorescence is limited to damaged cells. After 1, 3, 5 and 7 days of culture, hydrogels were removed aseptically washed with PBS and then Ethidium Homodimer (4 μM) and Calcein AM (2 μM) mixture in sterile PBS was applied and the samples were incubated for 30 minutes at 37 °C. After incubation the gels were washed with PBS several times to remove the excess dyes and observed under an epifluorescent microscope with two different emission filters. Images are collated using the Cell-F imaging program (Olympus, Japan). The green areas denote viable cell cytoplasm whereas red areas denoted damaged cells with exposed DNA; which is bound by Ethidium Homodimer. As negative control, empty hydrogels were stained to; to show the specificity of the staining.

3.2.3.6 Uniaxial Tensile Strength of Cell Encapsulated Cryogels

Further mechanical tests were carried out to assess the effect of different gelation procedure via encapsulation on cryogel mechanical properties. For this end, the optimized gel mixture was poured into Flexercell plates and gel formation was achieved by the gelation mixture in the presence or in the absence of the cells.

After gelation, empty gels were also exposed to cyclic biaxial strain using a Flexercell^{TM} Strain unit system, to assess the effect of fatigue or strain hardening of the gel mixture at a strain of 10% at 1 Hz frequency after 24 h. Dog bone shape cutter with a different dimension (length x width: 15 mm x 3 mm) was used, due to the size limitation of the Flexercell plates. Samples were cut for uniaxial tensile strength tests as described in section 3.2.1.10 ($n \geq 6$).

3.2.4 Application of Cyclic Strain to Encapsulated cells

Flexercell^{TM} Strain unit is capable of applying physiological level of cyclic stresses and strains by application of controlled vacuum beneath sterile tissue culture plates specifically designed to fit the moulds where the vacuum is applied (Figure 3.10). To apply cyclic strain to the encapsulated cells within PVA/Gelatin hydrogels; gels were formed within pronectin coated Flexercell culture plates and after thawing and addition of culture medium; plates were mounted onto the Flexercell system and sealed. A thin layer of initial PVA/Gelatin solution secured the attachment of the cryogels ontot he elastomer surface. Then, cyclic strain at the level of 10% was applied via the FX4000 controller system for a period of 24 hours which corresponds to 86400 cycles. Afterwards, cell number was determined by Alamar blue assay and cell distribution was assessed with Live-Dead cell viability assay and morphology of the cells was assessed with SEM.

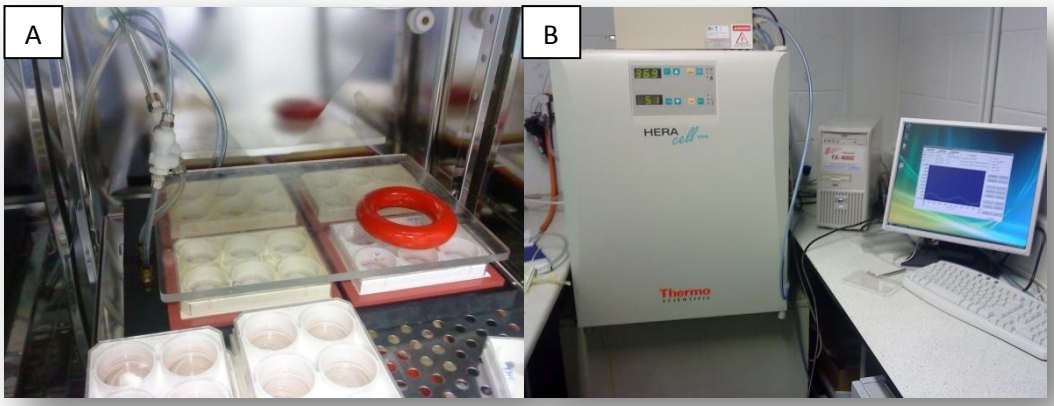

Figure 3.10 Flexercell system a) Mold base for the fitting of Flexercell culture plates. Application of controlled vacuum causes deformation of the elastomeric plate, deformation of the cryogels was achieved by the vacuum-driven platen movement beneath the plate b) Overview of the system which is composed of a vacuum pump, a controller system, computer interface and a CO_2 incubator.

3.2.5. Endothelial/Smooth Muscle cell co-culture experiments

Since the final aim is to produce a hydrogel structure where SMCs are encapsulated within and the endothelial cells cover the lumen surface, cell-laden hydrogels were seeded with endothelial cells to show that they support attachment and functionality of endothelial cells (Figure 3.11). To this end, by using the optimized smooth muscle cell encapsulation protocol, a co-culture system was developed. For full gel formation, at least endothelial cell seeding was done on to the cryogel surfaces after removal of medium and endothelial cells were allowed to attach to the surface. After the initial attachment period, the co-culture system was cultured under standard culture conditions. Since serum supplemented RPMI-1640 media was feasible for both cell types, medium composition did not need to be changed. After initial feasibility experiments, an experimental design was carried out to see the effect of three parameters.

3.2.5.1 Experimental Design for Co-culturing and NO synthesis

To determine the best conditions for commencement of endothelialisation, a general factorial design (two 3 level and one 2 level factor based, factorial design) was employed, for the parameters of (i) storage time (1 day,4 days, 1 week),(ii) SMC culture period (1day, 5 days, 10 days) and (iii) shear stress application(0 and 10 dyn/cm^2) (Table 3.1). The response selected was the NO synthesis level following two days of culture. All experiments were conducted in three blocks, in which there are at least 3 replicates (54 runs). Also levels of NO were checked after 5 days.

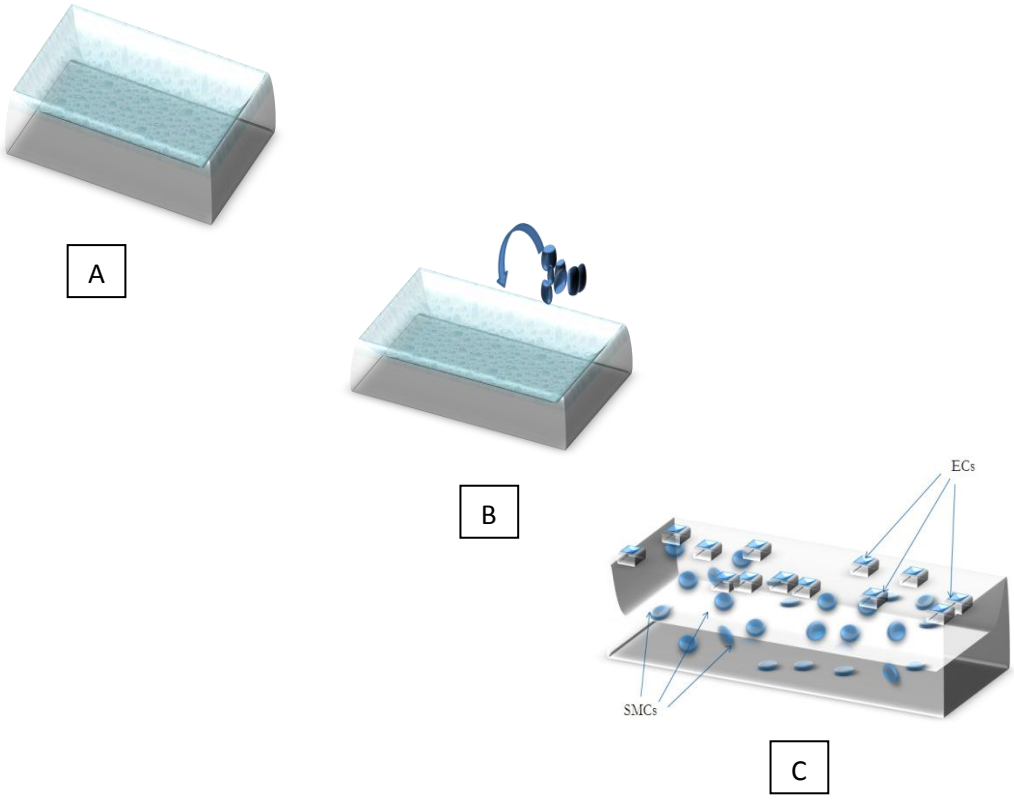

Figure 3.11 Schematic representation of co-culture experiments a) The optimum gelation solution is prepared with respect to the results obtained from encapsulation experiments b) Smooth muscle Cells are added to the mixture and gels were obtained after a Freeze-thaw cycle c) After gel formation, endothelial cells were added on to the top of the hydrogels for endothelialisation.

Table 3.1 List of the experimental combinations for co-culture experiments. The experiments conducted for determination of effect of cryogel storage time, SMC culture period and shear stress application on NO synthesis by BAEC in co-culture system.

Standard Run Number	Randomized Run Number	Storage Time	Culture Time	Shear
44	1	7 days	5 days	10
47	2	1 day	10 days	10
29	3	1 day	1 day	10
26	4	7 days	10 days	0
2	5	1 day	1 day	0
1	6	1 day	1 day	0
24	7	3 days	10 days	0
54	8	7 days	10 days	10
28	9	1 day	1 day	10
18	10	7 days	5 days	0
25	11	7 days	10 days	0
53	12	7 days	10 days	10
19	13	1 day	10 days	0
30	14	1 day	1 day	10
46	15	1 day	10 days	10
52	16	7 days	10 days	10
20	17	1 day	10 days	0
31	18	3 days	1 day	10
17	19	7 days	5 days	0
11	20	1 day	5 days	0
51	21	3 days	10 days	10
37	22	1 day	5 days	10
33	23	3 days	1 day	10
5	24	3 days	1 day	0
13	25	3 days	5 days	0
42	26	3 days	5 days	10
43	27	7 days	5 days	10
35	28	7 days	1 day	10
40	29	3 days	5 days	10
48	30	1 day	10 days	10
12	31	1 day	5 days	0
7	32	7 days	1 day	0
34	33	7 days	1 day	10
50	34	3 days	10 days	10
15	35	3 days	5 days	0
32	36	3 days	1 day	10
9	37	7 days	1 day	0
14	38	3 days	5 days	0
38	39	1 day	5 days	10
10	40	1 day	5 days	0
49	41	3 days	10 days	10
21	42	1 day	10 days	0
4	43	3 days	1 day	0
8	44	7 days	1 day	0

3	45	1 day	1 day	0
16	46	7 days	5 days	0
27	47	7 days	10 days	0
36	48	7 days	1 day	10
22	49	3 days	10 days	0
41	50	3 days	5 days	10
23	51	3 days	10 days	0
6	52	3 days	1 day	0
39	53	1 day	5 days	10
45	54	7 days	5 days	10

3.2.5.2 Indirect Immunofluorescence

In order to differentiate between endothelial cells and smooth muscle cells, indirect immunofluorescence was employed by using two distinct markers of endothelial and smooth muscle cells. eNOS (endothelial Nitric Oxide Synthase) is a protein responsible for synthesis of NO and it is an endothelial specific variant of NOS family. Smooth Muscle α- actin on the other hand is a smooth muscle actin specific member of actin cytoskeletal protein family, which is more prominently expressed in smooth muscle cell in contractile phenotype. As a negative control, a secondary dye was applied in the absence of the anti- α-Smooth Muscle actin antibody.

3.2.5.3 α-Smooth Muscle actin Staining

After two days of co-culture hydrogel samples were fixed with 3.7% formaldehyde for 30 minutes and then rinsed with PBS. The hydrogels were overturned and cells were permeabilised by treatment with 0.1% Triton X solution for 5 minutes. Following removal of Triton-X; samples were incubated in 1% PBS-BSA solution at 37 °C for 1 hour. Afterwards 1:300 dilution anti α-smooth muscle actin antibody produced in mice was applied to the samples and antibody was allowed to bind overnight. The next day, the cells were washed with PBS, the secondary antibody,

1:200 dilution of AlexiFluor 546 Anti-mouse IgG antibody was applied and the samples were stored in dark for 2 hours. After two hours, 1:1000 dilution of DAPI was added as counter nuclei stain and following an additional 15 minute incubation in the dark. Samples were observed under an epifluourescence microscope under single or multiple fluorescence modes (Olympus, Japan). As a negative control, a secondary die was applied in the absence of the anti- α-Smooth Muscle actin antibody.

3.2.5.4 eNOS staining

After two days of co-culture hydrogel samples were fixed with 3.7% formaldehyde for 30 minutes and then rinsed with PBS. Then the hydrogels are overturned and cells were permeabilised by treatment with 0.1% Triton X solution for 5 minutes. Following removal of Triton-X; samples were incubated in 1% PBS-BSA solution at 37 °C for 1 hour. Afterwards 1:300 dilution of anti eNOS type III antibody produced in mice was applied to the samples and antibody was allowed to bind overnight. The next day, after washing with copious amount of PBS, the secondary antibody, 1:200 dilution of AlexiFluor 546 Anti-mouse IgG antibody was applied and the samples were stored in dark for 2 hours. After two hours, 1:1000 dilution of DAPI was added as counter nuclei stain and following an additional 15 minute incubation in dark; samples were observed under an epifluourescence microscope under single or multiple fluorescence modes (Olympus, Japan). As a negative control, a secondary die was applied in the absence of the anti-eNOS antibody.

3.2.6 Statistical Analysis

Statistical analysis of experimental designs (ANOVA, model fitting, lack of fit analysis, Box-Cox plot and Normal Plot of Residuals) was done and analyzed in Stat-Ease Design Expert 8.0 software. For a two way comparison, the Student's t-test with significance level of $p \leq 0.05$ was used and, for multiple sample sets, one-way, two-way or three-way ANOVA analysis was carried out with Tukey's test or Holm-Sidak method to determine the variance between samples. Again, the level of significance was set as $p \leq 0.05$. These tests were done in Statistics package extension provided in MS Excel 2007.

CHAPTER 4

4 Results

The results chapter is divided into 3 sections. Prior to cell culture experiments, physical characterisation was done to elucidate the mechanism and the effects of the 2-step cryogelation protocol on the final cryogel structure. Thus, section 4.1.1-4.1.3 presents results related to the physical characteristics of the cryogels and how these characteristics are affected by freeze-thaw cycle number, coagulation bath treatment and changing the additive used to obtain the composite structure, together with their influence on cell culture studies. The second section, deals with the evaluation of endothelial cell behaviour and it is further divided to sections 4.2.1 and 4.2.2 which are related to characterization of endothelialisation on the hydrogels under static and shear stress conditions, respectively. The third section is about the development of the novel encapsulation technique via cryogelation and subsequent co-culture experiments. Sections 4.3.1 and 4.3.2 deals with the development and optimization of the encapsulation technique and the application of cyclic loading and finally, section 4.3.3 describes the results of the co-culture experiments.

4.1 Physical characterisation of cryogelation

Due to the fact that, the presence of additives interferes with the cryogelation process, coagulation bath treatment was used as a method to ensure the retaining of the additives and also as a reinforcement system. Because of the availability of

previous body of work on PVA/Chitosan cryogels produced with this method, initial programme of experiments used this cryogel formulation, to investigate some effects of coagulation bath treatment and freeze/thaw cycles which have not been determined before. In the next set of experiments involving cryogels with other additives some of the experiments were repeated, to understand any changes due to the presence of different additives. Statistical significance is stated for discussion purposes and for the cases where it was not stated the detailed analysis is given in Appendix F.

4.1.1 Effect of Coagulation bath treatment (PVA/Chitosan hydrogels)

As a physical crosslinking method, freeze-thawing results in stronger gels when applied in multiple cycles. When PVA solutions are exposed to several cycles, it tends to form more crystals which define the properties of the hydrogel. The presence of additives might change this process. Also the additives are not as liable to crosslink under freezing and thawing as PVA and thus an additional crosslinking step is needed. In this study coagulation of the polymers in a supersaturated solution was used as a secondary physical crosslinking procedure. During the experiments it was noticed that the physical changes due to the coagulation bath were drastic and since there was no available data in the literature to assess these changes, the characterisation of cryogels before and after coagulation bath was carried out with PVA/Chitosan cryogels initially. The first aim was to improve the cryogels mechanical properties and also their degradation resistance while trying to keep other desirable properties within the acceptable limits. One such property is the water content of the hydrogels, which should be above 80% for better imitation

of the mechanical properties of the vessels. Initially, several coagulation bath solutions were tried, but due to the excessive decrease in water content in high coagulation bath concentrations (down to 56.16 ± 4.01), a 1M KOH and 1M Na_2SO_4 bath composition was used (concentrations between 0.5 and 2 M were tried). The most persistent change induced by coagulation bath treatment was the decrease of water content of the hydrogels ($p=0.0001$), which was observed for all cycle numbers and also for different additives and PVA concentrations (Figure 4.1a). The 6/0.9 ratio was the previous ratio utilized in literature and the water content for it was measured to determine the effect of lower PVA and chitosan concentrations. The most dramatic change was observed on 3rd cycle PVA/Chitosan hydrogels (from 86.34± 1.00 % to 68.04± 1.36%). Lower concentration PVA/Chitosan gels (6/0.9 w: w ratio, as used previously) had higher water contents (90.72 ± 1.06% and 85.38 ± 3.41%), before and after coagulation bath respectively, $p\leq0.001$); However they were considerably harder to handle. For this reason, they were not assessed any further.

The coagulation bath had a distinct effect on the rehydration capacity of the hydrogels as measured by the change in weight upon rehydration. Rehydration and reswelling which decreased for 1st cycle PVA/Chitosan gels from 2.16± 0.17 to 1.37± 0.20 times. Rehydration is a property related to the robustness of the gel structure and even though there was a considerable decrease. Two-step crosslinked hydrogels were still able to rebound up to their original volume. It also affected the morphology of the hydrogels both microscopically and macroscopically. The initial hydrogels were hard to handle, fragile and transparent; whereas upon application

of the coagulation bath, highly resilient and opaque gels were obtained fulfilling the set aims (Figure 4.2).

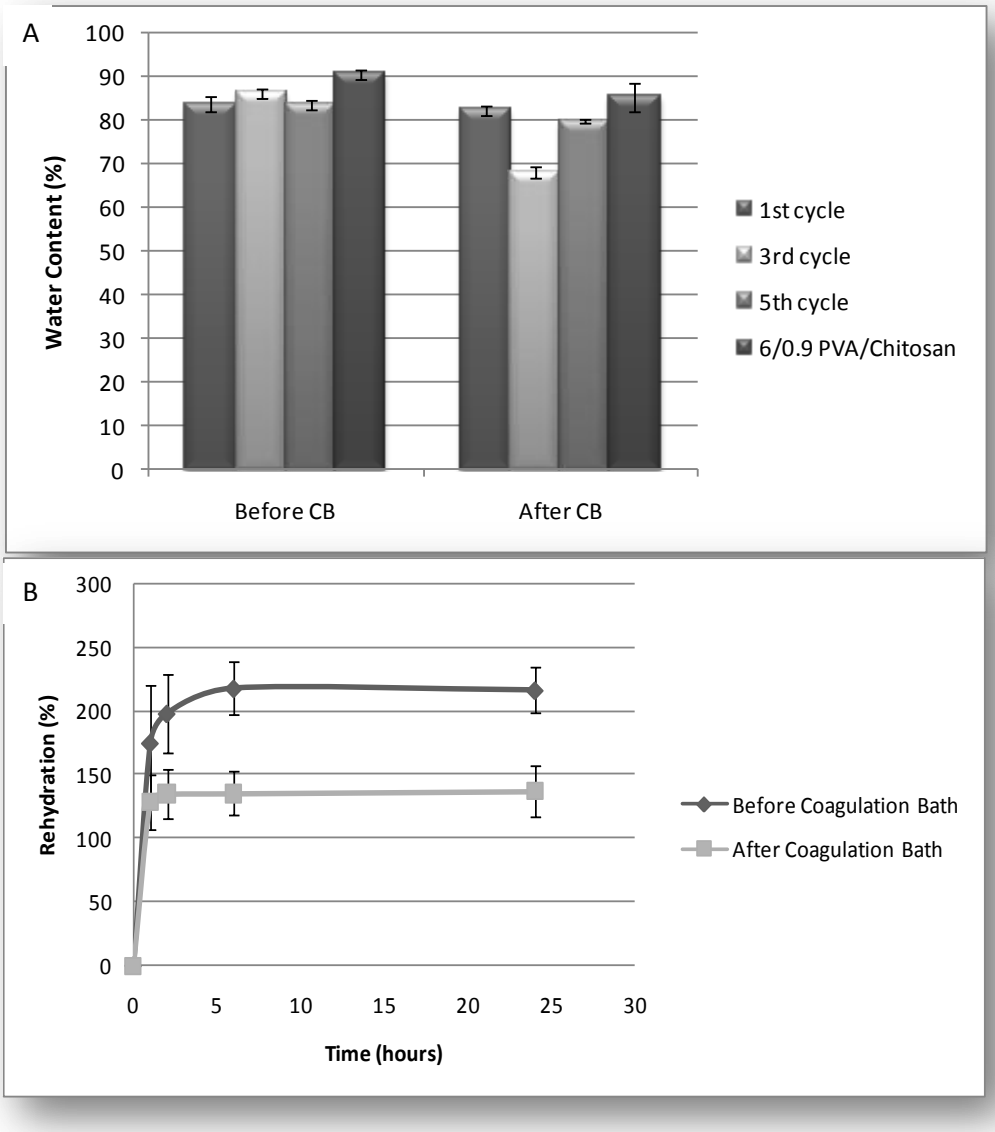

Figure 4.1 Water content and reswelling behaviour of PVA/Chitosan hydrogels a) effect of coagulation bath on the water content after different freeze-thaw cycles and a different composition (6/0.9 ratio instead of 9/1, after 1st cycle), in all cases there was a significant decrease after coagulation bath treatment ($p \leq 0.001$), whereas the difference between the samples were not significant before coagulation bath treatment b) Rehydration behaviour of PVA /Chitosan first cycle hydrogels before and after coagulation bath (n=6); before coagulation bath hydrogels have nearly 2 fold more swelling capacity ($p \leq 0.001$).

Initially, long fibres of meshed together polymer chains were visible under SEM observation, but upon coagulation bath treatment the surface porosity of the gels decreases and surface became more uniform (Figure 4.2 b) Surface changes inflicted by the coagulation bath treatment also affected the surface hydrophilicity of the hydrogels, where the surface was considerably more hydrophilic following application of coagulation bath.

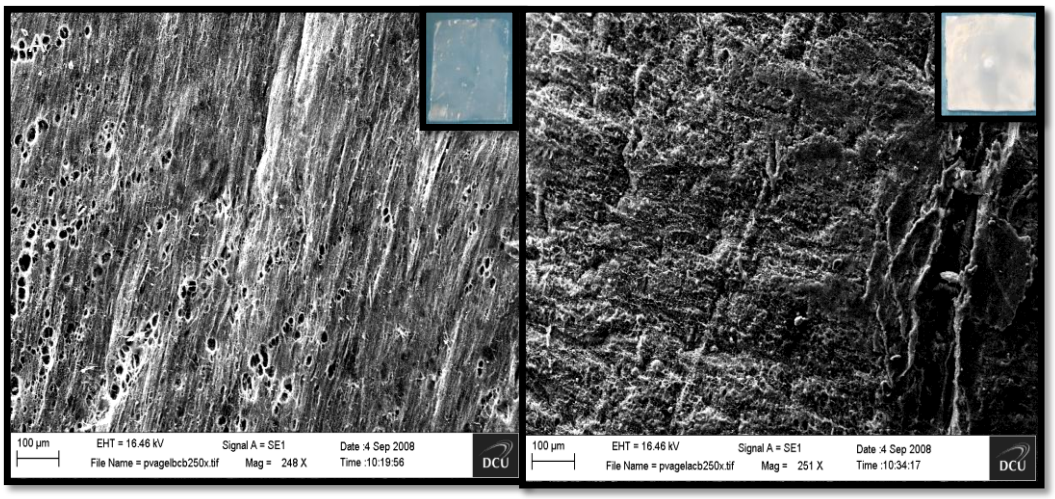

Figure 4.2 Surface morphology of the cryogels a) Before b) After coagulation bath treatment. There is a distinct decrease in surface porosity and the fibre orientation was lost. Insets) Macroscopic views of the hydrogel surfaces; coagulation bath converts transparent weak hydrogels to compacted, opaque structures. Representative of 3 independent observations.

Surface hydrophilicity of the hydrogels was dependent on the number of freeze-thaw cycles and also the type of the additive used. However, after application of the coagulation bath, the contact angle measurements converged which might indicate formation of a thin PVA enriched layer on the surface since PVA is the more hydrophilic component (Figure 4.3, 4.4). This was the main disadvantage of the

secondary crosslinking, since it is known that highly hydrophilic surfaces are unfavourable for cell attachment.

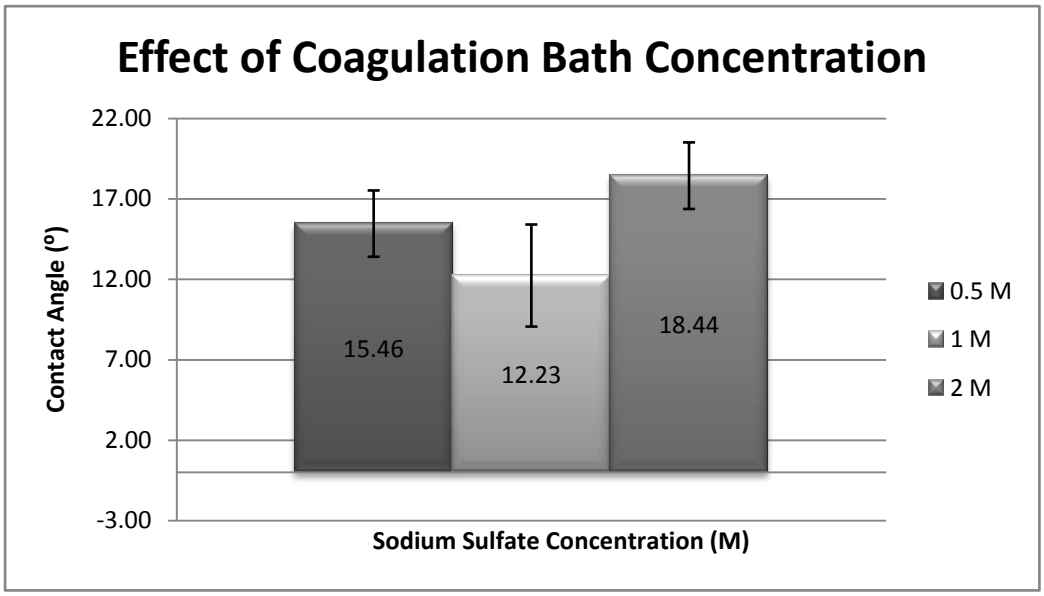

Figure 4.3 Change in water contact angle of PVA/Chitosan hydrogels (n=6) For different concentrations of coagulation bath (0.5 M, 1 M, 2 M) after 1 freeze-thaw cycle, the difference between the different coagulation bath concentrations was not significant (p= 0.23).

The change in the crystalline structure of the gels after successive freeze-thawing cycles, via the presence of more crystallites is reported in the literature several times, and would also determine the way they respond to coagulation bath treatment. This also had an effect on their degradation. The application of the coagulation bath effectively decreased the weight loss, probably due to dissolution and removal of the sol (solution) part of the hydrogels. To see whether these effects are general, in the next step different freeze-thaw cycle numbers were used to determine changes in physical properties.

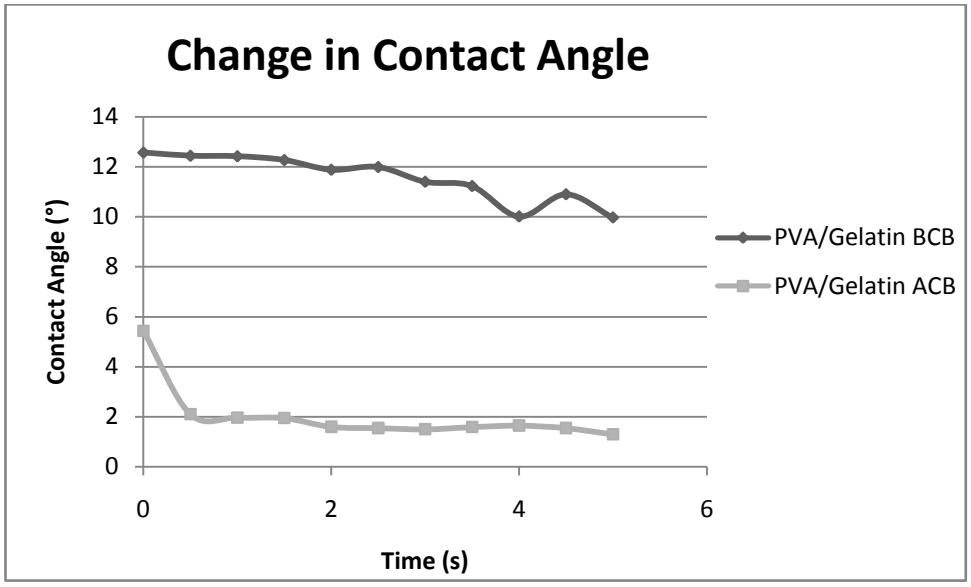

Figure 4.4 Wettability of PVA/Gelatin hydrogels a) Before b) After Coagulation bath treatment The average contact angle for PVA/Gelatin hydrogels were 12.57± 1.09 before coagulation bath and 5.44 ± 1.05 after coagulation bath (n=3)

4.1.2 Effect of Freeze-Thaw Cycles (PVA/Chitosan)

One important advantage of cryogelation technique is its versatility in producing scaffolds with different physical properties with small modifications, such as increasing freeze-thaw cycle number. As the cycle number increases, cryogels become stiffer and since it is shown that stiffer matrices are better for cell attachment, in this part of the study the physical changes induced by freeze-thaw cycles and the subsequent effect on cell attachment was evaluated.

The decrease in surface hydrophilicity after coagulation bath treatment was persistent for all cryogels produced via different number of freeze-thaw cycles (Figure 4.5). As it is desirable to have more cell attachment, promoting protein adsorption from serum is important. Increasing the number of freeze thaw cycles had a positive effect on the protein adsorption capabilities of the gels for 3rd vs. 1st cycle hydrogels, It also caused increased stiffness and strength and was also significantly effective in increasing cell attachment and proliferation, albeit not excessively. After 1 freeze thaw cycle, aligned fibres of PVA clumps in the surface morphology were observed. This is lost after the 3rd cycle. Instead huge blobs of polymer patches were visible. The decrease in surface porosity was more visible for 1st cycle hydrogels and in cross-sections the inner porosity of the hydrogels was similar (Figure 4.6).

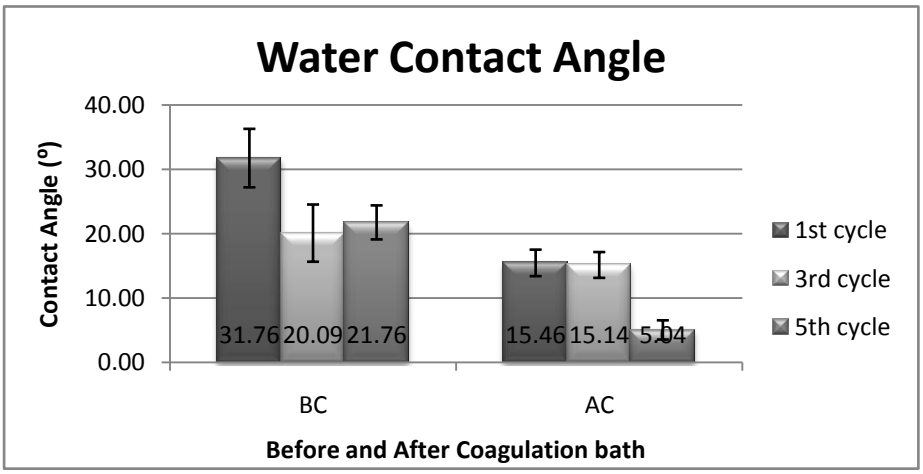

Figure 4.5. Change in hydrophilicity with respect to Freeze-thaw cycle and coagulation bath treatment for PVA/Chitosan cryogels (n=6). The water contact angle was significantly lower upon coagulation bath treatment ($p \leq 0.001$).

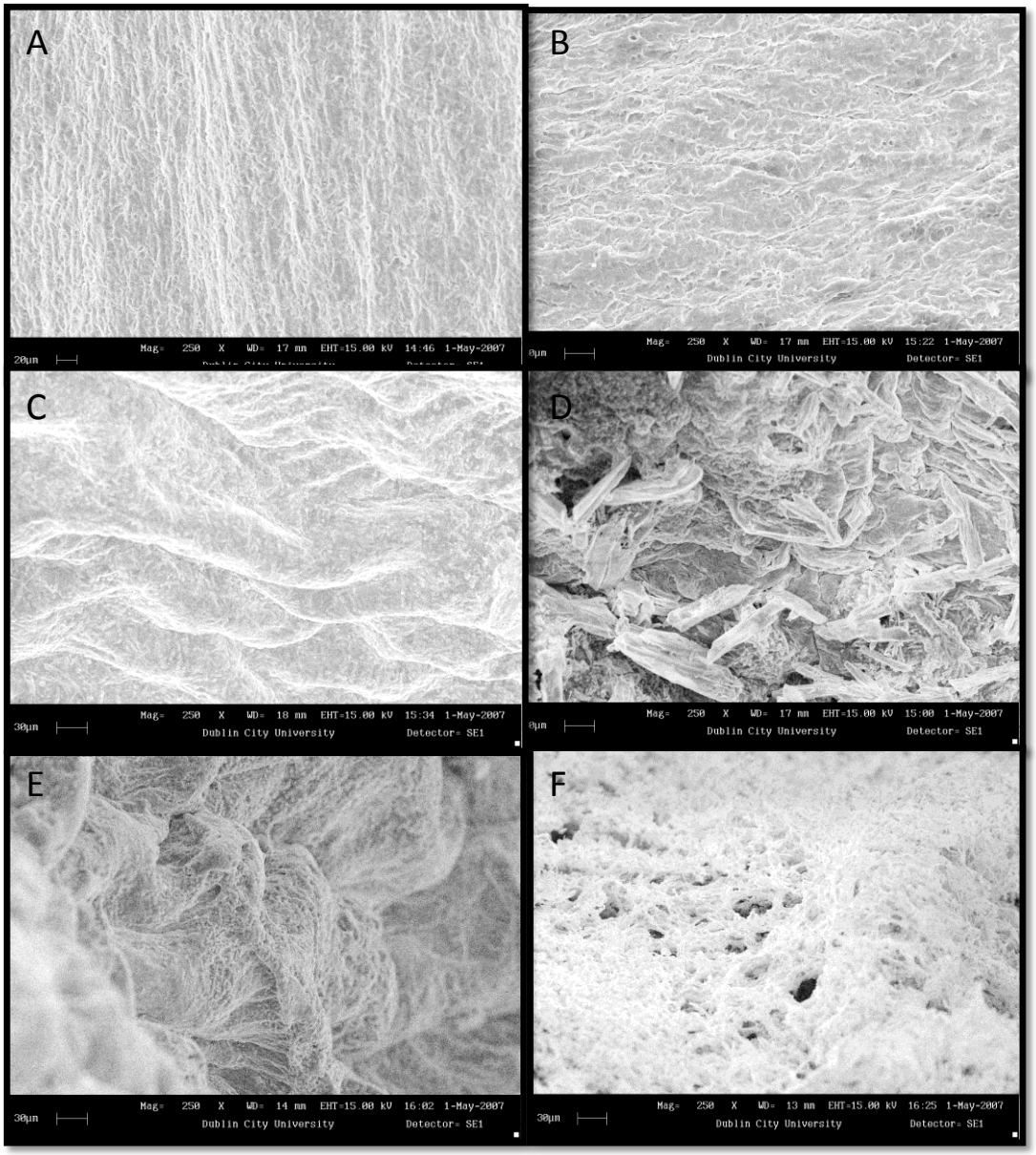

Figure 4.6 Effect of Freeze-thaw cycle number and coagulation bath on surface morphology of the PVA/Chitosan hydrogels a) 1st cycle BCB, orientation of the PVA filaments are apparent b) 3rd cycle BCB, filament orientation as lost and islands of polymer meshes has formed c, d) 1st and 3rd cycle ACB respectively, a significant loss of PVA alignment occurred, surface undulations have appeared due to the shrinkage effect of the coagulation bath; the surfaces were noticeably rougher. e, f) Cross-sectional views after coagulation bath for 1st and 3rd cycle respectively, the appearance was similar to the surface, only more porous. Representative of 2 independent observations.

The 3rd cycle hydrogels were more susceptible to degradation (weight loss) both before and after coagulation bath treatment for a 3 week period, where coagulation bath treatment caused a significant decrease in the degradation for each time point (Figure 4.7a).

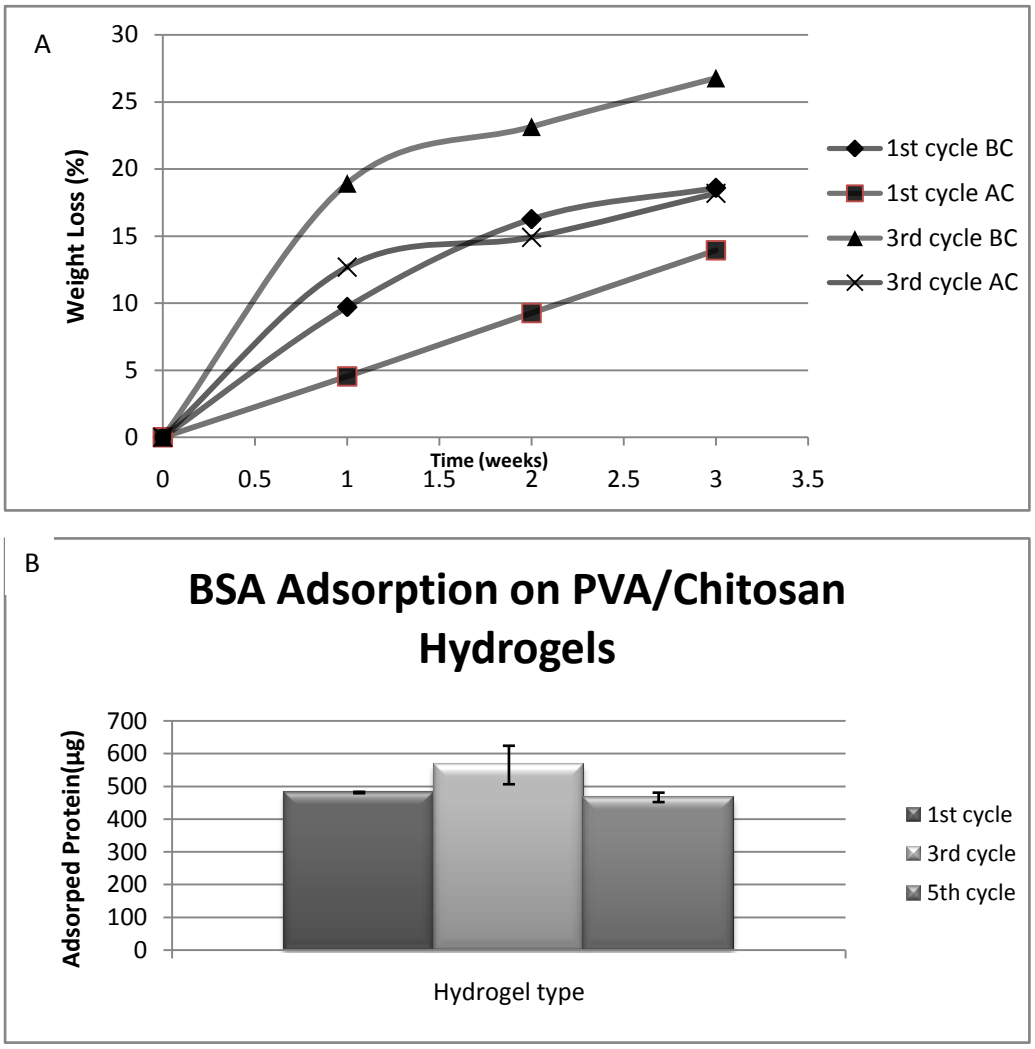

Figure 4.7 a) Degradation profile of 1st and 3rd cycle PVA/Chitosan hydrogels before and after coagulation bath (n=6). The lowest degradation rate was achieved by 1st cycle ACB (13.92±5.79 %) whereas the highest rate was with 3rd cycle BCB samples (26.77± 0.46 %) b) Adsorption of Bovine Serum Albumin (BSA) onto the PVA/Chitosan hydrogels after different numbers of freeze-thaw cycles, the adsorption level on 3rd cycle hydrogels was significantly higher (p= 0.006).

The 3rd cycle hydrogels had a lower level of degradation compared to the first cycle before and after coagulation bath, believed to be mainly due to decreased sol composition. Degradation was both freeze-thaw cycle number and additive dependent. Together with resistance to degradation the most important aspect of coagulation treatment was the significantly improved mechanical properties. As for protein adsorption, quantified by using Bovine Serum Albumin for each cycle number, the highest adsorption was observed on 3rd cycle hydrogels where 1st and 5th cycles had similar adsorption values (Figure 4.7 b).

The 3rd cycle hydrogels were also effective in promoting cell attachment and improving proliferation both for arterial smooth muscle cells and endothelial cells (Figure 4.8). Coating of the surface of the hydrogels with Collagen type I caused a slight increase in attachment and proliferation. To see whether immobilization of proteins covalently would be more effective than adsorption a crosslinker (glutaraldehyde) was used for collagen coating. However, the improvement in attachment and proliferation was slighter compared to the Collagen type I adsorped samples. Endothelial cell attachment was much more pronounced in all cases compared to that of smooth muscle cells, which will be discussed in more detail in the next section. Due to this low level of attachment, lag of proliferation with respect to tissue culture polystyrene (TCPS) was more significant in the case of smooth muscle cells (Figure 4.8), where the Alamar Blue reduction on TCPS on day 7 was 41.37 ± 5.45 % compared to 3rd cycle collagen coated hydrogels' 23.71± 0.10 %; which had the highest cell number between the samples.

The improvement of cell attachment and proliferation was also observed by fluorescent microscopy that demonstrated the endothelial attachment was much more effective than smooth muscle cell attachment. Endothelial cells grew and reached near confluence by 10 days. 3rd cycle hydrogels attained higher cell numbers, validated by cell number counts with CellF program (Olympus, Germany) (Figure 4.9). Endothelial cells spread and covered most of the surface on PVA/Chitosan hydrogels without any need for surface coating. Thus, PVA composite surfaces can be used for endothelialisation experiments with different kinds of additives to further improve physical properties that might facilitate cell attachment and proliferation.

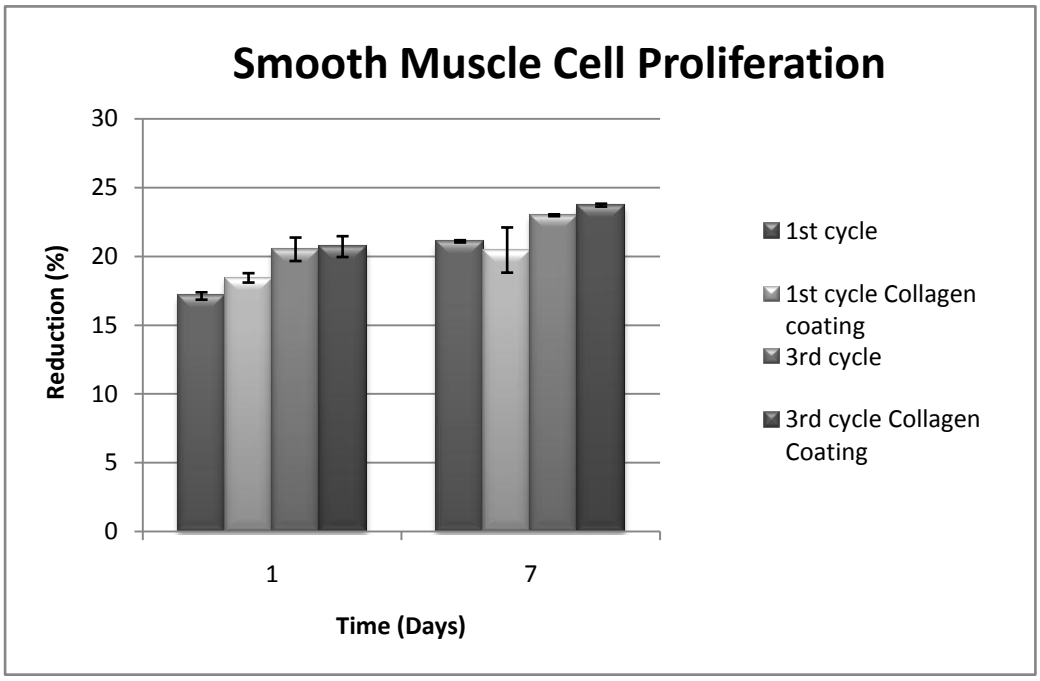

Figure 4.8 Proliferation of Arterial Smooth Muscle cells on PVA/Chitosan cryogels, depending on surface coating (chemical, physical or absent) and freeze-thaw cycle number. The highest cell number was attained on 3rd cycle hydrogels with collagen coating. Inset) The whole range of measurements for 1 week period; it can be seen that smooth muscle cell growth was slower compared to TCPS (n=3), see Appendix F.

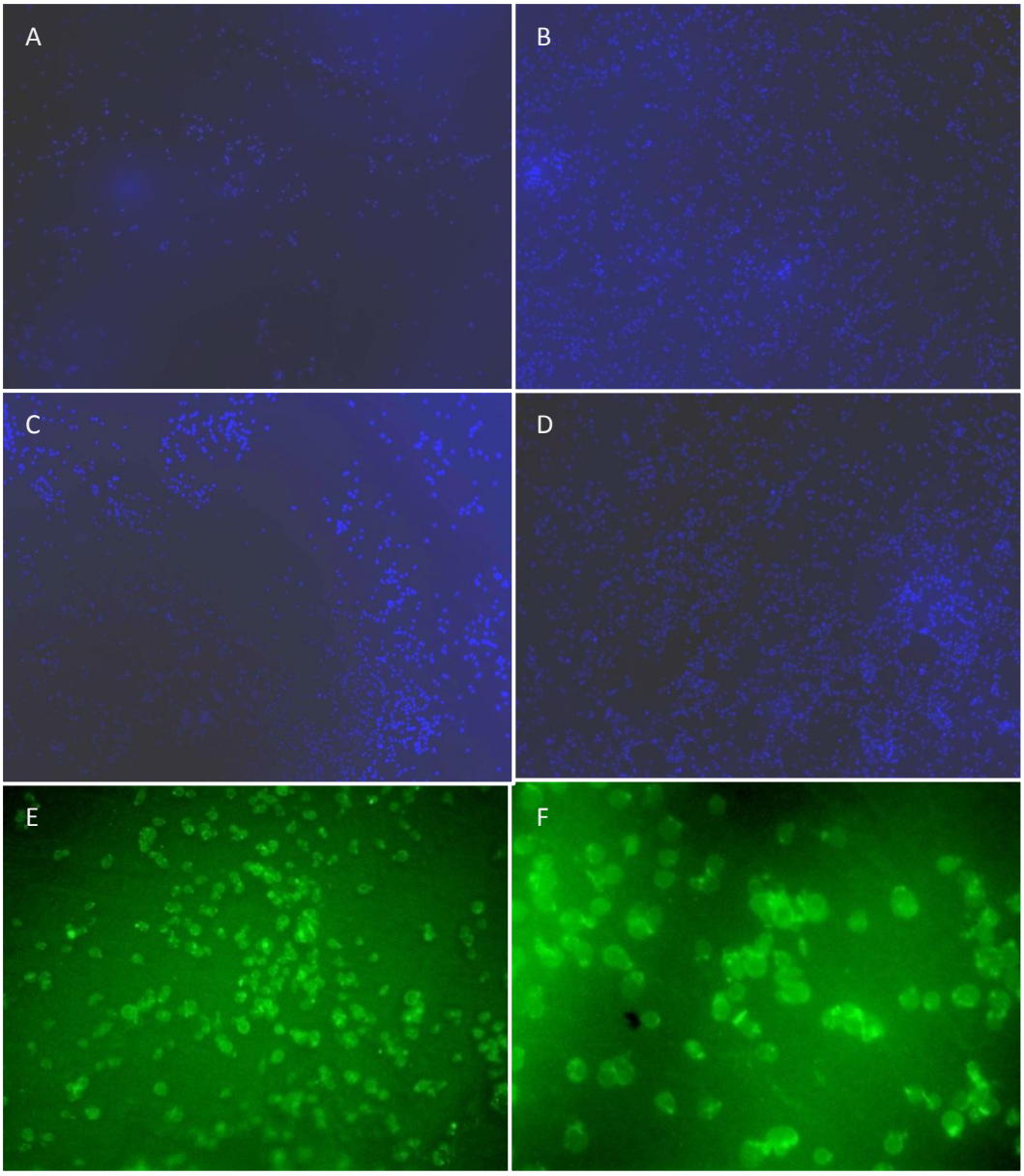

Figure 4.9 Growth of Endothelial cells on 1st and 3rd cycle PVA/Chitosan hydrogels (n=6) a, b) 1st cycle hydrogels after 5 and 10 days of culture respectively (Magnification 10x). c, d) 3rd cycle hydrogels. Cell numbers were significantly higher in 3rd cycle hydrogels compared to 1st cycle samples, validated by cell counts in 10x and 20x magnification images. Cell counts were done with at least 6 micrographs per treatment at 20x magnification. e-f)Cells were spread on the hydrogel surfaces on day 5 as observed by Phalloidin staining (10x and 20x magnification).

Even though, successive freeze-thaw cycles are beneficial, the extent of benefits did not compensate for the prolonged cryogel production time (72 hours vs. 24 hours). However, this system can be utilized to observe stiffness effects on cell behavior in the future. In other words, even though increasing freeze-thaw cycles did not have practical value for the current application in terms of cell response; it is a valuable tool to clarify the effects of stiffness change on cell behavior without the need of changing the concentration of the starting solution, as done in most of the current literature.

4.1.3 Additive Effect (Chitosan, Gelatin, Starch)

To determine whether the process is reproducible for other additives and to see whether a better option can be developed, composite cryogels with 3 different additives were prepared. Experiments showed that, the additives had a profound effect on the gelation process. Each additive caused a different level of water content. The change in terms of mechanical properties to the coagulation bath was also different for each composite hydrogel type. For this reason, coagulation bath effect experiments were repeated for each cryogel type. The highest water content was attained by PVA/Starch hydrogels (93.05±0.24 %) and the greatest decrease in water content following coagulation was observed with PVA/Starch hydrogels (to 80.95 ± 3.59 %) (Figure 4.10). Surface morphology was extremely different in the presence of the different additives. For PVA/Starch composite surface was covered with clumps rich with starch, which were also observed macroscopically while PVA/Chitosan retained the aligned fibrillar appearance of the pure PVA cryogels

only with thicker chains. PVA/Gelatin hydrogels had a layer of fibres on their surface with more apparent porosity (Figure 4.11).

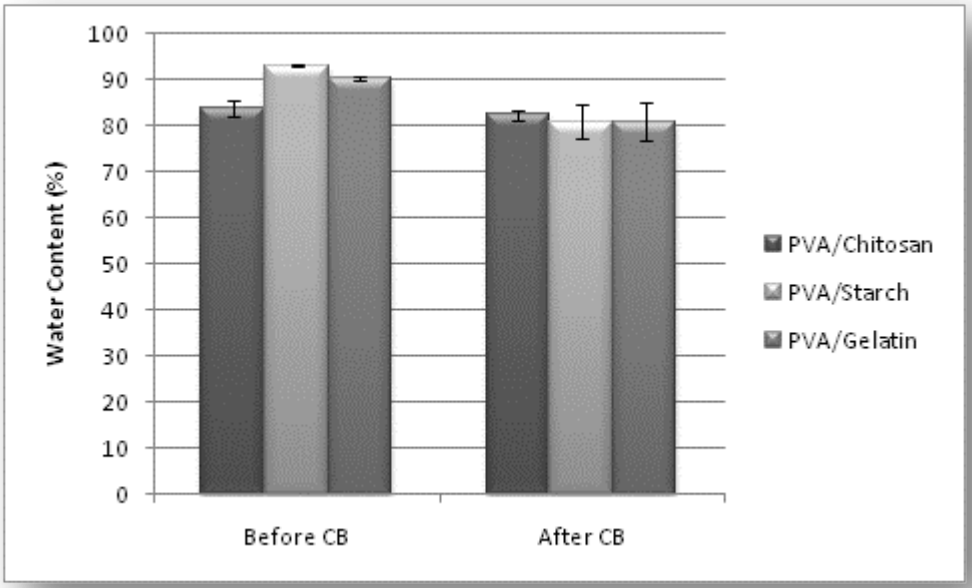

Figure 4.10 Change in water content with respect to additive type and application of coagulation bath (n=6). Even though PVA/Starch hydrogels had significantly higher water content before coagulation bath treatment (p=0.004), after coagulation bath treatment, water content values for all three composites were similar (p=0.69).

The coagulation bath treatment caused changes in dimensions and shrinkage of all hydrogel types. This effect was most visible for PVA/chitosan and PVA/Starch cryogels; whereas shrinkage was significantly less in PVA/Gelatin cryogels (Table 4.1). Differences in surface morphology (Figure 4.11) together with the intrinsic differences between the additives ability to absorb proteins led to a significant difference between the protein adsorption capacities of the hydrogels. To test whether the differences are protein-type dependent, two types of proteins were utilized, collagen type I and bovine serum albumin. Although the amount of protein adsorbed depended on the protein type; the trend was the same between cryogels;

the highest adsorption was observed with PVA/Gelatin cryogels (Table 4.1). Since protein adsorption is the first step in the attachment of the cells to a synthetic surface, a composites ability to absorb protein is beneficial. Compared to the pure PVA cryogels, which were only able to absorb 31.82±24.17 µg of collagen out of 1 mg, on PVA/Gelatin cryogels could absorb 338.52± 55.21 µg.

Table 4.1 Dimension Change and Protein adsorption on PVA based cryogels after coagulation bath treatment, * denotes p≤0.05 (n=6)

	PVA/Gelatin	***PVA/Chitosan***	***PVA/Starch***
Dimension Change (Diameter) (%)	**7.65±0.82***	16.28±0.28	16.49±0.41
Dimension Change (Height) (%)	**10.30±0.11***	25.10±0.90	35.32±0.42
Adsorbed BSA (µg)	**558.59±108.60**	487.50±36.74	490.62±85.25
Adsorbed Collagen type I(µg)	**338.52±55.21***	190.97±10.63	188.65±17.54

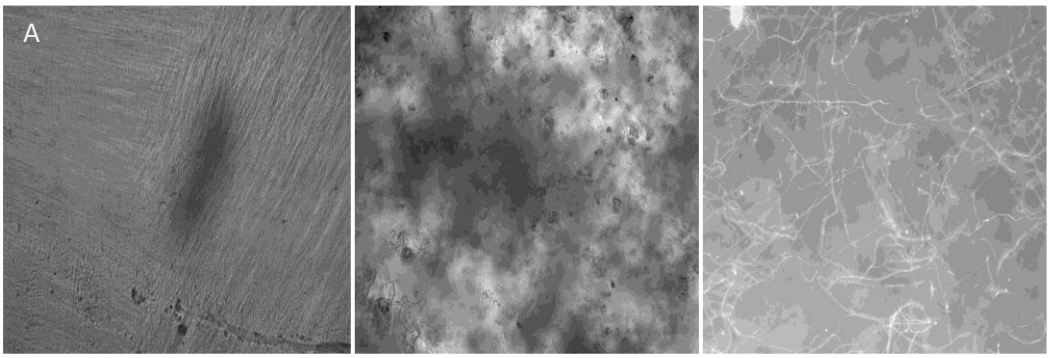

Figure 4.11 Surface morphology of PVA based composites observed with a Phase Contrast microscope (Magnification 100x). a) PVA/Chitosan, b) PVA/Starch c) PVA/Gelatin. PVA/Chitosan hydrogels had the conventional aligned layers, whereas PVA/Starch hydrogels had areas of starch accumulation and PVA/Gelatin surface was filled with fibrous gelatin (n=6).

The degradation rate was also strongly affected by the type of additive, especially without the application of the coagulation bath treatment. The treatment was again effective for all additives over 2 weeks and measurements after 1 month period still showed a lower degree of degradation for all types of hydrogels. After application of the coagulation bath treatment, the differences between the degradation rates of different composites were statistically insignificant (p= 0.59) (Figure 4.12). The highest degradation rate without coagulation bath treatment was observed with PVA/Starch hydrogels (40.13±3.03 %).

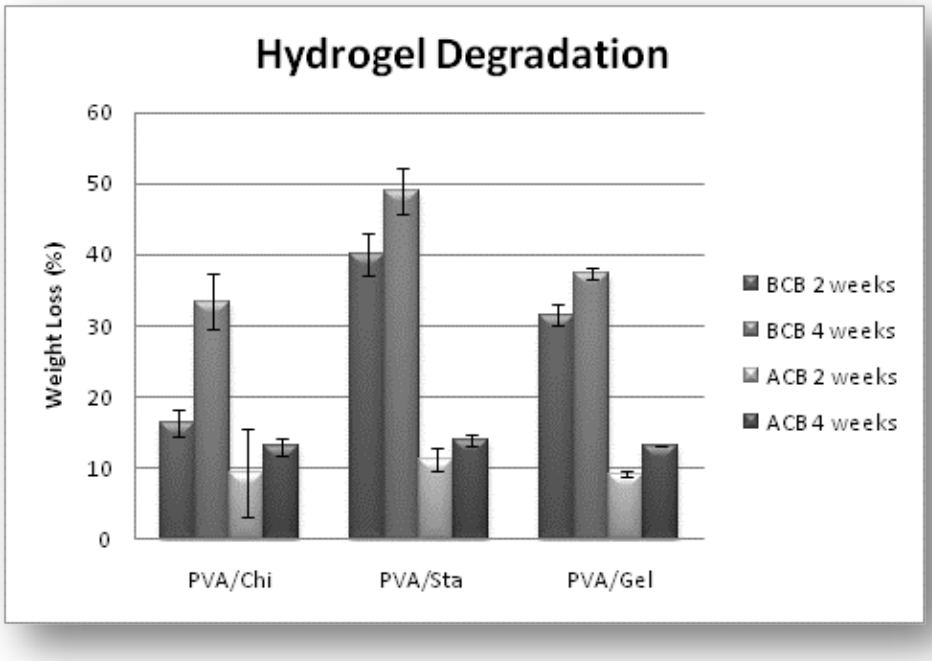

Figure 4.12 Degradation profiles of PVA composites before and after coagulation bath treatment up to 1 month. Coagulation bath effectively decreased degradation for all three types of composites and also slowed down level of degradation over a month compared to the samples before coagulation bath treatment (p≤0.001) (n=3).

The most apparent effect of both freeze-thaw cycle number and coagulation bath was a distinct increase in ultimate tensile strength and an increase in extensibility

(p≤0.001). This effect was observed in all three composites, but to different degrees (Table 4.2). The contribution of the coagulation bath treatment to the increase in tensile strength was significantly more than that of increasing the freeze-thaw cycle number. Also, the number of freeze-thaw cycles had a negative effect on extensibility except in the case of pure PVA cryogels. The highest tensile strength was attained by 3rd cycle for pure PVA cryogels after coagulation bath treatment, but PVA/Starch and PVA/Gelatin cryogels both had similar strengths.

Table 4.2 Ultimate tensile strength and Elongation at break values for pure PVA and composite hydrogels (n=6)

Cryogel Type	*UTS(MPa)*	*L_{max}(%)*
PVA 1st BCB	0.05±0.03	111.59±34.80
PVA 3rd BCB	0.48±0.17	124.49±28.88
PVA 1st ACB	0.48±0.23	147.21±57.72
PVA 3rd ACB	**0.96±0.25**	140.13±25.17
PVA/Chi 1st BCB	0.05±0.01	129.09±33.43
PVA/Chi 3rd BCB	0.30±0.08	92.84±8.24
PVA/Chi 1st ACB	0.44±0.12	137.44±58.58
PVA/Chi 3rd ACB	0.44±0.08	111.97±26.25
PVA/Sta 1st BCB	0.20±0.06	178.58±27.48
PVA/Sta 3rd BCB	0.51±0.10	158.94±22.65
PVA/Sta 1st ACB	0.70±0.10	**205.79±18.56**
PVA/Sta 3rd ACB	**0.93±0.23**	166.66±28.72
PVA/Gel 1st BCB	0.07±0.02	171.63±22.39
PVA/Gel 3rd BCB	0.84±0.30	135.15±28.32
PVA/Gel 1st ACB	0.60±0.14	**200.35±30.41**
PVA/Gel 3rd ACB	**0.92±0.18**	149.62±17.79

The most important aspect of the composites is their capability to promote cell attachment and proliferation. All three composite were suitable for cell attachment, but PVA/Gelatin hydrogels had significantly higher cell numbers after the initial attachment period (Figure 4.13). Cell attachment was concentrated on certain areas of the gel, which were probably rich in the additive. On pure PVA gels, there was nearly no cell attachment such that after 10 days no proliferation was observed. The proliferation rate for the composite hydrogels exhibited a similar trend with protein adsorption studies; i.e. PVA/Gelatin hydrogels had the highest

rate of proliferation and had significantly higher cell number by day 10 (Figure 4.14). The cells reached confluency on PVA/Gelatin by day 15 with an initial seeding concentration of 1x 10^5 cells/cm^2. PVA/Chitosan hydrogels also supported the growth of the cells with near confluence was apparently reached at a slower rate; whereas PVA/Starch hydrogels lagged behind significantly.

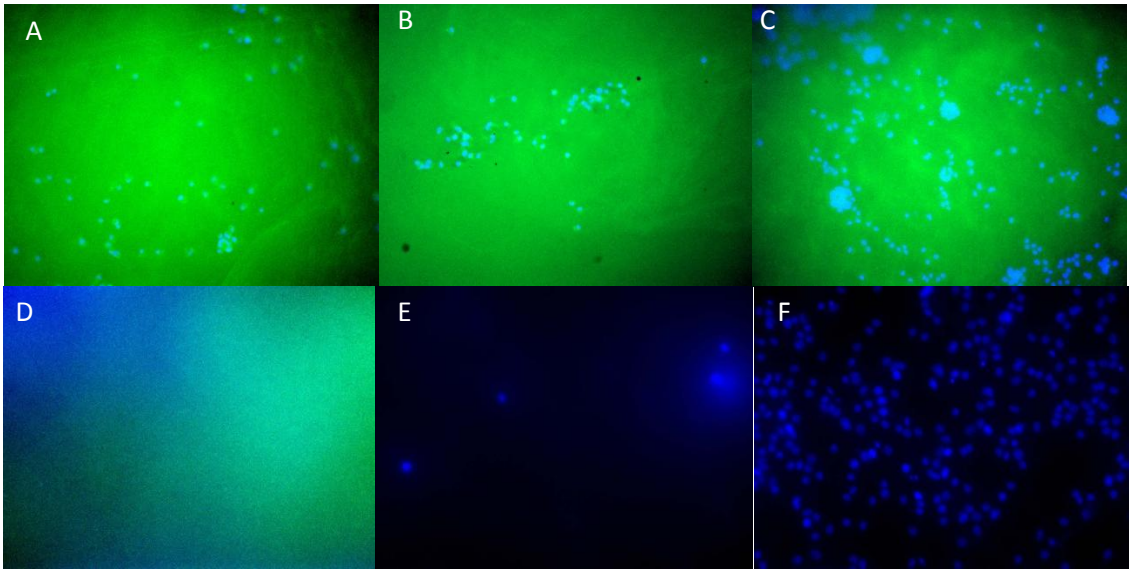

Figure 4.13 Initial attachment of Bovine endothelial cells to a) PVA/Starch b) PVA/Chitosan c) PVA/Gelatin hydrogels, 1st cycle after coagulation bath (Magnification 4x), the highest cell number was observed on PVA/Gelatin hydrogels d) Only few cells are observed on pure PVA gels, with most of the gel surface empty e) PVA gels after 5 days of culture, cell growth was retarded and cell presence was sparse f) PVA/Chitosan hydrogels after 5 days of culture, cell number has substantially increased (Magnification 20x).

Finally, to evaluate their feasibility as encapsulating materials, all three composites were used for preliminary encapsulation tests with arterial smooth muscle cells. Again, PVA/Gelatin was the most suitable candidate because of its ability to form much more stable cryogels under drastically different gelation conditions of encapsulation and also the improved cell distribution and numbers achieved. PVA/Starch cryogels did not encapsulate many cells, whereas with PVA/Chitosan

hydrogels, there were large smears of DNA which indicated cellular damage (Figure 4.14).

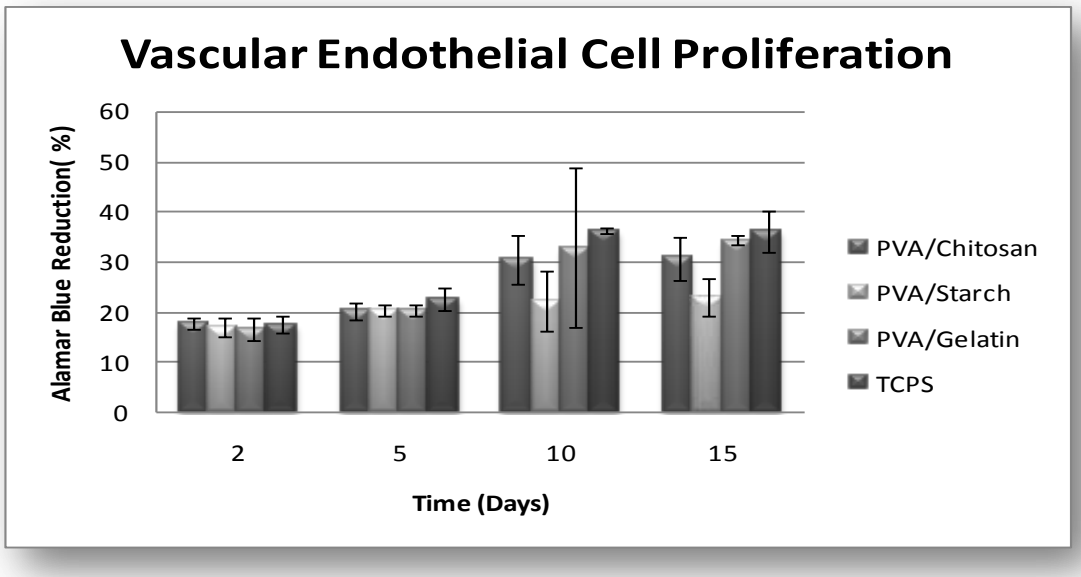

Figure 4.14 Endothelial cell proliferation on 1st cycle PVA based composites and TCPS. Confluency was reached on TCPS between days 5-10, whereas for hydrogels it was after 10 days. PVA/Chitosan and PVA/Gelatin presented more suitable surfaces for endothelial cell attachment and proliferation (n=6). PVA/Gelatin had significantly higher number of cells compared to other composites by day 15,see Appendix F for Statistical analysis.

To summarise, water content of all composite cryogels were similar, the highest protein adsorption for both BSA and Collagen was on PVA/Gelatin cryogels, the difference between the degradation rates of all the composite cryogels were statistically insignificant, the strongest cryogels were PVA/Starch cryogels but PVA/Gelatin cryogels had comparable strength, whereas PVA/Gelatin outperformed the other two composites both in endothelial proliferation and also smooth muscle cell encapsulation. All these results suggest that the PVA/Gelatin hydrogels are the most suitable option to pursue more elaborate characterization of endothelial cell

behavior and the development of a cell encapsulation system. They adsorbed the highest amount of protein, showed the least shrinkage and had the highest proliferation rate. Their water content, degradation profile and mechanical properties were superior or comparable with the other composites. Thus the PVA/Gelatin combination was selected for detailed investigation in this study at this point. Due to this decision, detailed endothelial cell behavior studies were performed with 1st cycle PVA/Gelatin cryogels after coagulation bath treatment, which has improved the mechanical properties and degradation resistance as expected. The higher freeze-thaw cycle numbers were not pursued, because in the encapsulation experiments conducted simultaneously, increasing freeze-thaw cycle number was detrimental to the cell viability within the cryogels. Protocols were adjusted accordingly for the increased surface hydrophilicity due to the coagulation bath treatment(longer incubation at 37 °C to improve protein adsorption).

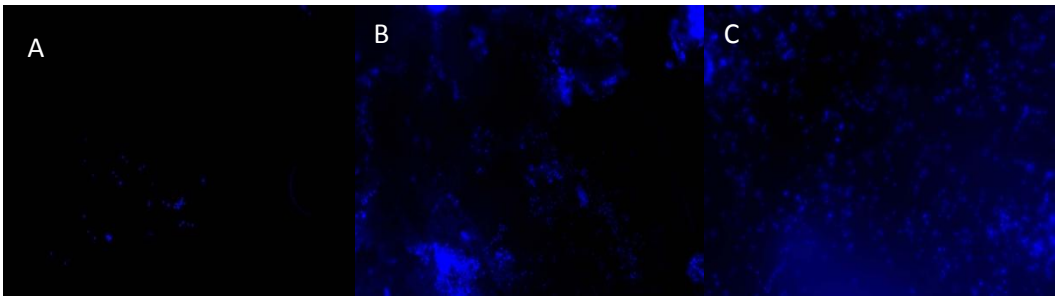

Figure 4.15 Preliminary Encapsulation tests with the composite cryogels a) PVA/Starch b)PVA/Chitosan c) PVA/Gelatin. PVA/Gelatin supported the most cells with minimum apparent damage and also resulted in a much more stable gel. The cell presence on PVA/Starch cryogels was minimal; whereas big smears were observed in PVA/Chitosan cryogels which indicated extensive cell damage (n=3).

4.2 Endothelial cell characterization on PVA/Gelatin hydrogels

4.2.1 Under Static Conditions

After selection of PVA/Gelatin as the most promising composite, the scaffolds were first tested to evaluate their suitability as a basement for endothelial cells. To assess endothelialisation of the scaffold surface, four main characteristics of endothelial cells were investigated:

1) Whether there is a significant difference between proliferation of endothelial cells on tissue culture polystyrene and the PVA/Gelatin surface (FACS, CFDA cell proliferation assay)
2) Whether presence of the hydrogel surface or mode of attachment to the hydrogel surface induces short term or long term apoptosis (FACS, Vybrant Apoptosis assay)
3) To assess the cell-cell contact molecules related to inflammatory response(ICAM and Selectin P) and also endothelial barrier formation (PECAM) to determine the effect of attachment to hydrogel surface with respect to control, tissue culture polystyrene (Real time RT-PCR)
4) Determination of NO (an endothelial cell health marker) synthesis and release and the difference in its level between the hydrogel surface and TCPS.

Endothelial cells grew at a steady pace on PVA/Gelatin hydrogels and started to establish cell-cell contacts. By day 10, the surface was nearly fully covered by endothelial cells (Figure 4.16). These results were consistent with previous Alamar

Blue assays (Figure 4.14). Low seeding density was selected to ensure that the growth of the cells could be monitored.

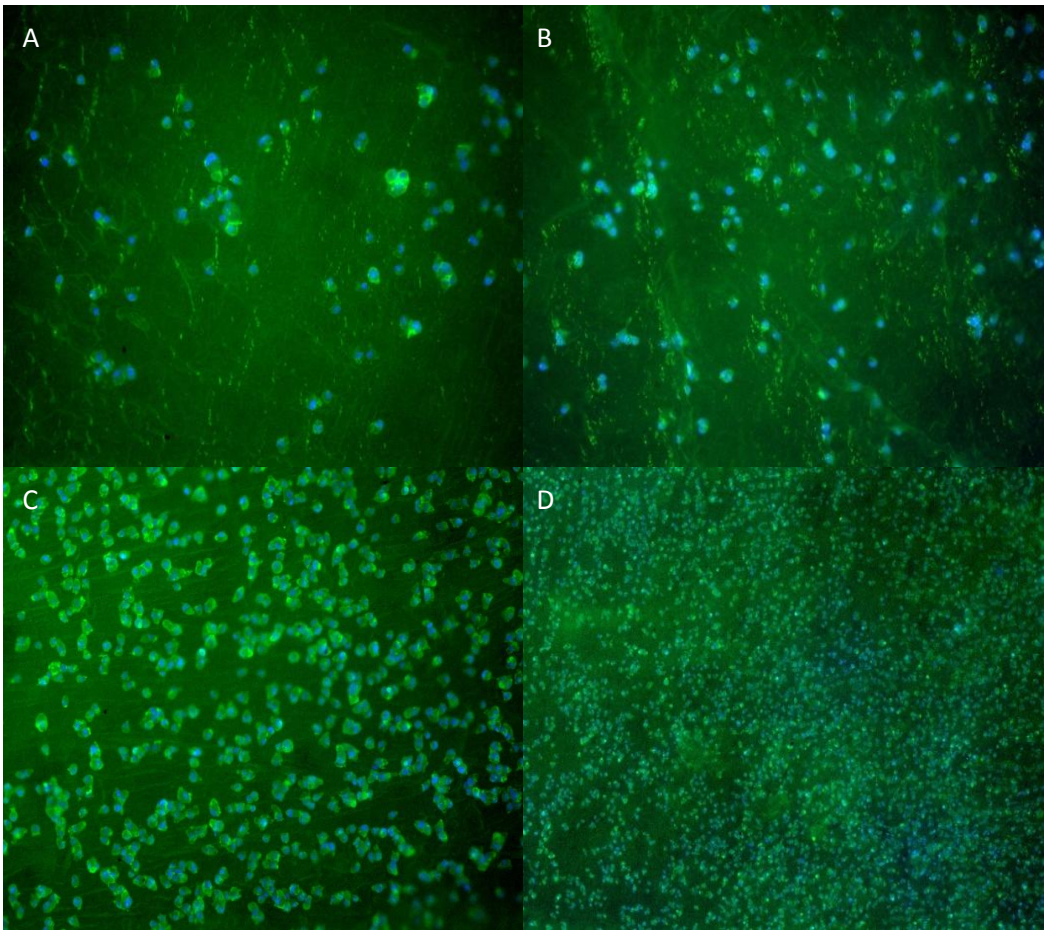

Figure 4.16 Endothelial cell proliferation and spreading on PVA/Gelatin cryogels observed by DAPI/FITC-Phalloidin staining (n=3) a) Day 2, b) Day 5 c) Day 10 (Magnification 10x). There was a steady growth. d) Near confluence of endothelial cells on PVA/Gelatin cryogels after 10 days of culture (Magnification 4x).

CFDA is a non-toxic dye which penetrates into the cells. The decrease in its fluorescence intensity is indirect evidence of the division of cells. Since it does not rely on any metabolic activity, cell type to cell type differences and differences due to cell attachment on to different surfaces (such as TCPS and the hydrogel in this

case) is minimal. CFDA results showed that aside from the initial attachment period, the proliferation rate between PVA/Gelatin cryogels and the TCPS was not statistically significant by day 10 (Figure 4.17a).

Both conditions exhibited an exponential growth that peaked by day 15. The distribution of the cells was also observed to determine whether there are sub-populations of cells that grew at a faster pace (Figure 4.17b).

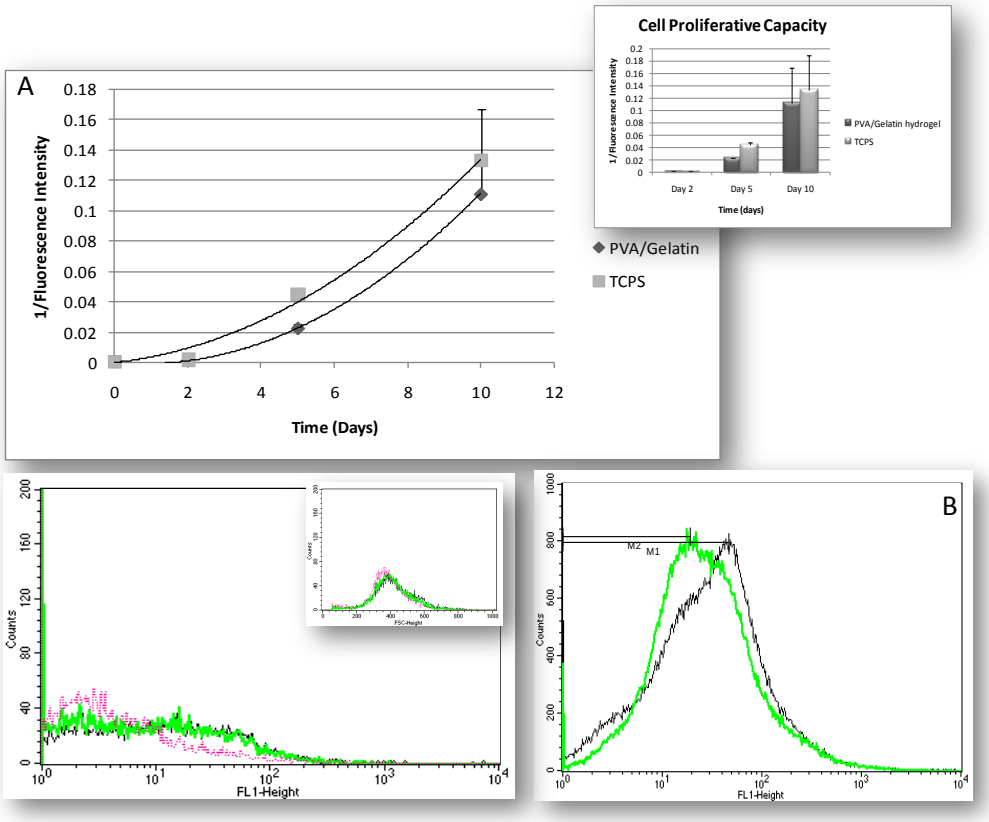

Figure 4.17 a) Proliferative capacity of endothelial cells on PVA/Gelatin hydrogels vs. TCPS. Starting points for the graphs are 0.00048 and 0.00051, respectively corresponding to readings taken just after introduction of CFDA. Inset Bar graphical representation, on day 5 TCPS had significantly higher proliferation b1) Representative reading for day 10; pink line (TCPS), Black and Green (PVA/Gelatin) Inset) Forward Scatter counts to prove that the average cell size was the same; all curves coincided b2) Representative FACS read-outs on day 5, green line (TCPS) black line(PVA/Gelatin). PVA/Gelatin curve skewed around 10^1 fluorescence range, implying a faster proliferating sub-population (n=3).

On day 5, on PVA/Gelatin hydrogels, a small bump can be seen on the distribution which indicated a group of cells with higher proliferative capacity. These cells were located on gelatin rich areas of the surface that would give them an advantage in spreading and subsequent proliferation, whereas the cells on TCPS showed a wider distribution.

Attachment to a surface does not guarantee long term survival for cells, since lack of necessary signals may induce apoptosis. Long-term and short-term apoptosis was examined using FACS analysis. Apoptosis results shed light to the initial high number of cells observed on TCPS, because there was a significantly higher number of necrotic cells observed on the PVA/Gelatin cryogels after day 2 (Table 4.3). However, this number decreased significantly by day 5 and only a small group of early and late apoptotic cells was present for both PVA/Gelatin cryogels and TCPS surface. This trend continued up until day 20 of culture where in both cases the number of apoptotic cells was minimal (Figure 4.18).

Table 4.3 Viable, Necrotic and Apoptotic Cell counts for PVA/Gelatin cryogels and TCPS over 20 days, * denotes p≤0.005 (n=9)

	Viable (%)	***Necrotic (%)***	***Early Apoptotic (%)***	***Late Apoptotic (%)***
Day 2 PVA/Gel	84.70±1.88	**14.65±1.35***	0.15±0.07	0.48±0.26
Day 2 TCPS	96.66±1.26	**3.31±1.25***	0.01±0	0.02±0
Day5 PVA/Gel	97.22±0.16	2.28±0.64	0.25±0.26	0.24±0.20
Day 5 TCPS	95.89±0.23	3.90±0.14	0.11±0.04	0.10±0.04
Day 10 PVA/Gel	99.67±0.20	**0**	**0.33±0.19***	**0**
Day 10 TCPS	99.95±0.03	**0**	**0.05±0.03***	**0**
Day 20 PVA/Gel	98.63±0.09	1.33±0.10	0.03±0.01	0.01±0.02
Day 20 TCPS	99.79±0.24	0.20±0.25	0.01±0	0±0

Attachment of the cells to the compliant PVA/Gelatin cryogel surface did not induce any significant apoptotic response. Rather, the only effect was a decreased

effectiveness of cell attachment when compared to the TCPS surface. During the initial culture period, necrosis of the unattached or loosely attached cells was observed more on PVA/Gelatin cryogels. After that, TCPS and cryogel surfaces behaved in a similar way, suggesting that PVA/Gelatin cryogels can sustain long-term culture of endothelial cells, which is crucial for long term bioreactor tests to develop long-term compliance. The results of apoptosis assays corroborate well with proliferation assays and it appears that the initial necrotic cell count must be responsible for the lag observed in proliferation on PVA/Gelatin cryogels.

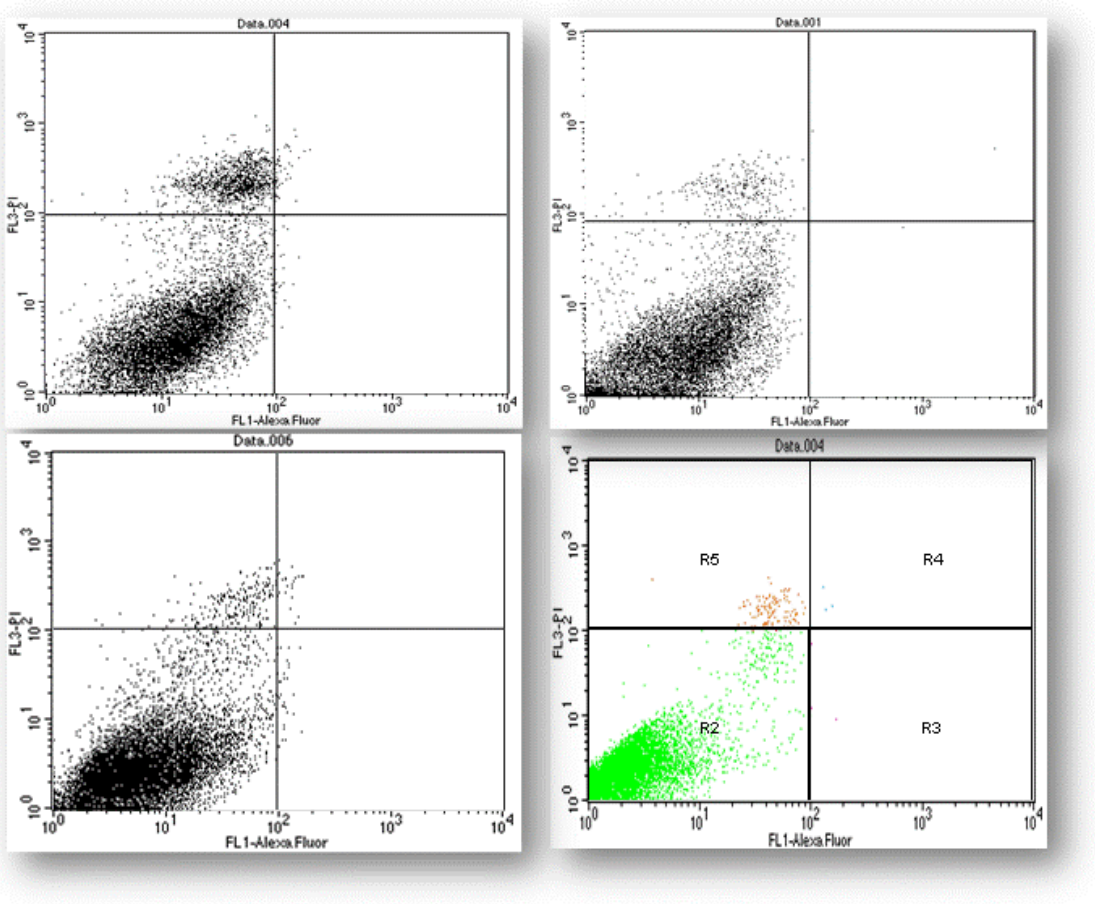

Figure 4.18 Propidium Iodide vs. AlexiFluor conjugated AlexiFluor FACS readings (n=3) for a) PVA/Gelatin cryogels day 2 b) TCPS Day 2 c) PVA/Gelatin Day 5 d) PVA/Gelatin Day 20. Decrease in the counts in regions 3, 4 and 5 showed a decrease in apoptotic and necrotic cells. The necrotic and apoptotic counts were higher in the beginning of the culture and subsided later.

The main function of the endothelium is to act as a selective barrier and the first line of response in the case of injury or infection. Endothelial cells achieve this function through cell-cell contact molecules which either hold the integrity of the endothelial barrier (such as PECAM I, an endothelial cell specific cell-cell contact molecule) or recruiting white blood cells or platelets for commencement of coagulation or inflammation (such as ICAM and Selectin P). These 3 proteins were selected to evaluate how attachment to hydrogel surfaces would affect endothelial response with respect to the standard tissue culture conditions. GADPH was used as an endogenous housekeeping gene. Over a 10 day period, expression of PECAM mRNA steadily increased which indicated more cell-cell contacts (Figure 4.19). ICAM is a constitutively expressed protein whose expression levels increase during inflammation. The ICAM levels were steady for the first 5 days and after that there was only a slight increase in its expression. At the beginning of the culture Selectin-P expression on hydrogels was significantly higher than that on TCPS, but the expression came to the similar levels by day 10.

The average C_t values for PECAM on PVA/Gelatin hydrogels decreased (which indicates a higher level of expression) from 22 to 16.8 over 10 days whereas the expression on TCPS in the same period was steady. For ICAM the decrease was smaller (from 26.9 to 24.1).

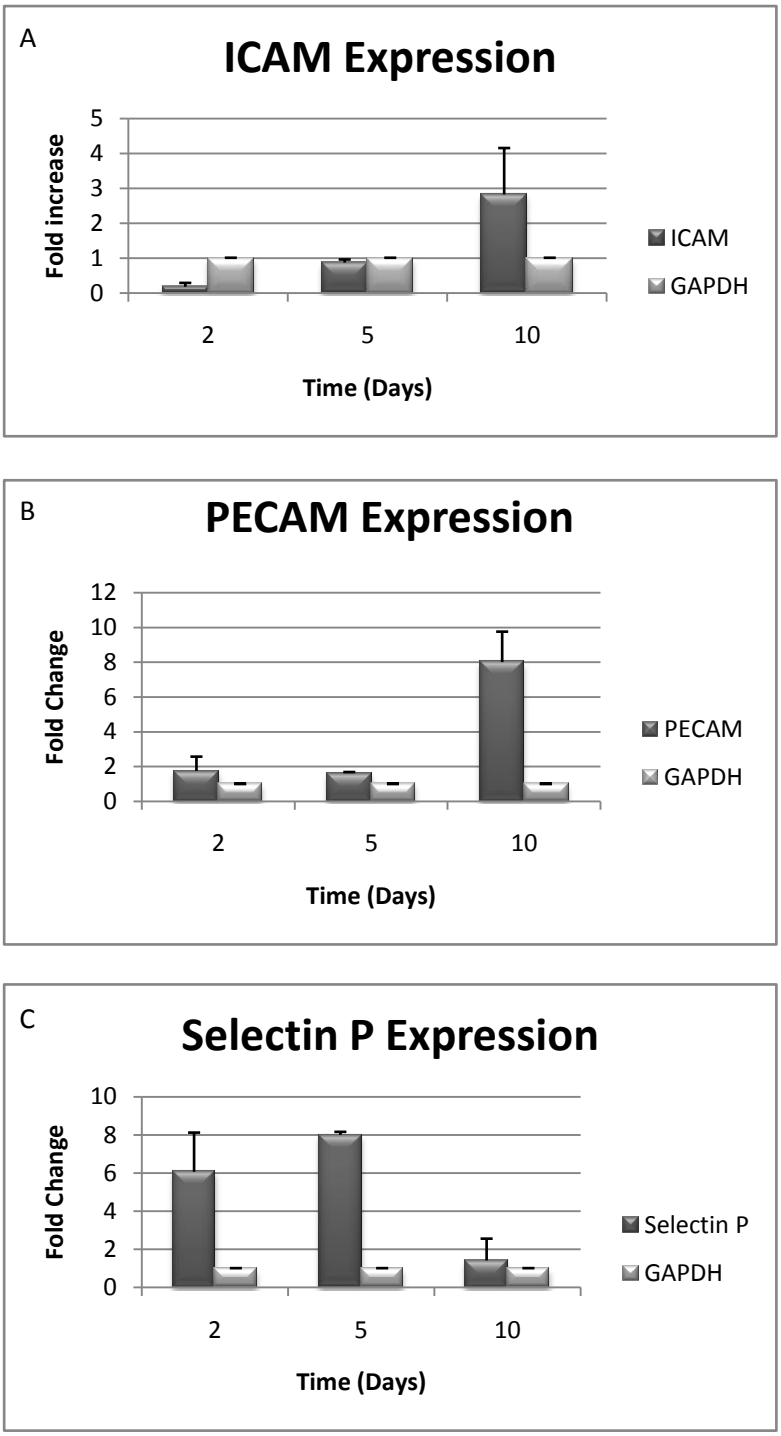

Figure 4.19 Fold change in expression of ICAM, Selectin-P and PECAM respectively on PVA/Gelatin cryogels vs. TCPS for a 10 day period. Fold change was calculated by $2^{-\Delta\Delta Ct}$ method, Fold change with respect to time was significant for ICAM for all time points, whereas change in PECAM and Selectin P was significant for day 10 (n=3).

Lastly, the level of NO (Nitric Oxide) synthesis on PVA/Gelatin cryogels was measured by the Griess method. The concentration of NO for the cell seeded on TCPS was 0.70±0.02 μM in the beginning of the culture and it was raised to 10.20± 1.39 μM by day 5. During the same time interval, NO production on the PVA/Gelatin cryogels was 0.28±0.05 μM and 5.26± 0.83 μM, respectively. These results represent concentration within the medium, thus the cell number difference had an effect on the NO levels. However secretion of NO was detectable for both cases. NO secretion levels are highly affected by the presence of shear stress and this will be discussed in the next section.

The results presented suggest that a healthy endothelial lining with NO synthesis, cell-cell contacts and full confluency with low levels of apoptosis can be achieved on PVA/Gelatin cryogels. The mode of attachment on PVA/Gelatin cryogel surface was slower due to the surface properties, which led to initial high levels of necrotic cells. But, eventually cells were able cover the cryogel surface and the high level of expression of ICAM and PECAM and depressed expression of Selectin-P were good indicators of the ability of PVA/Gelatin cryogels to sustain the endothelialisation. The next question was, whether this layer would be strong enough to withstand physiological shear stress conditions. Thus, the next section deals with how shear stress can be used to improve the endothelialisation.

4.2.2 Effect of Shear Stress

Endothelial cells are under constant shear stress due to the blood flow under *in-vivo* conditions and the shear stress conditions have a significant effect on their behaviour. This can be exploited under *in-vitro* conditions to facilitate the endothelialisation of scaffolds. Also, even though blood flow is generally laminar, under certain circumstances such as plaque formation or surgical intervention, it can become turbulent and the varying shear stresses generated by turbulent flow have markedly different effects on endothelial cells (Chien 2008a). With this in mind, endothelialisation on 1st cycle PVA/Gelatin cryogels after coagulation bath treatment was evaluated under shear stress conditions induced by an orbital shaker motion which creates a turbulent flow. The underlying hypothesis was that, since this kind of flow is known to induce proliferation, the endothelialisation period can be shortened even for small initial seeding numbers.

The shear stress level was selected as 10 dyn/cm^2 and this was applied either directly or via ramping in 3 steps. Direct application of shear caused widespread detachment of the cells from PVA/Gelatin surfaces, while cells stayed on the surface of TCPS under these conditions, confirmed by microscopical observations. This underlines the initially weaker attachment of endothelial cells to PVA/Gelatin cryogels (Figure 4.18). However, when the shear stress is applied by ramping, cell detachment was prevented and considerably higher number of cells were observed even on day 2 (Figure 4.20).

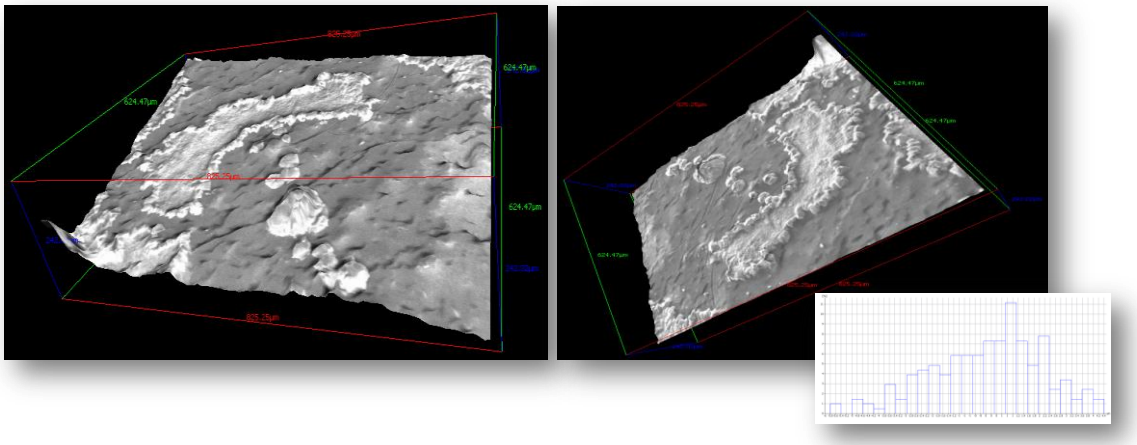

Figure 4.20 3D reconstruction of endothelial cell seeded PVA/Gelatin cryogel surfaces after 24 hours of direct application of shear stress. Detachment of the cells and empty areas was the result of direct shear stress application. Shear stress caused a decrease in surface roughness. Under these conditions the surface roughness was calculated from the images in random areas of 4x4 μm^2 areas and 10 µm length lines by Alicona Max Image analysis program as 1.63 ±0.11 µm with the highest peak observed being 8.17 µm. In comparison, surface roughness of unseeded empty cryogels which were exposed to shear stress was 2.11± 0.56 µm. Inset) Representative distribution of surface roughness values.

The increase in cell number and proliferation was quantified by both CFDA assay and Alamar Blue assay and in both cases there was a significant increase in the cell number compared to the static culture conditions (Figure 4.22). The coagulation bath treatment of the PVA/Gelatin cryogels caused an increase in surface roughness of the cryogel surfaces from 5.30± 0.96 µm to 10.93±1.21 µm. After application of shear stress the surface roughness decreased down to 2.11± 0.56 µm (n=6).

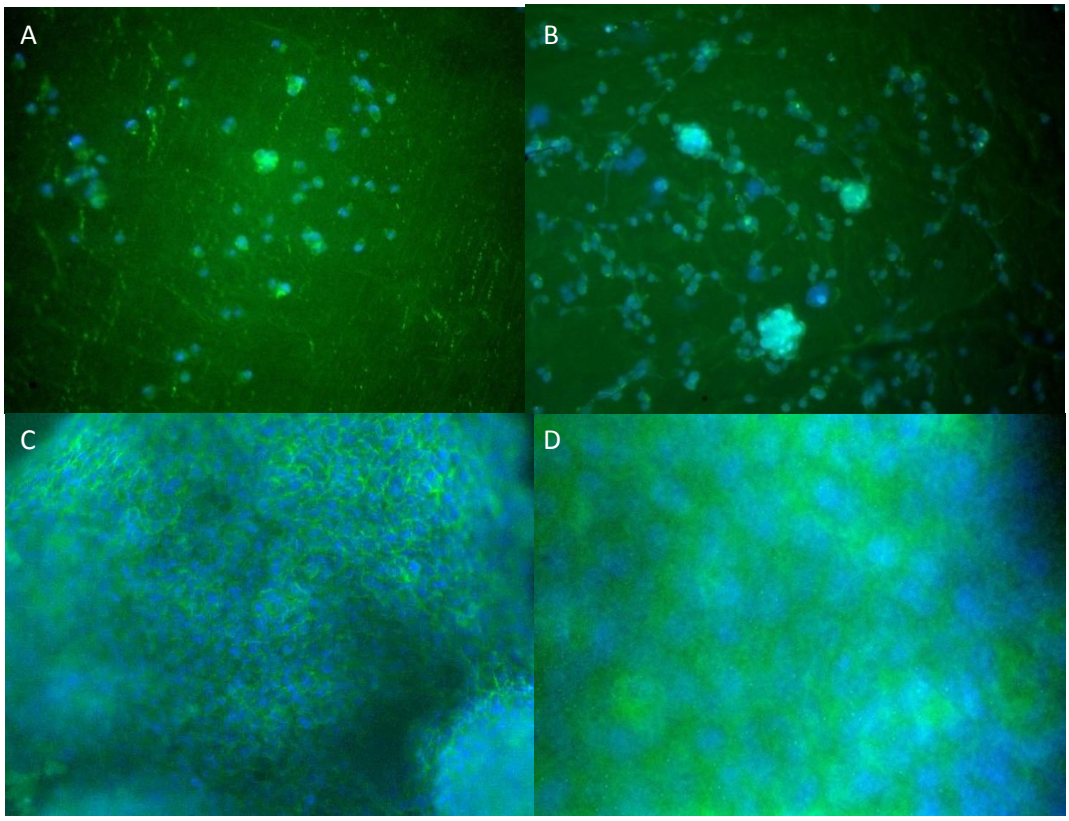

Figure 4.21 Proliferation of endothelial cells under ramped shear stress conditions (n=3) (DAPI/FITC Phalloidin staining a) Static culture 2 days b) Ramped shear stress 2 days (Magnification 10x, Inset Magnification 20x) , cell number and cell spreading was dinstinctly higher c) Lining formation on ramped shear stress samples after day 5; most of the surface was covered by a layer of endithelial cells (Magnification 10x) d) Lining formation, endothelial cells were connected within the lining (Magnification 60x)

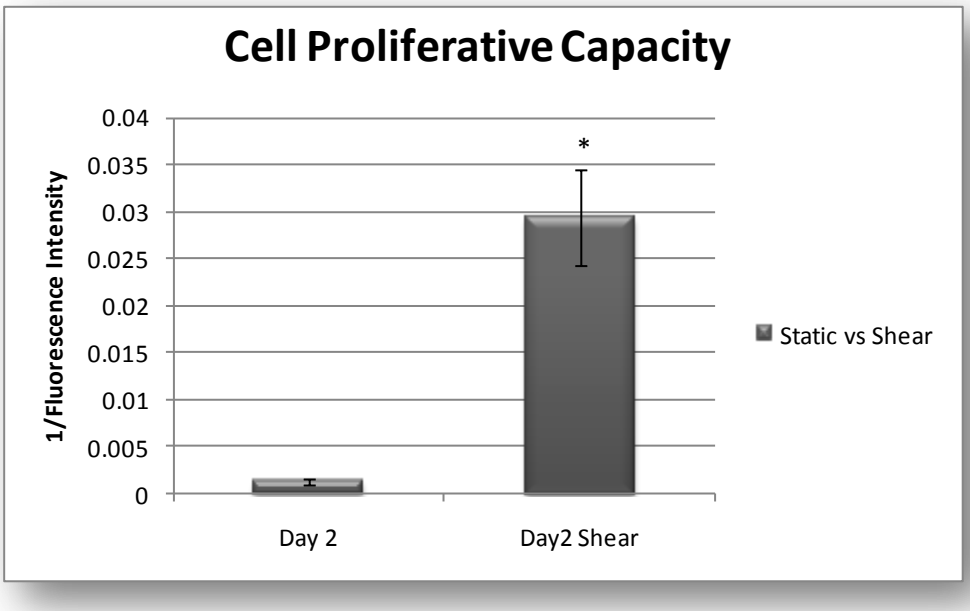

Figure 4.22 CFDA results for endothelial cells under ramped shear vs. static conditions. There was a significant increase in proliferation (p= 0.0006) and the increase in proliferative capacity was nearly 10 fold.

After application of ramped shear, there were no necrotic cells observed by apoptosis assay 99.33± 0.05 % of the cells were viable whereas there was a population of 0.67± 0.05 % early apoptotic cells. Application of shear stress not only increased the proliferation but also decreased the level of necrosis due to lack of attachment during the initial period of culture observed earlier. Gene expression levels under shear stress conditions demonstrated that the differences between PVA/Gelatin surface and TCPS was reduced as shear became the dominant factor. Under ramped shear stress, the only significant increase was in ICAM expression (Figure 4.23 a), but when compared to the expression levels at static culture conditions, all genes were upregulated, most notably Selectin-P and ICAM (Figure 4.23 b). The level of PECAM expression has also increased indicating improved cell

to cell contacts even from the onset. The lining formation was also observed by SEM, and showed cellular coverage of most of the cryogel surface. The alignment of cells was not widespread but visible; the decrease in the roughness of the surface was also observed in non-cell seeded cryogels (Figure 4.24).

Application of shear stress also had a marked effect on NO secretion. Initially, NO levels of cells under shear stress were higher for both TCPS and PVA/Gelatin cryogels, however after day 5 the direct shear samples exhibited lower levels of NO compared to static and ramped shear samples (Figure 4.25). At this point ramped shear stress samples still had slightly higher NO secretion. It is known that application of shear stress has the ability to increase NO levels, so the depression after 5 days under directly applied stress was due to the diminished number of cells due to detachment under simultaneous high levels of shear stress, as observed by the SEM too. Both SEM images and fluorescent microscope images demonstrated a slight directionality for the cells but the circular nature of the flow prevented a higher degree of orientation.

Laminar physiological shear stress is important for keeping the homeostasis of endothelial cells and involves low level of proliferation and secretion of NO. However, in tissue engineering a high rate of proliferation is important during the commencement of product manufacture and sub- or supra-physiological shear stresses can be used to induce these high initial rates of growth. Ability to utilize non-physiological conditions can be an important tool in tissue engineering, since such conditions can be created *in-vitro*.

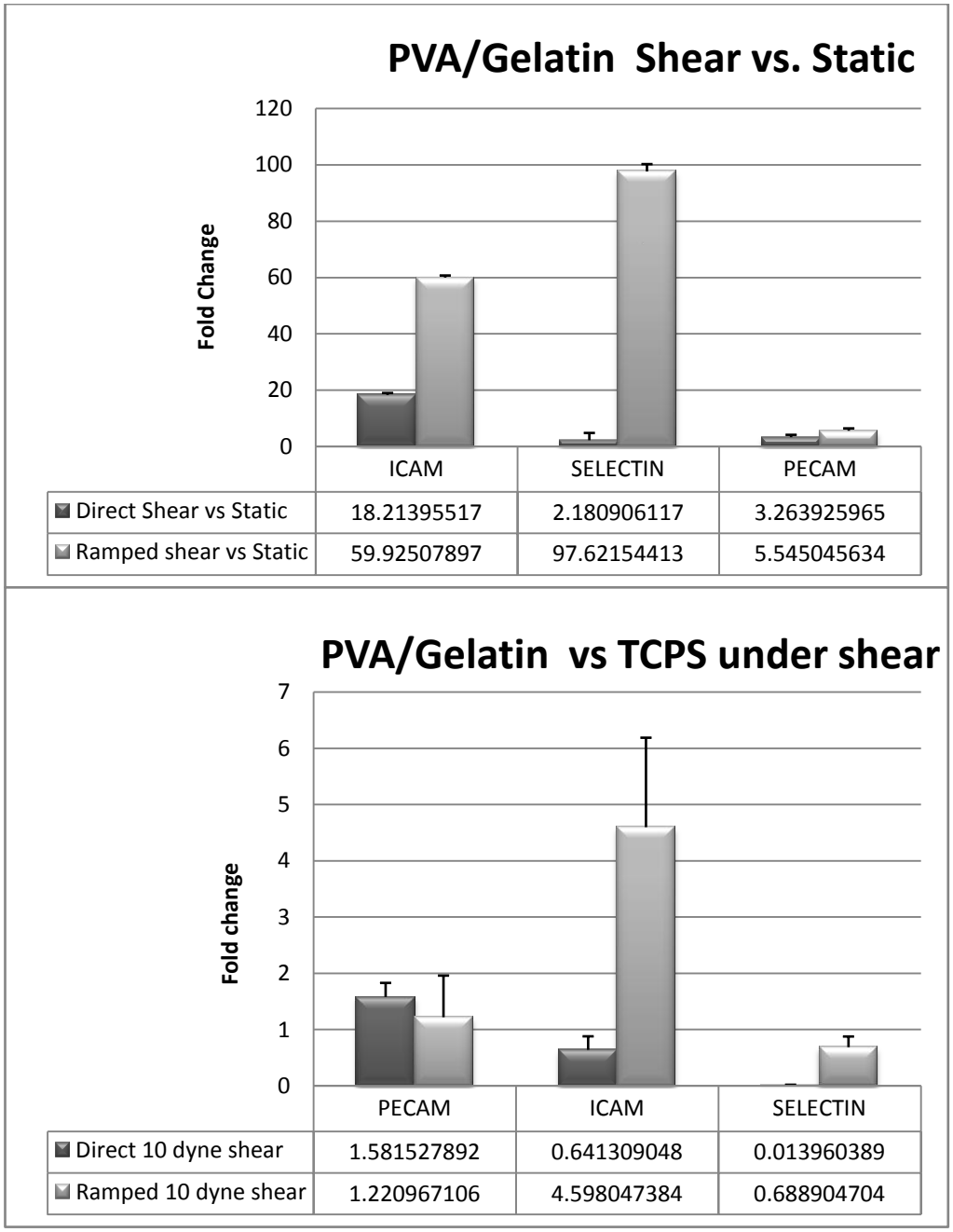

Figure 4.23 Real time RT-PCR results for endothelial cells on PVA/Gelatin exposed to ramped shear stress at 24 h (n=3) a) Fold change with respect to Static PVA/Gelatin Control. b) Fold change with respect to TCPS control under shear Under shear stress, the difference between TCPS and PVA/Gelatin disappeared and the shear became the dominant factor; only in ICAM there was a statistically significant increase in expression on cryogels compared to TCPS ($p<0.001$). When compared to the static conditions, both ramped and direct shear application caused substantial increase in all three genes' expression.

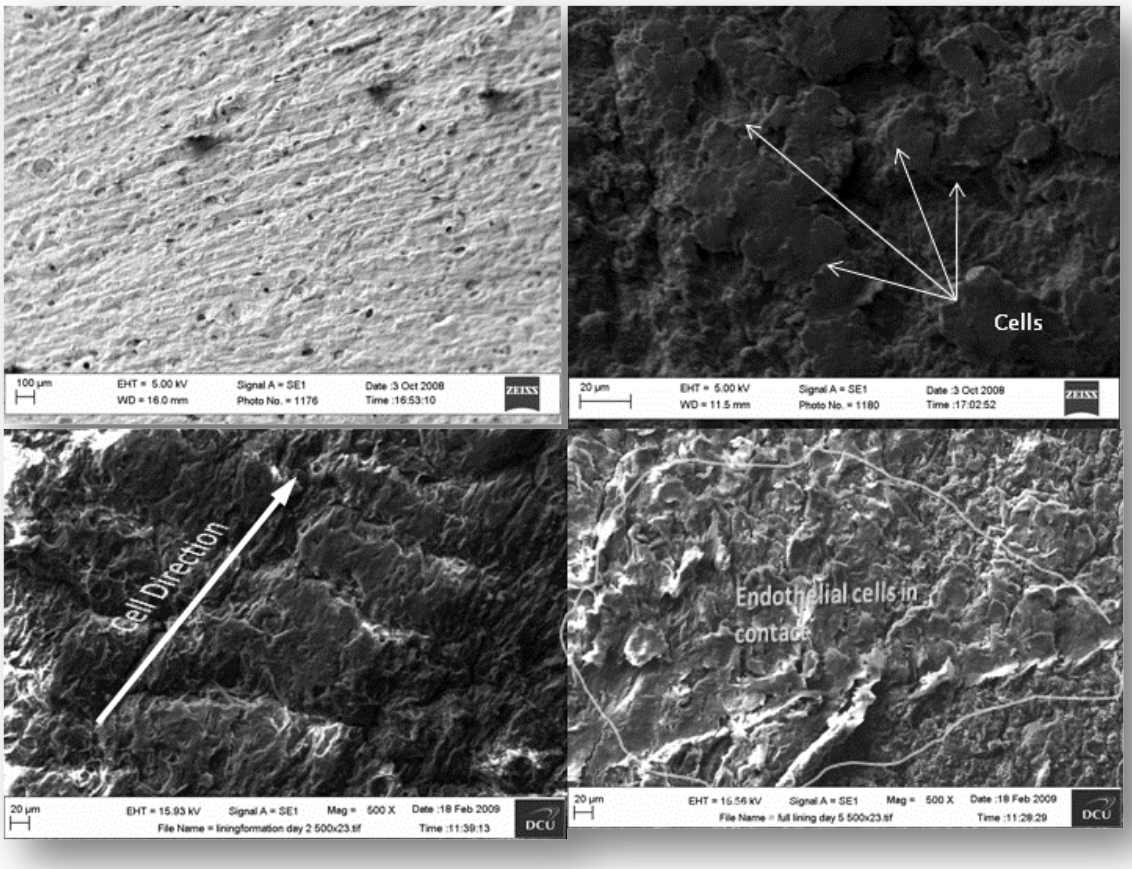

Figure 4.24 SEM images of endothelial cell seeded cryogels a) Unseeded PVA/Gelatin cryogel after application of shear, resulted in a smoother surface for the hydrogel b) SEM images of endothelial cells on PVA/Gelatin cryogel under static conditions, day 5 c) Endothelial cells on PVA/Gelatin cryogels under shear after 2 days; cells showed some orientation d) Endothelial cell coverage of the cryogel surface under shear after 5 days, shows a group of cells in contact that has covered most of the surface of the gels.

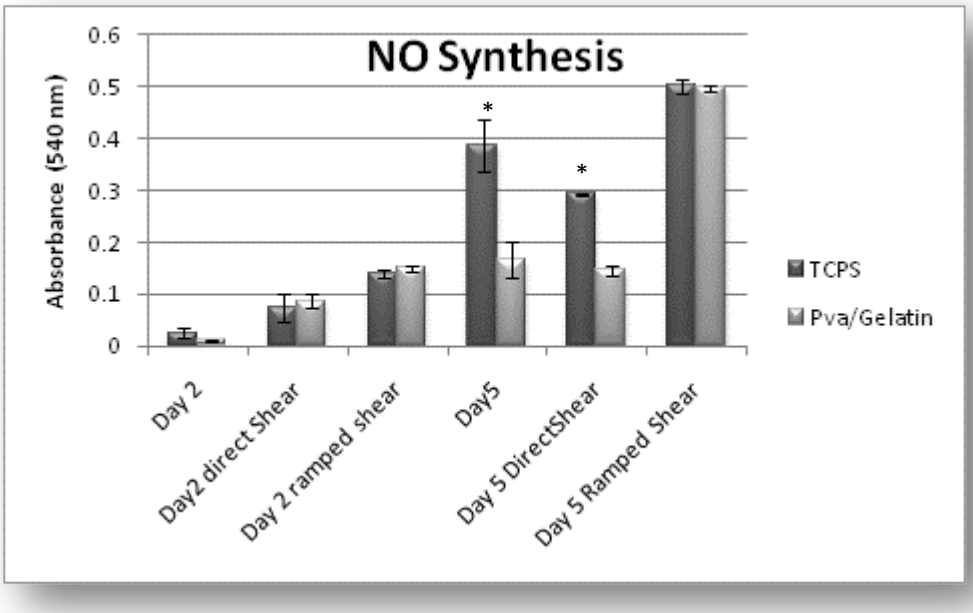

Figure 4.25 NO synthesis by endothelial cells on TCPS and PVA/Gelatin cryogels under static or shear stress conditions. NO synthesis has increased over time for both static and shear stress conditions. NO synthesis on PVA/Gelatin cryogels was comparable to TCPS on static and ramped shear conditions on day 2; whereas on day 5 static conditions and direct application of shear caused a decrease in NO synthesis compared to TCPS ($p \leq 0.001$), where as the difference between cryogels and TCPS was not significant under ramped shear conditions.

4.3 Encapsulation of Smooth Muscle Cells via Cryogelation

Since arterial smooth muscle cells are embedded in a thick extracellular matrix under *in-vivo* conditions, smooth muscle cells should be cultured in 3D environment. Since the effective pore size of the hydrogels are generally smaller than cell size, for hydrogel systems it is preferable to encapsulate cells in-situ during the formation of the scaffold. The freeze-thawing procedure presents a unique opportunity in this sense, since the gelation starts at sub-zero temperatures. The gel formation step can be also used as a storage media. However, conditions must

be such as to ensure cell viability and functionality and this part of the study is focused on development of such a protocol for cell encapsulation and storage via cryogelation.

Pure PVA solution in distilled water has a slightly acidic nature, and the addition of all three additives caused a further drop in pH of these solutions (Table 4.4) Moreover, since thick specimens are targeted, utilization of these solutions in distilled water was detrimental for initial nutrient transfer, which resulted in lower cell viability in experiments. To stabilize the pH at physiological levels and also to provide cells with nutrients during the initial phase of encapsulation PVA- additive solutions were dissolved in HEPES buffered RPMI-1640 cell culture medium and pH stability was attained (pH 7.22± 0.10).

Table 4.4 ph and viscosity values of pure PVA and PVA/Additive solutions (n=6)

	PVA	***PVA/Gelatin***	***PVA/Starch***	***PVA/Chitosan***
pH	6.22	5.84	5.93	5.24
Viscosity(mPa.s)	2175.1±9.0	2054.9±9.0	1376.4±13.1	2141.7±32.5

Four parameters were selected to investigate their effect on cell viability after encapsulation, (i) PVA viscosity (300.9± 5.8 and 2175.1±9.0 mPa.s, respectively), (ii) Coagulation bath treatment after addition of cells, and addition of (iii) DMSO and (iv) Serum into the encapsulation media. The results were evaluated by Design Expert 7.0 after 3 blocks of experiments of all possible combinations were done in triplicates (288 runs) in random order. From the results, it was possible to derive a model for encapsulation ($p \leq 0.0001$). The viability results showed that there was a

strong dependence on all factors with several interactions between the factors having a significant effect on final cell viability (Equation 4.1).

$$c = 0.76x + 0.77y + 6.88z + 8.09w + 0.023xy + 0.41xz + 0.42xw + 0.20yz + 0.67yw - 4.42zw - 0.39xyz - 0.11xyw + 0.078xzw + 0.1yzw - 0.53xyzw + 19.41 \quad \text{(Equation 4.1)}$$

Where c is cell viability, x is DMSO concentration, y is serum concentration, z is coagulation bath treatment and w is PVA viscosity (For Full ANOVA table see Appendix D). By themselves and with two factor interactions, all parameters had a positive effect on cell viability. However, including all process variables caused a significant lack of fit. By only including the biggest impact factors, the coagulation bath treatment and PVA viscosity, and with a square root transformation, the equation turns to Equation 4.2:

$$\sqrt{c} = 0.054z + 2.66 \times 10^{-3}w - 2.29 \times 10^{-5}zw - 0.80 \quad \text{(Equation 4.2)}$$

This significant effect of coagulation bath treatment and PVA viscosity was mainly due to the difficulty of obtaining cryogels with the low viscosity PVA without coagulation bath treatment (cell viability was undetectable with low viscosity PVA cryogels without coagulation bath). From Equation 4.1, it can be seen that the correlation between high level of coagulation bath and high level of viscosity by itself is negative which indicates that it is not necessary to use the coagulation bath for high viscosity PVA samples (Figure 4.26). For cell-free cryogels, with both viscosities, coagulation bath treatment was necessary to obtain robust samples for

1st cycle cryogels, whereas with this mixture strong gels were obtained even after one cycle. This huge effect of coagulation bath and PVA viscosity on cryogel stability has aliased the effects of DMSO and Serum addition, even though these factors are positive contributors to the cell viability. Moreover, the coagulation bath caused clumping of the cells on the outer layers of the gel, so an additional model was created to see how Serum and DMSO concentration affected the cell viability in the absence of the coagulation bath treatment with the high viscosity cryogels. Since DMSO is also a solvent of PVA it was important to know the structural effect versus the cell viability properties in the presence of increasing DMSO concentrations.

Both DMSO and Serum significantly increased the viability of the samples; and the interaction parameter was also effective (Equation 4.3). Increasing the concentration of the serum and DMSO simultaneously resulted in higher viability ($p \leq 0.0318$). The model input was the result of 39 centred experimental runs, so it was possible to derive quadratic relations between serum and DMSO versus cell viability. However, ANOVA tests demonstrated a linear relationship instead of a quadratic one. The increase in cell viability as the concentration increased was rather steep and it can be said that this increasing trend would continue with higher concentration of Serum. However increasing their concentration would decrease the overall PVA content which would inhibit the gelation process; thus higher concentrations of DMSO and Serum was not further examined (Figure 4.27).

$$c = 0.26xy - 0.17x - 0.16y + 26.44 \quad \text{(Equation 4.3)}$$

Where x is DMSO concentration and y is Serum concentration and c is cell viability.

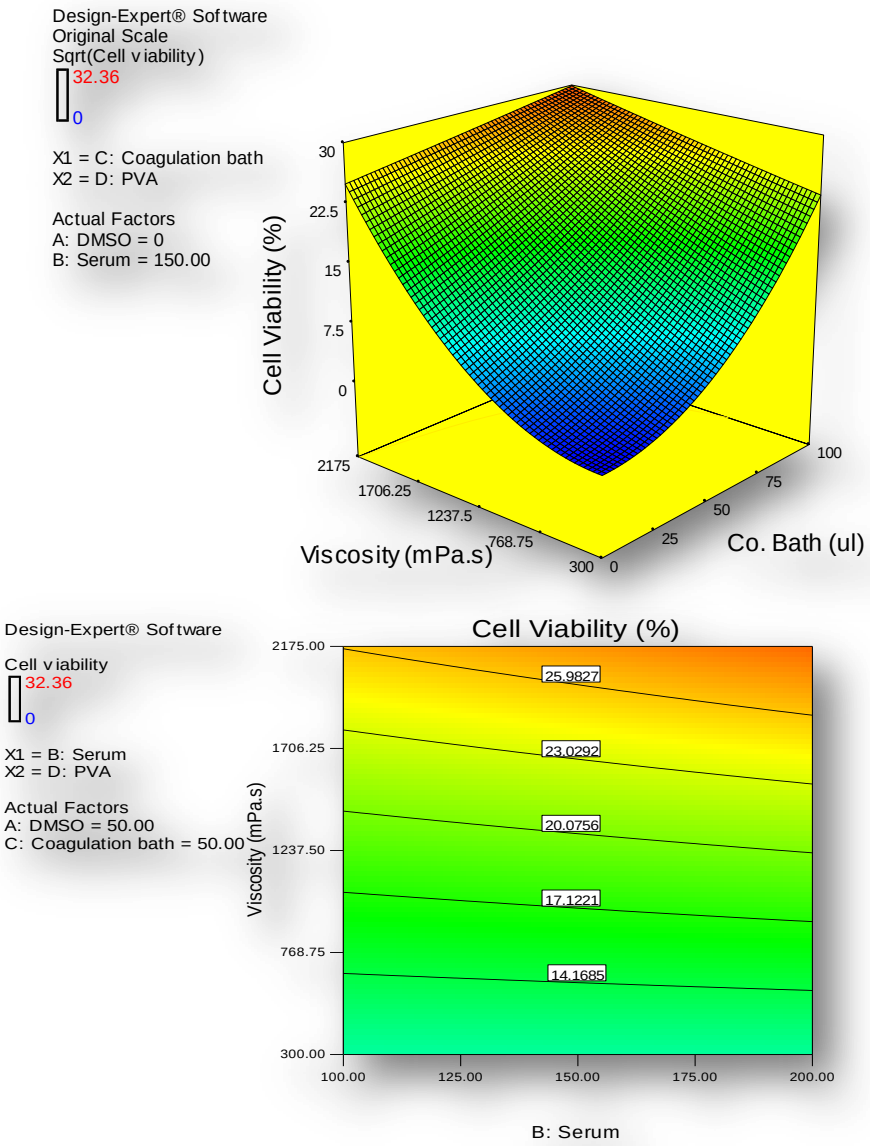

Figure 4.26 Analysis of Design of Experiment results a) Response Surface for Cell viability within PVA/Gelatin cryogels with respect to PVA viscosity and coagulation bath treatment at constant values for Serum and DMSO. In the absence of DMSO, viability dipped to 0 for low viscosity, no coagulation bath conditions. B) Contour graph of relationship between PVA viscosity and Serum concentration, increasing serum concentration in the presence of DMSO and Coagulation bath had little effect, since the behaviour was dominated by viscosity.

These results, together with the previous results suggest that the sole addition of either DMSO or Serum would not be sufficient for cryoprotection in a gel mixture

system and both are necessary to obtain improved viability. When present together they caused a synergistic effect, for no coagulation bath and high viscosity conditions. Since all the constituents added to gelation mixture would cause freezing point depression, the bulk of the gelation would happen during the thawing stage. This was the reason for the dry incubation of the gels at 37 °C prior to the addition of pre-warmed culture media. This interval is important for the development of the mechanical properties of the gels, so the feasible time interval for this waiting period was checked within a 30 minute interval of thawing before the addition of the culture media. Direct addition of the media impeded the gel formation and, in turn, viability; whereas a 10-15 minute thawing period had increased cell viability. However further incubation caused a slight decrease; suggesting that cells are prone to osmotic shock if the swelling of the cryogel was not facilitated (Figure 4.28).

Another aspect of the method requiring consideration was whether it was safe to store cells within the encapsulation mixture under frozen conditions and whether this would affect their viability. Cells were stored at -70°C for up to one month and upon thawing, no significant difference in cell viability was observed between different storage periods (Figure 4.28 inset). The recovery rate has improved up to 80%. Also no significant differences in gelation were observed for these different storage time points, which indicated that the system can be used for storage purposes.

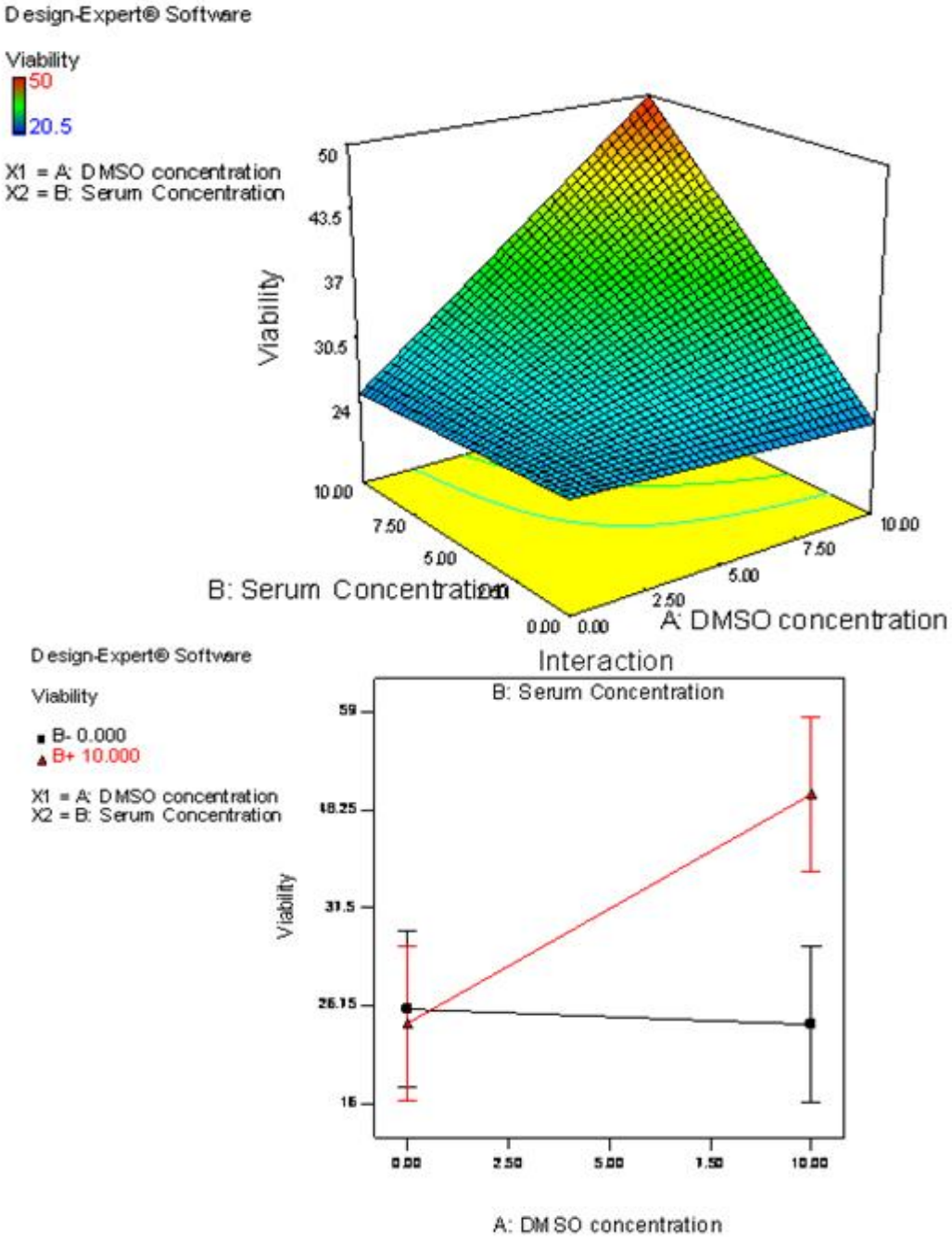

Figure 4.27 Analysis of Box-Behnken viability design a) Response surface for the effect of DMSO and Serum concentration on viability of encapsulated cells. Interaction of Serum and DMSO caused an increase in cell viability b) Interaction curves between DMSO and Serum; in the absence of serum increasing the DMSO concentration caused a slight decrease in viability whereas if DMSO concentration was increased in the presence of 10% serum, there was a significant increase in cell viability.

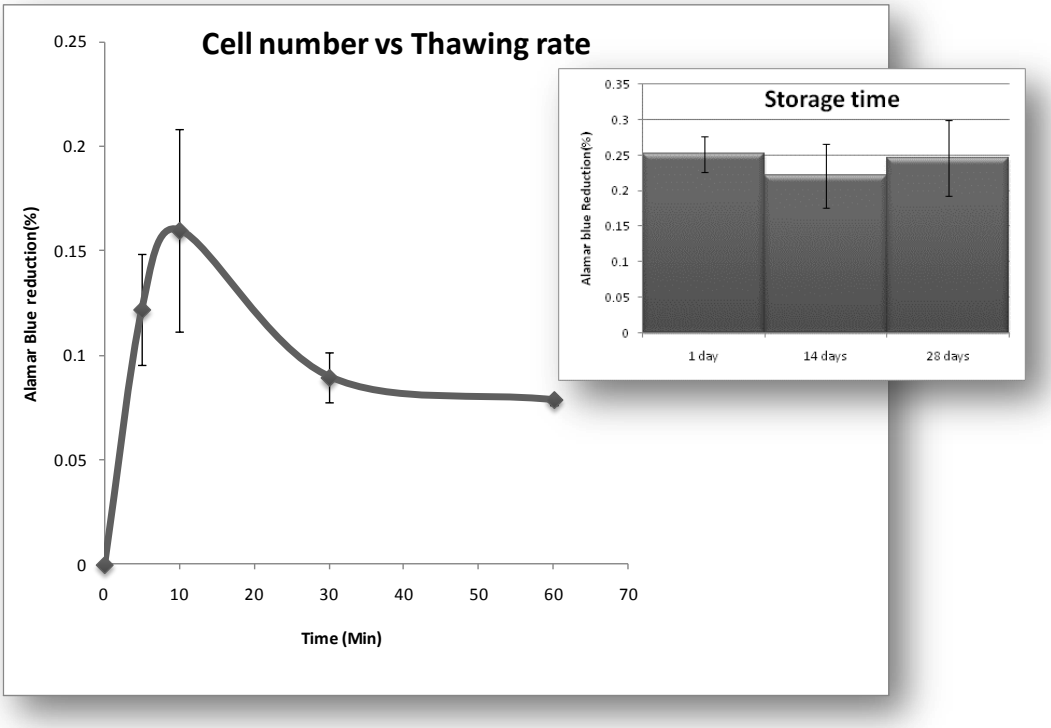

Figure 4.28 Effect of thawing rate on cell viability in encapsulated state. Direct addition of medium disrupted gel formation at time point 0 min, the peak of cell viability for thawing rate was around 10 minutes, the thawing without addition of medium was essential, but after a period of time it induced further cell death .Inset) Effect of storage period in frozen state on cell viability, no significant differences was observed for up to 1 month period (p=0.27).

The presence of the cells, medium and other additives on the gelation mixture cause dramatic morphological changes in the final cryogel structures. These have been observed by optical microscopy and SEM. After initial exchange of medium to ensure the removal of excess DMSO the gels were fragile. However, after 2nd or 3rd change of the medium the same day, the cryogels were much stronger indicating that gelation continued to proceed during culture time, since the time interval was too short for any kind of cell induced change. The final gels were much more

transparent and porous compared to conventional PVA/Gelatin cryogels (Figure 4.29). Cells generally resided in these pore areas, sometimes in clumps.

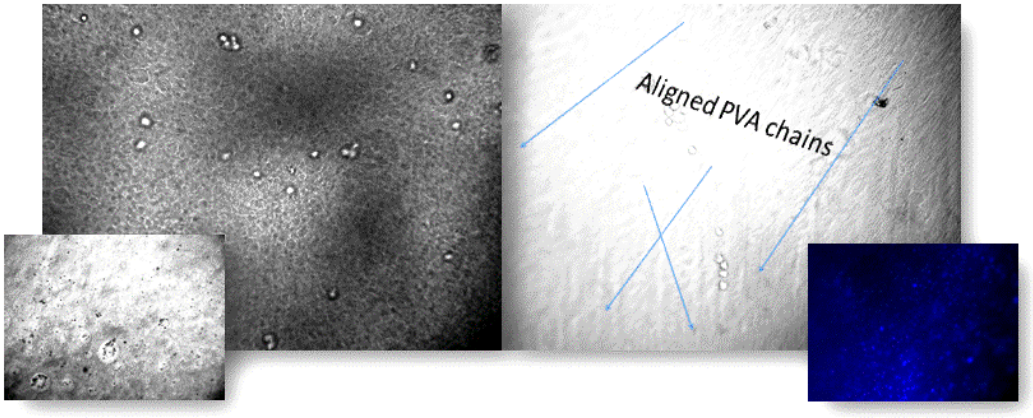

Figure 4.29 a) Surface morphology of cell encapsulated PVA/Gelatin cryogels; the surface was much more porous compared to the empty cryogel's structure in which cells can be observed as spherical bodies initially, Inset) there were even bigger pores in which cells could be observed. b) The criss-cross aligned fibrous orientation of PVA filaments was observed under these conditions too. However, the orientation had many more different directions, Inset) DAPI staining of the encapsulated cells; cells could be observed throughout the depth of the cryogels, either individually or as groups of cells, entrapped within the pore structures as observed in phase-contrast microscopy.

Cryogels with encapsulated cells were distinctly more porous, in that PVA oriented in a spider web-like structure of thick strands with a quite uniform distribution of circular pores (Figure 4.30). Pores are up to 100 µm in diameter, and their size narrowed down at the interior parts of the hydrogels. In SEM observations, the

spread cells or groups of cells were observed within and around these pores and migration of cells into or out of the inner parts of the hydrogel was observed. The spread cells suggested that the encapsulation did not restrict the movement of the cells and encapsulation environment was porous enough to allow nutrient and oxygen transport (Figure 4.31).

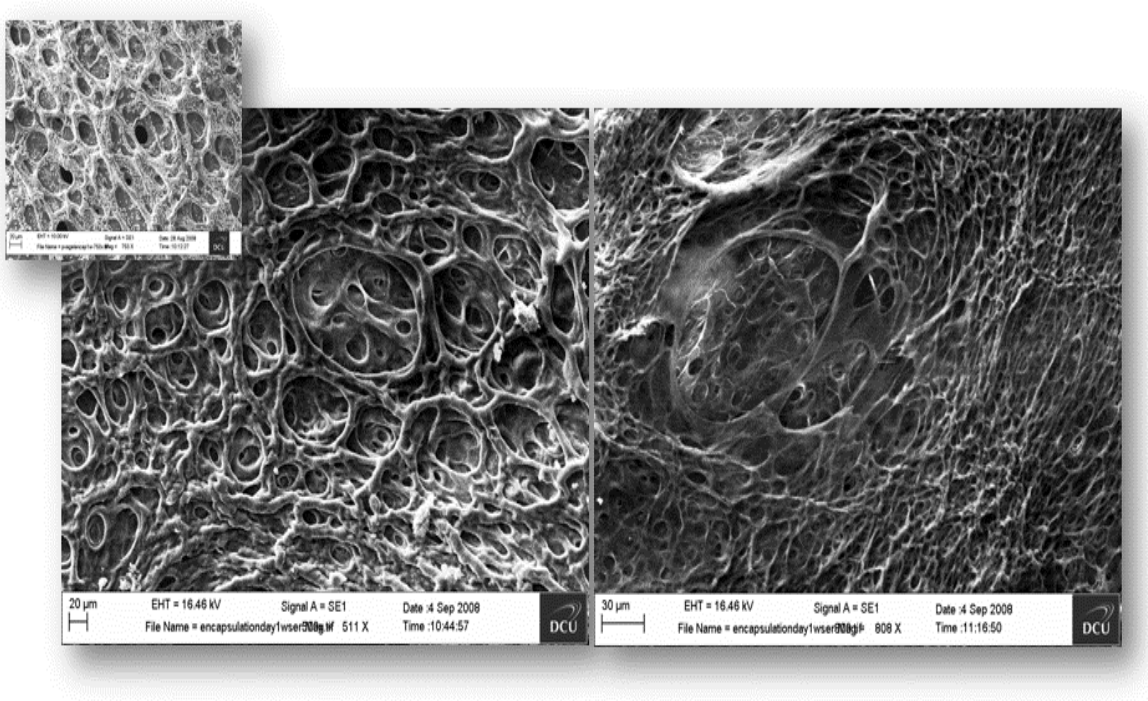

Figure 4.30 The microscopic structure of cell encapsulated PVA/Gelatin cryogels. a) Top b) Bottom side of the same batch of cryogels fixed after 1 day of culture under standard tissue culture conditions. Cryogels were more porous than the conventional cryogels and exhibit interconnected porosity throughout the structure. Pore sizes ranged from 10 to 100µm and pores were circumvented with thick layers of polymer strands

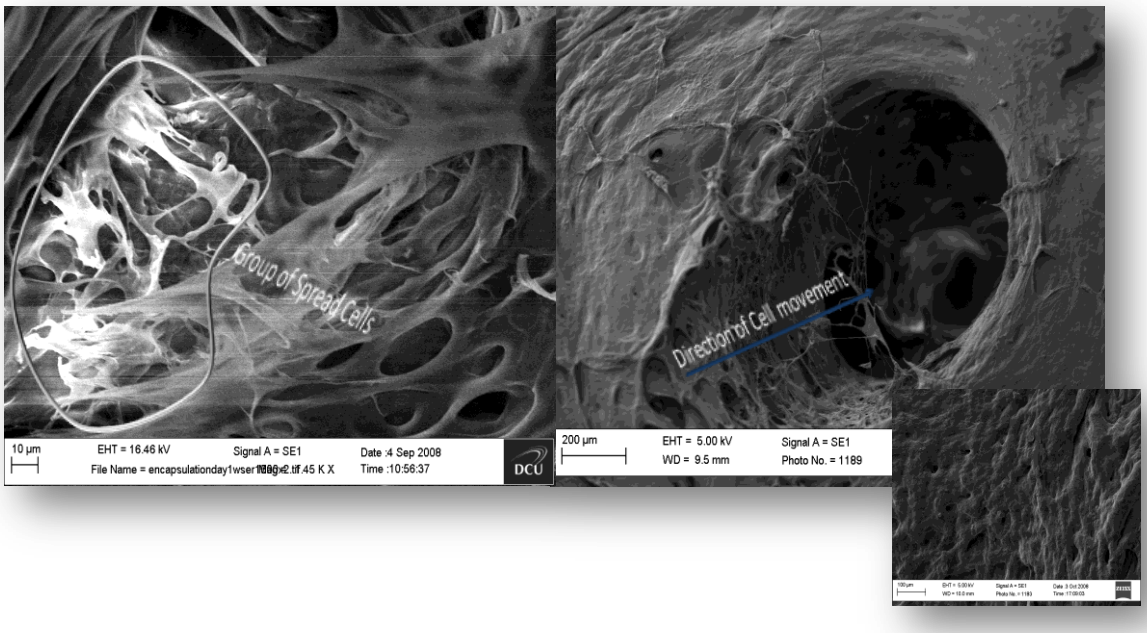

Figure 4.31 Distribution of encapsulated cells within the cryogel structure. a) Spread cells could be seen within the pore structures mostly in groups of 5-10 cells b) The connectivity and the size of the pores enable cell movement in and out of the cryogel structure, with pores going deep into the hydrogel core. Inset) Surface pores were closed up over time (in a week) of culture by cellular activity

In order to have a better idea of cellular distribution and morphology, haemotoxylin-eosin staining was carried out with 4 µm thick sections of hydrogels. As seen in the SEM micrographs, cells generally resided within the bigger pores as interconnected clumps, due to the segregation effect of the polymer chain movement during freezing. Histology results also showed the inferiority of the gels

produced in the absence of DMSO and Serum, in which the cell number was distinctly less, and further validated the previous cell counting results (Figure 4.32). It was possible to locate cells throughout the depth of the cryogels, so stratification of the system had not occurred and the cells were evenly distributed within the scaffolds. To ensure that the cells observed were indeed viable, Live/Dead Cell viability assay tests were performed on unfixed samples immediately after removing them from culture conditions. The Live/Dead assay indicated that most of the cells were viable and metabolically active, as evidenced by their ability to metabolize Calcein-AM to Calcein (Figure 4.33). Only, in some areas Ethidium Homodimer permeable cells were also observed, but the cells were predominantly viable, showing that there was little damage to the cells under the processing conditions.

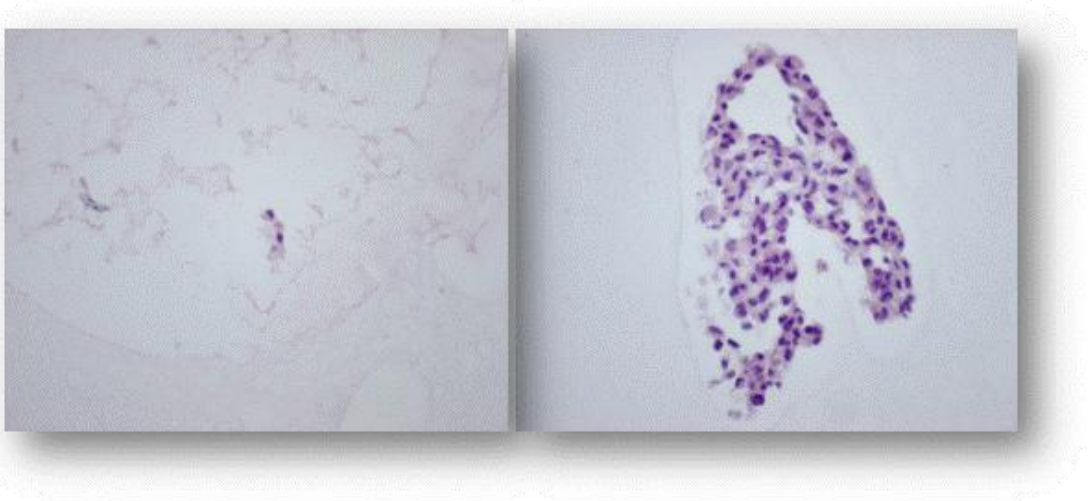

Figure 4.32 Haemotoxylin-Eosin Staining of PVA/Gelatin cryogel cross-sections a) In the absence of DMSO and Serum b) 10% DMSO and 10% Serum. DMSO and Serum presence resulted in higher cell yield. Cells were mainly observed in big pores produced during the freezing and thawing process

In order to see the protective effect of the encapsulation process, confluent monolayers of smooth muscle cells were frozen in a DMSO and Serum supplemented medium. Under these conditions there was a huge cell loss on monolayer culture conditions (Figure 4.34a), indicating that the cryodamage protection resulting from the gel formation. In order to see whether this method can be used with other cell types, three more different cell types were encapsulated using freeze-thawing; namely rat arterial smooth muscle cells, bovine arterial endothelial cells and MC3T3 mouse osteoblast-like cells. Rat SMCs was used to see whether the system could be translated to other species and MC3T3 and endothelial cell experiments were done to show the viability of the process for cells of different size and morphology. All these cells were successfully encapsulated within the cryogel structures with similar macroscopic cryogel appearance (Figure 4.34b-d).

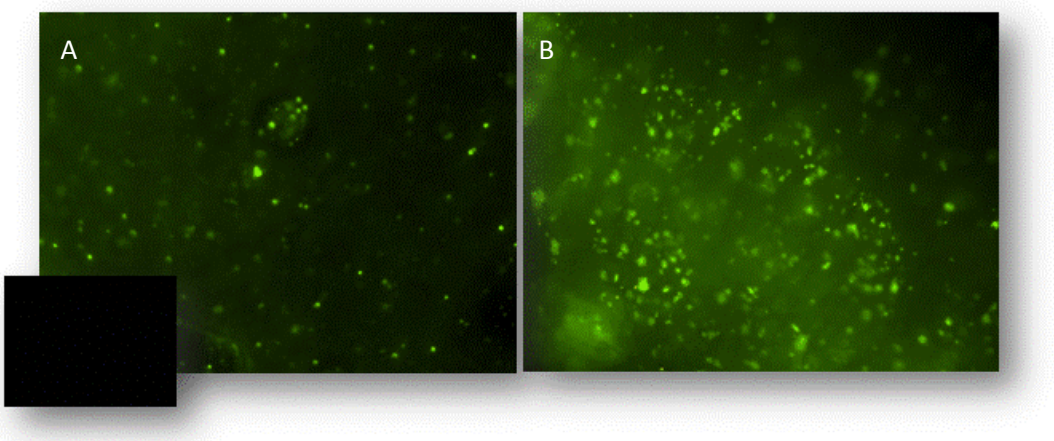

Figure 4.33 Live/Dead Staining of Smooth Muscle cells in PVA/Gelatin cryogels; a)Day 3 b) Day 7. Green dots denote live cells, while areas of red denote necrotic cells. Yellow areas were seen when there are dead cells within the cell clumps. Inset) Negative control staining of empty cryogel; no unspecific staining or cleavage of the Calcein-AM without cell activity was observed.

Steady cell growth was attained with encapsulated cells over a period of two weeks. The cell number nearly tripled in this period, which showed that encapsulation did not hinder cell proliferation. Over the culture period, cryogels became translucent and stiffer, due to both cell activity and ongoing crystallite formation, which ensured the stability of the cryogels. Hydrogels also had a slightly reddish colour at the beginning due to the presence of phenol red in the culture medium. Also after a 3 day culture period, contraction of the cryogels was generally observed which further indicated the activity of smooth muscle cells as they migrate and interact with the cryogel the stress applied by their actions caused gel contraction. Encapsulation of the smooth muscle cells following this encapsulation procedure resulted in distinctly different cryogel morphology. The results presented in this section proved that with necessary modifications, cryogelation can be used for cell encapsulation and storage and the system can be modified for different cell type. The next step was to quantify the change in mechanical properties caused by the new cryogel morphology in the presence and the absence of the cells, and also under cyclic strain and how this would affect cell proliferation and orientation, and potentially the overall cryogel structure. These results will be presented in the next section.

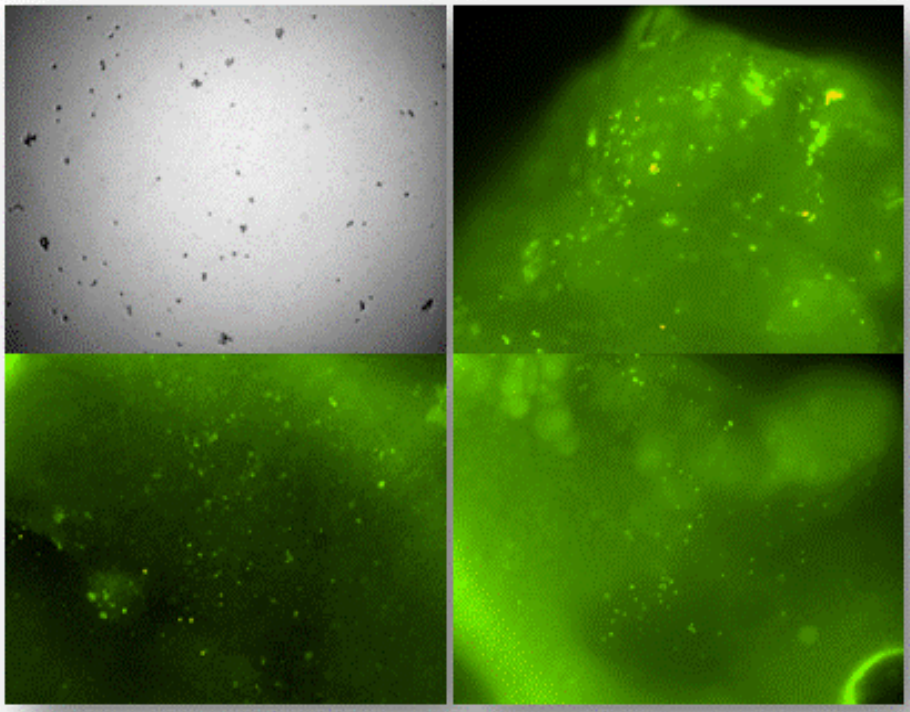

Figure 4.34 Live/dead staining with other cell types a) Remaining cells after a monolayer of smooth muscle cells were exposed to freeze-thawing cycles in the absence of the PVA/Gelatin cryogel; most of the layer has been removed during the process. b-d) Live/Dead viability assay staining of cell encapsulated in PVA/Gelatin cryogels b) Endothelial cells c) Rat Smooth muscle cells d) Mouse MC3T3 osteoblast like cells

4.3.1 Effect of Cyclic Strain on Encapsulated Smooth Muscle Cells

The mechanical properties of the cryogels produced by the encapsulation methodology (10% Serum, 10% DMSO with high viscosity PVA) were evaluated with uniaxial tensile strength test for both cell laden and cell-free conditions. In order to observe the effect of cyclic biaxial strain on the cryogel structure, both cell-laden and empty cryogels were tested after application of 10% cyclic loading for 24 h. Cryogels produced from the cell encapsulation mixture both in the presence and absence of the cells had similar mechanical properties with the conventional PVA/Gelatin cryogels (Table 4.5).

Table 4.5 Ultimate Tensile Strength and Elongation at break values for cryogels with or without cells produced with encapsulation mixture, * denotes p≤0.05 (n=6).

Cryogel Type	*UTS(MPa)*	*L_{max}(%)*
Conventional PVA/Gelatin	0.07±0.02	171.63±22.39
Encapsulation mixture with Cells	0.10±0.04	110.46±22.56
Encapsulation Mixture without Cells	0.08±0.02	190.99±17.60
Encapsulation mixture with Cells after cyclic strain	0.03±0.01*	**314.94±66.74**
Encapsulation Mixture without Cells after cyclic strain	0.12± 0.07	189.12±33.85

The change induced by the gelation mixture with and without cells demonstrated that, both the additive (proteins in the serum, the osmolarity of the media and

DMSO) and cell presence had effects on the cryogel physical properties. Application of the cyclic strain did not cause any damage to the cryogels; on the contrary it caused a slight increase in mechanical properties for the cryogels without cells. On the other hand, the ultimate tensile strength of the cryogels with cells decreased after application of the cyclic strain and the cryogels became extremely extensible. It should be noted that these cryogels did not go through an acclimatization period for the cells, thus the decrease observed could be related to this fact. Application of cyclic strain caused a slight increase in cell proliferation compared to the static counterparts, however by day 7 this effect levelled off and the cell numbers was closer although the difference was still significant (Figure 4.35).

The application of cyclic strain did not affect the viability of the smooth muscle cells, which was observed with Live/Dead viability assay. There was no distinct difference between static versus cyclic strain samples in this regard. Cyclic strain treatment caused orientation of the cells in primary strain directions (Figure 4.36). Groups of cells were observed aligned along the horizontal and vertical axes with a slight distortion of the shape of the pores of the cryogel structure. The cryogel's ability to withstand cyclic strain is a good indicator of their feasibility to be used under pulsatile flow conditions.

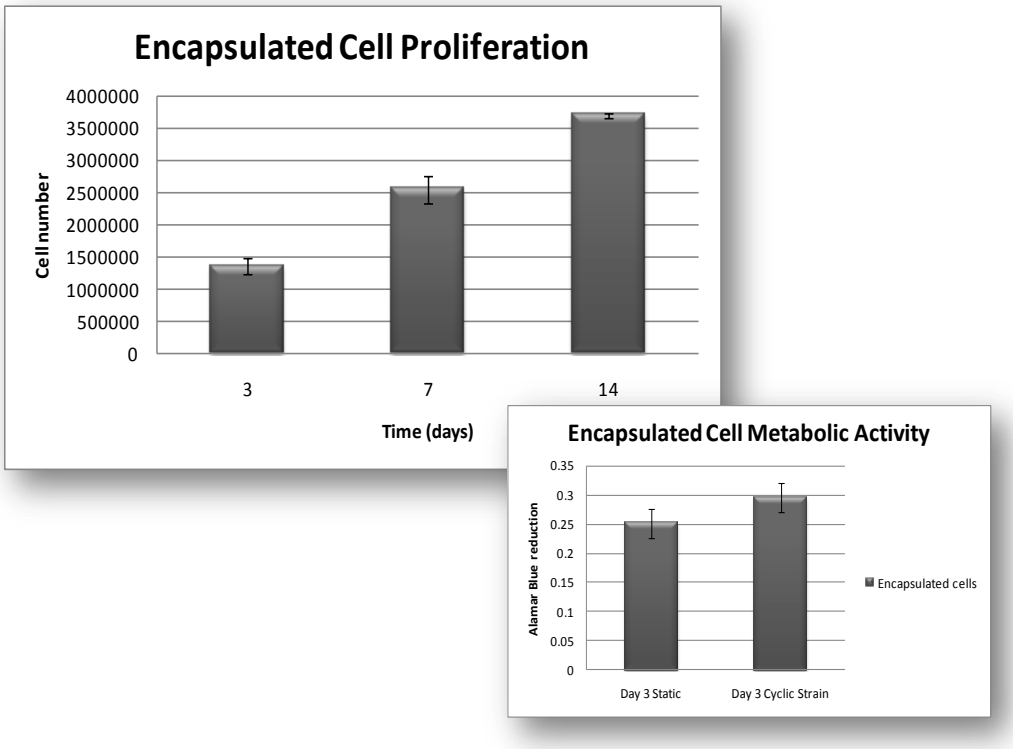

Figure 4.35 Smooth muscle cell proliferation within PVA/Gelatin cryogels measure by Alamar Blue proliferation assay; cells showed steady growth under static conditions ($p \leq 0.001$) and application of cyclic strained caused a slight increase ($p=0.004$); which levelled off by day 7 (34.05± 0.06 % Alamar Blue reduction versus 35.76± 0.02 %.) (n=6).

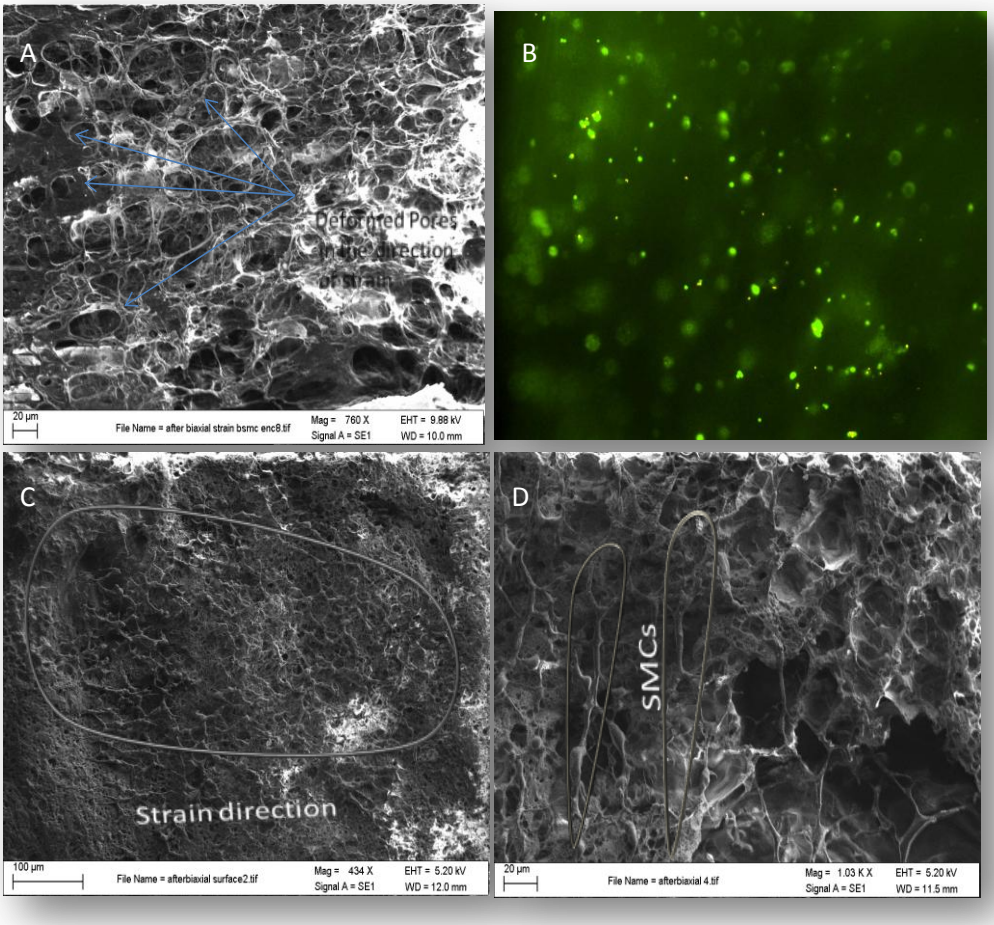

Figure 4.36 Effect of cyclic strain on PVA/Gelatin cryogel morphology and Smooth muscle cells. a) Cyclic strain converted most of the spherical pores into ellipsoidal shape b) Live/Dead viability assay results were quite close to that of static conditions. Orientation of the Smooth muscle cells c) in horizontal d) in vertical direction

4.3.2 EC/SMC Co-Culture Experiments

After establishing the encapsulation procedure and characterization of the endothelial cell response on the cryogels, the next step was to characterize the conditions for the co-culture of endothelial and encapsulated smooth muscle cells. For this end, an experimental design based on 3 factors was conducted and the Nitric Oxide (NO) synthesis level was selected as the response, as it is a good indicator of endothelial health and is an endothelial specific signal which cannot be interfered by the smooth muscle cells, unlike in the case of most of the previous methods used. The factors were the storage time (1-7 days) for smooth muscle cells before seeding endothelial cells, culture time (1-10 days) and application of ramped shear stress (0-10 dyn/cm^2) which corresponded to 54 runs. The level of NO was quantified by Griess Reagent. A statistically significant model for NO synthesis and dependence on these 3 factors at the level of 3 factor interaction was obtained ($p \leq 0.0001$) and the model suggests that both culture time and the application of shear stress had a strong positive effect on NO synthesis and their interaction confounded this effect. The storage time had a slight negative effect on NO synthesis (Figure 4.37). The increase in NO synthesis was steep after 1 day storage, whereas the slope of the line for the 7th day storage was smaller. However, NO synthesis increased steadily, and for shear samples it reached similar or higher levels than that achieved with the empty cryogels (12.56±0.29 μM for 1 day storage and 10 day culture of Smooth muscle cell samples versus the 13.79 ±0.20 μM on empty cryogels, n=6). To ensure the compartmentalization of the endothelial and smooth muscle cells, both cell types were stained with markers; namely endothelial Nitric oxide Synthase (eNOS) for endothelial cells and Smooth muscle α-actin for

Smooth muscle cells. e-NOS staining was restricted to the top surface of the cryogels where endothelial cells were forming small islands, possibly due to the presence of big pores, which were also observed via SEM (Figure 4.38). However expression of the eNOS indicated that they were functional.

It was observed that not all of the Smooth muscle cells within the cryogels were expressing Smooth muscle α-actin, which should be expected due to their proliferative phenotype, evidenced by cell proliferation. However, there were cells within the pore structures that were positive for α-Smooth muscle actin which showed that conversion to contractile phenotype from synthetic phenotype was indeed possible within the cryogel structure (Figure 4.39).

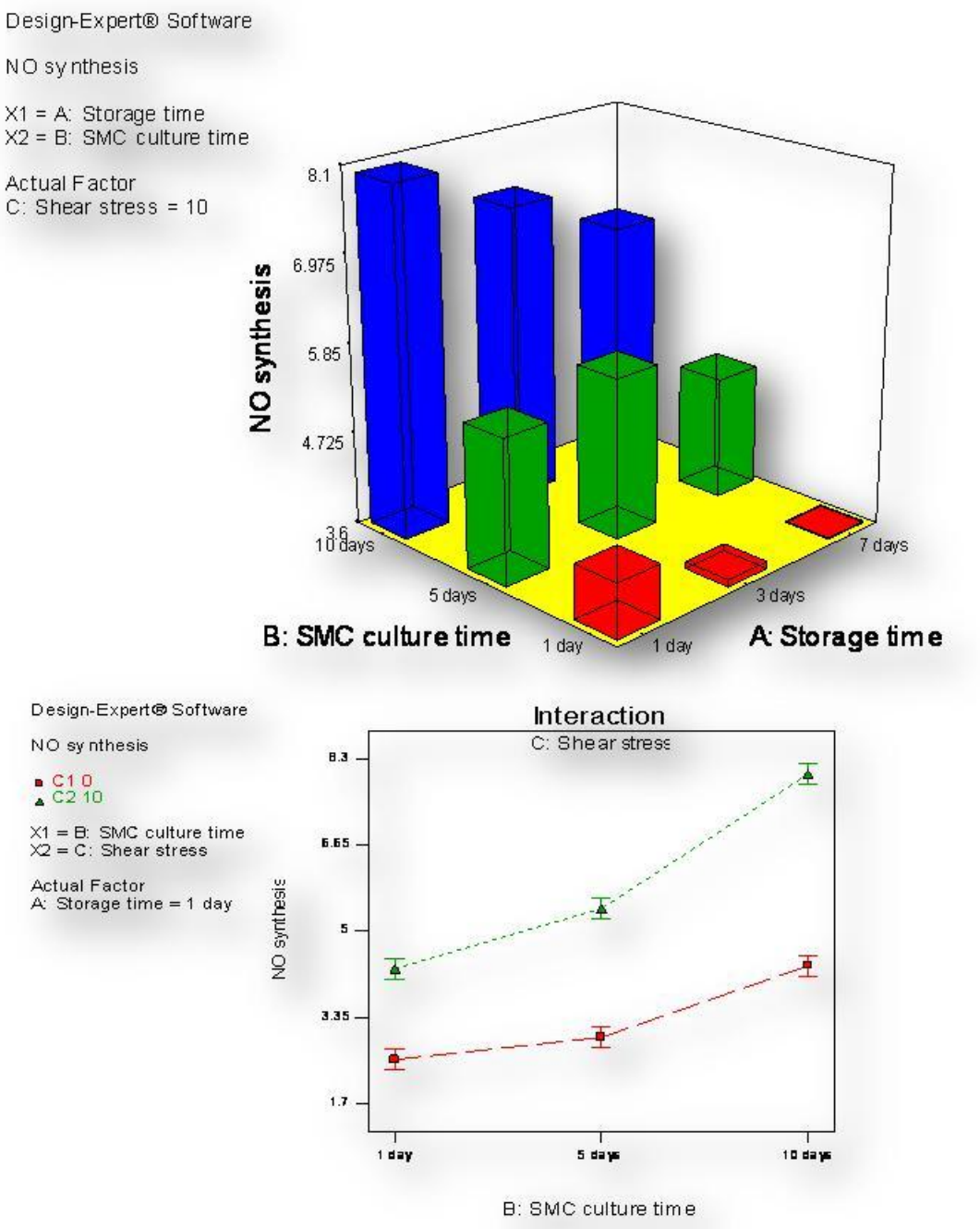

Figure 4.37 Effect of SMC culture time and cryostorage time on NO synthesis by endothelial cells under co-culture conditions. Increasing SMC culture period has a positive effect; while storage time had a negative effect b)Interaction between shear stress application and SMC culture time; longer the culture time higher the effect of shear stress.

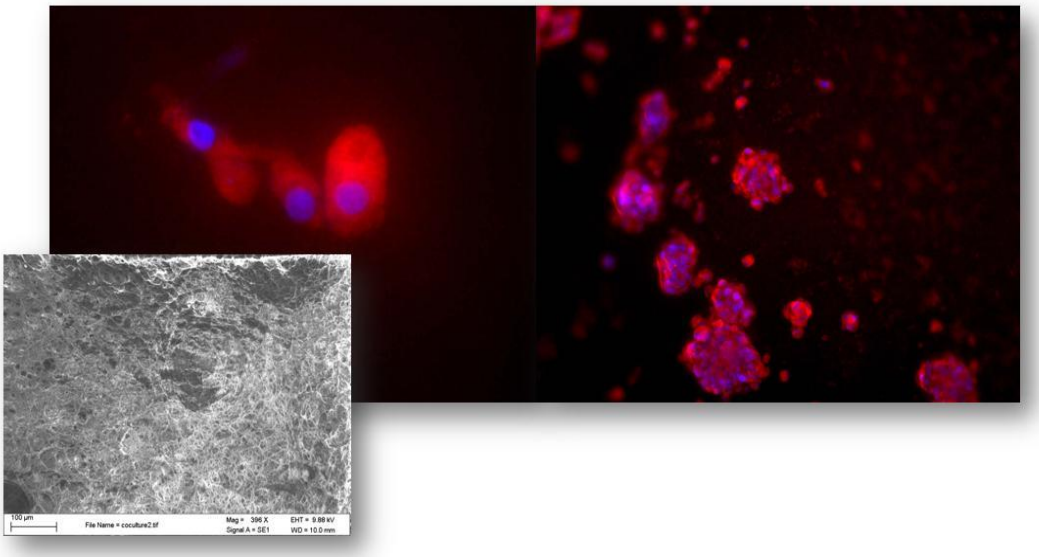

Figure 4.38 eNOS immunostaining of Endothelial cells seeded on to the cryogels after 7 days culture of encapsulated smooth muscle cells, cells were positive for eNOS expression (Magnification 60x) and they were mostly restricted to the surface of the cryogel (b Magnification 20x); similar patterns were also observed with SEM(Inset).

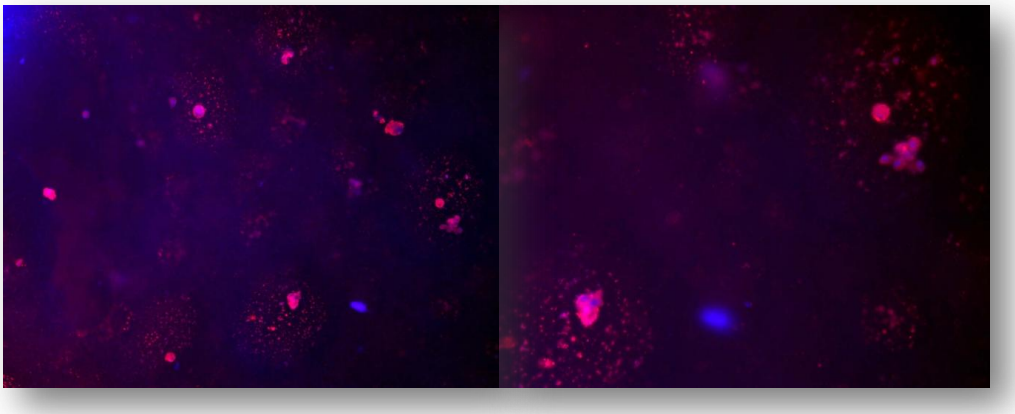

Figure 4.39 Alpha-Smooth Muscle actin expression by encapsulated Smooth Muscle cells under co-culture conditions a) 10x b) 20x magnification; not all of the SMCs were expressing smooth muscle actin and cells were confined within the pores.

4.4 Summary of Results

Briefly, it was possible to develop cryogels with distinctly different physical properties by using three different biomacromolecules as additives and coagulation treatment as a secondary physical crosslinking method, which provided additional strength and resistance to degradation. Amongst the additive used, PVA/Gelatin cryogels were optimal with respect to endothelial cell response and physical properties. It was possible to obtain a healthy endothelial lining with strong cell-cell contact protein expression and diminishing inflammatory response related gene expression. Utilization of a turbulent flow for inducing shear stress was able to greatly facilitate endothelium formation, which indicate that sub- or supra-physiological stress/strain conditions can be used for the benefit of scaffold maturation. Cryogelation can be used as a novel encapsulation and storage medium for different cell types, and the system can be made more cell-friendly by stepwise addition of DMSO and Serum. Newly formed cryogels had similar mechanical properties compared to conventional cryogels and they were able to resist cyclic strain. Finally, it was possible to obtain a co-culture of endothelial cells and smooth muscle cells by using cell encapsulated cryogels; endothelial cell response was dependent again on shear stress but also on the culture time interval for smooth muscle cells and also the storage time in frozen conditions for the cryogels. Interpretation of these results and their possible applications while be discussed in the next chapter.

CHAPTER 5

5 Discussion

The results of this study suggested that different additives can change the morphology and physical properties of cryogels and that coagulation bath treatment caused significant physical effects for all kinds of composites studied. The first aim of the study was to assess the feasibility of using different additives to support cell attachment on PVA based cryogels and determination of the effect of different additives on the physical properties of the cryogels. In the discussion of these results, previous results of composite cryogel formation trials will be compared with the results of the current study. The effect of additives on cryogelation will be explained and the mode of action of coagulation bath treatment and its effects on cryogel properties will be evaluated in light of previous modelling and physical characterization work.

For vascular tissue engineering an endothelial lining of the scaffold surface is essential. To facilitate endothelialisation, the proposition of the study was that the turbulent flow induced shear stress can be used to improve the rate of endothelialisation, which was supported by the results. The probable reasons for this observation and supporting *in-vitro* and *in-vivo* results are presented. Apoptosis, proliferation, cell-cell contact and NO synthesis results on PVA/Gelatin cryogels were compared with the results of other scaffold types.

Another proposal was that PVA cryogelation can be used for formation of scaffolds containing encapsulated cells, such as tunica media substitutes with encapsulated vascular smooth muscle cells. The effect of each additive used, the reasoning behind this and their overall effect on the gel behaviour is discussed with respect to other encapsulation techniques and the properties of cryogels. The results presented before were aligned with the discussions as presented below (Figure 5.1).

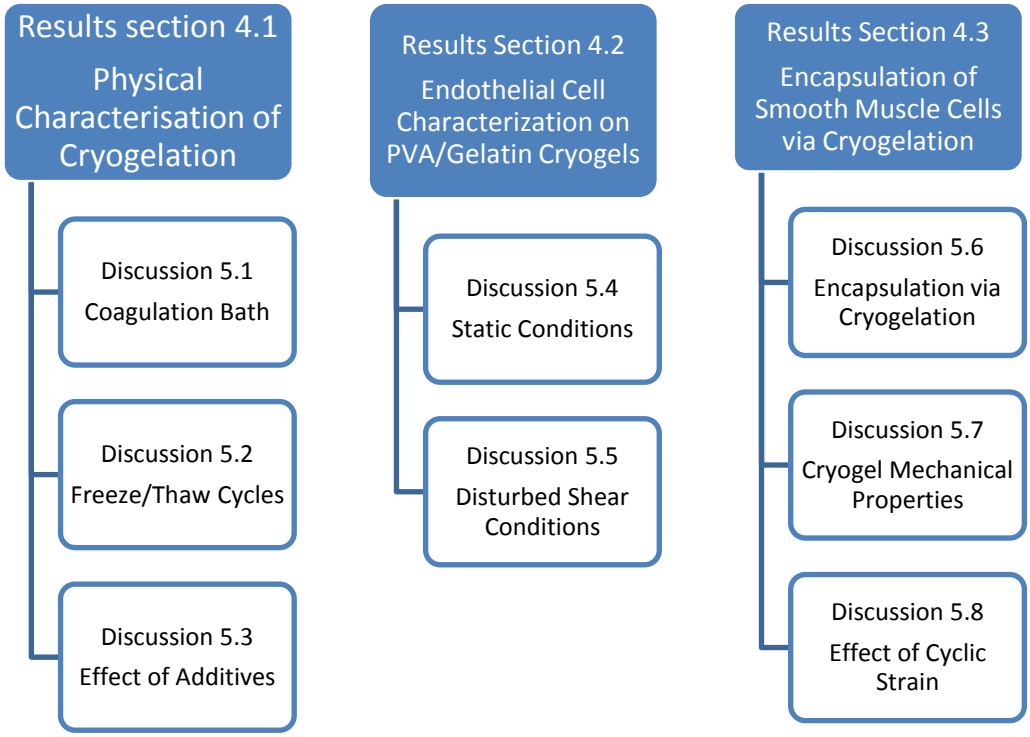

Figure 5.1 The corresponding parts between results and discussion chapters

5.1 Physical Effects of Coagulation Bath

The coagulation bath treatment was effective in decreasing degradation of the cryogels and it also imparted a substantial improvement in mechanical properties. By-products of the process were a significant increase in surface hydrophilicity, changes in cryogel dimensions and a loss of PVA cryostructuration. Gel formation

via crystallite growth by PVA is a useful property that can be exploited to form strong hydrogels. It has been shown that these hydrogels are stronger and more elastic than conventional chemically crosslinked hydrogels or irradiation-produced hydrogels, mainly due to the presence of the crystallites (Katta et al. 2007, Hassan, Peppas 2000a). However, in the cases where composite cryogels are needed, such as for cell culture applications, the presence of the additives necessitates a secondary crosslinking method (Hennink, van Nostrum 2002). Moreover, use of a secondary crosslinking method instead of just increasing the number of freeze-thaw cycles would decrease the production time. Since using chemical crosslinking would negate the biocompatibility advantages of the cryogels, coagulation treatment was selected as the method of secondary crosslinking in this study. Since the non-solvent system used is only an over-saturated basic (measured pH 13.16) water soluble salt solution (saturated Sodium sulfate solution in 1M KOH), there would be no problem of biocompatibility since these components can be easily removed by PBS washes. The reason for using a basic solution was to improve coagulation since all components are acidic molecules (mixture pHs in solution ranged from 5.24 to 6.22). Their solubility is even lower under basic conditions; thus they will be more prone to precipitation. The process can be defined as phase inversion precipitation, which is precipitation of a polymer system by introduction of a miscible non-solvent which causes abrupt precipitation of polymer chains due to the decrease in their solubility (Termonia 1995). This process is even more effective if used on a formed polymer structure such as a hydrogel since precipitation would occur within the architecture presented by the original hydrogel.

The coagulation process depends on the interaction of the polymer system with the non-solvent solution. As a non-solvent solution permeates through the hydrogel it causes local precipitates that result in strengthening and increasing the degradation resistance of the hydrogels. The form and the rate of local precipitation depend on coagulation bath temperature, concentration and rate of stirring. The overall effect of coagulation is also time dependent (Zhou, Powell 2006). The mode of action can be like extending fingers within the gel system or can form immediate precipitates, such as a skin layer, on the surface (Figure 5.2).

Since the coagulates are clumps of two constituents of the composite, an increase in the roughness of the surface after coagulation treatment is a predictable result. An increase in surface hydrophilicity can be explained by the increased PVA concentration on the surface, since it is the more hydrophilic constituent. The coagulation process can happen in two modes, either by delayed demixing or instantaneous demixing. Delayed demixing occurs when the solvent/non-solvent couple miscibility is low and it causes thick skin layers (Termonia 1995).

However, in this system the non-solvent is only a saturated aqueous solution, so the demixing was instantaneous and the result was a thin skin layer, which was observed in SEM images as the less porous surface. However, this did not affect the cell behaviour negatively, but prolonged the initial attachment period. Cryogels formed by freeze thawing have three separate phases, crystalline polymer rich regions, water rich (polymer poor) regions and amorphous polymer rich regions (Lozinsky 2002). The coagulation bath treatment increases the polymer ratio, and while doing so it decreases the crystallinity of the system while at the same time

improving the mechanical properties due to the shrinkage of the system and the associated increase in density. All these precipitation processes cause a decrease in porosity, as there is a decrease in water content. Crystallite parts of the gel act as junction points and are important to the elastic behaviour of the gel as the stretched amorphous regions recoil with respect to the network backbone provided by these crystallites (Mangiapia et al. 2007). In this regard, cryogels are shape memory systems that improve their usefulness under cyclic loading conditions, such as in the case of arterial pulsatile flow, since they are highly elastic.

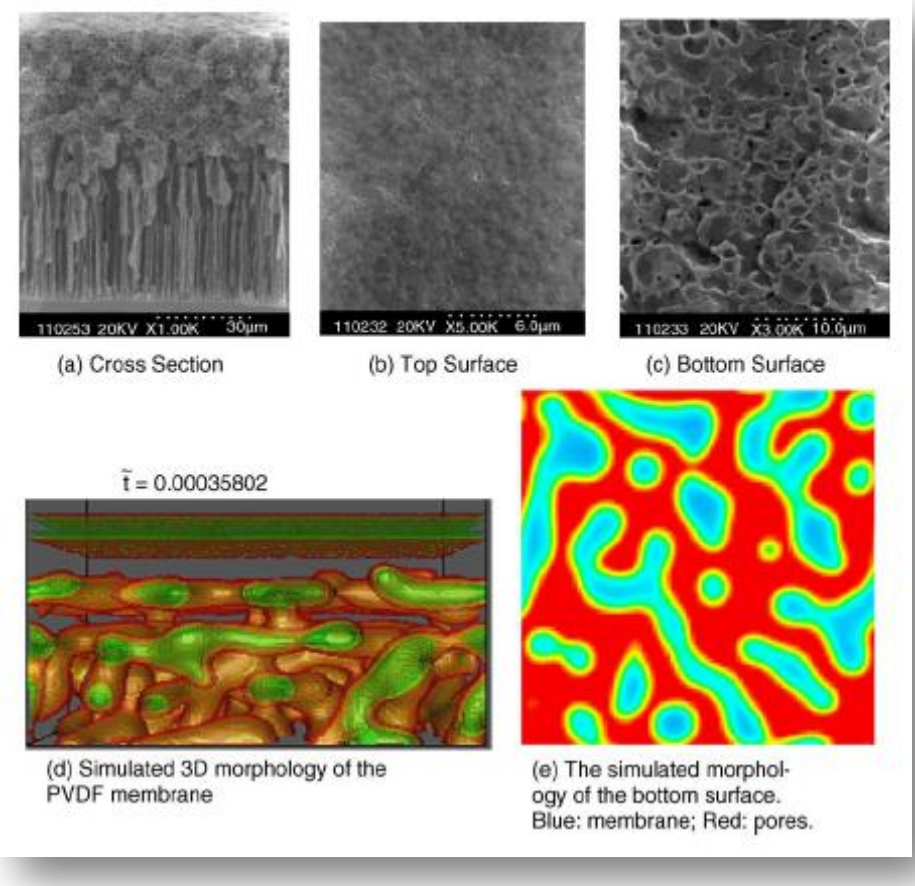

Figure 5.2 Effect of coagulation bath on a hydrogel system; the nonsolvent coagulation solution either produces long columns or finger like extensions during its penetration inside the hydrogel; this results in precipitation and strengthening of the hydrogel structure (Zhou 2006)

Degradation of hydrogels is the result of three different forces acting, depending on the constituents; (i) dissolution, (ii) hydrolysis of polymer chains and/or (iii) enzymatic cleavage (Brandl, Sommer & Goepferich 2007). Obviously, there are no enzymes that can cleave some synthetic polymers and for some polymers, hydrolysis is very limited or not at all possible. Even though there are microbial enzymes that can fully degrade PVA (Solaro, Corti & Chiellini 2000), PVA is not biodegradable in the human body in the sense that it is not hydrolytically cleaved and there are no active enzymes present that can degrade it (Wang 2007). Thus any degradation is entirely due to physical erosion of the cryogel structure and dissolution. Crystallization during the freeze-thawing process is very much dependent on the processing conditions such as freezing rate, thawing rate, final freezing and thawing temperatures, and duration of freezing and thawing (especially thawing for PVA cryogelation), and the properties of the initial PVA solution such as PVA concentration, PVA tacticity, presence of additives etc . (Willcox et al. 1999). It is plausible that just one freeze-thaw cycle might not be enough for full gelation of the structure. In fact, even after several freeze-thaw cycles the system may still be in a sol-gel state. In this sense, another degradation process is the removal or leaching out of the solution of part of the final sol-gel system after a freeze-thawing cycle, instead of dissolution of already formed gel parts. It has been also reported (Nugent et al. 2005a) that in the long term, crystallite dissolution can also be observed. However this was generally compensated by secondary crystallite formation in the swollen hydrogels so its

effect should be a long-term one. This sol-gel ratio should decrease with successive freeze-thaw cycles due to higher crystallization degree. There is an upper limit to the degree of crystallization that can be achieved with a given concentration of PVA which also defines degradation behaviour. Application of the coagulation bath is effective in that it protects crystallite regions by precipitating the sol part of the structure around it. Even though it disrupts crystallites and subsequently increases the amorphous regions, it causes a distinct increase in mechanical properties. A solubility based degradation is beneficial in that there are no by-products of degradation, which are known to have adverse effects in some cases, basically through triggering chronic inflammation (Williams, 2009).

Other important parameters that affect the degradation of the hydrogels are the degree of crosslinking and the water content of the hydrogel. A decrease in water content can cause an increase in the resistance of hydrogels to degradation, which was the case for the hydrogels in this study after application of the coagulation bath treatment. This is especially important for physically crosslinked hydrogels which are more prone to dissolution based degradation due to the temporary nature of the crosslinks that hold the structure together (Van Tomme et al. 2006). The degradation observed in the present study was mainly due to erosion and also the sol-gel separation within the hydrogel. The presence of the additives affected the gelation process and caused different degradation profiles. Degradation occurred through water uptake and subsequent dissolution of the polymeric components. Similar dissolution results obtained in this study were also obtained with pure PVA cryogels (Hassan, Peppas 2000a). In a sense, coagulation

stabilizes the PVA chains that haven't contributed to crystallite formation and would possibly have been dissolved in the absence of coagulation. Increased numbers of freeze-thaw cycles for pure PVA cryogels lead to an increased number of crystallites and less sol part for dissolution. However, the presence of the additives caused a phase separation that led to an additive-rich region that was more prone to degradation. This might explain the higher degradation results for 3rd cycle cryogels obtained in this study. The mere presence of the additives had a significant effect on the degradation profiles of the PVA cryogels. The highest degradation rate observed was for PVA/Starch cryogels, which was mainly due to the higher solubility of starch in aqueous solutions. However, after coagulation bath treatment there was no significant difference in degradation profiles of the three composites, which suggests that the coagulation bath treatment successfully precipitated biomacromolecules within the system. The results with pure PVA cryogels also showed that the coagulation bath treatment was even more effective on PVA compared to the composite formulations (3.59±0.99 % weight loss for pure PVA after 2 weeks compared to 9.20±0.48 % for PVA/Gelatin). Overall, stable cryogels which resist degradation for long-times were obtained by the two step crosslinking system used in the study.

5.2 Effect ofNumber of Freeze-Thaw cycles (PVA/Chitosan)

The effect of thermal cycles was most prominently visible in the mechanical properties of cryogels where crystal formation is thought to be the main trigger. The gels become stiffer and stiffer as the number of freeze-thaw cycles increases. The main determinant is the prolonged thawing duration, as all the previous

experiments showed that it promotes more crystallite formation. The freezing duration is less effective in conferring stiffness, compared to the effect of thawing rate (Wan et al. 2002). The mechanical change with respect to the increasing freeze-thaw cycle number is a versatile way to fit the properties of the cryogel with the target tissue (Figure 5.3). Mechanical property related changes also affected the cellular activity properties. Smooth muscle cell attachment and proliferation was slightly higher on 3rd cycle hydrogels and surface coating with collagen type I also caused an improvement. This improvement can be related to two effects observed in 3rd cycle cryogels. The first is the improved protein adsorption compared to first cycle cryogels; most probably due to increased surface roughness, which would affect the adsorption of both serum proteins and also collagen. The second effect is the increased substrate stiffness. A positive effect of increased substrate stiffness has been reported for other polymeric systems too (Tan, Teoh 2007, Balgude et al. 2001) and it has been proposed that a stiffer substrate promotes better cell adhesion and subsequently spreading (Engler et al. 2004). The stiffening effect of freeze-thaw cycles could contribute in the same way to provide cells with a stiffer substrate. This effect was observed both for endothelial and smooth muscle cells and indicated an underlying physical difference related change. In order to permanently fix the collagen onto the PVA based composite surface glutaraldehyde was used as a crosslinker, as it can interact with both PVA and Chitosan. However this treatment did not cause a significant improvement and because of a general concern about toxicity it was abandoned. In a high water content hydrogel system glutaraldehyde would be hard to remove completely. The poorer attachment by smooth muscle cells was overcome by adsorption of fibronectin to the surface as

initial trials suggested. However since fibronectin is not a part of the basement membrane ECM (Dejana, Corada & Lampugnani 1995), this was not used for endothelial cells (Figure 5.4).

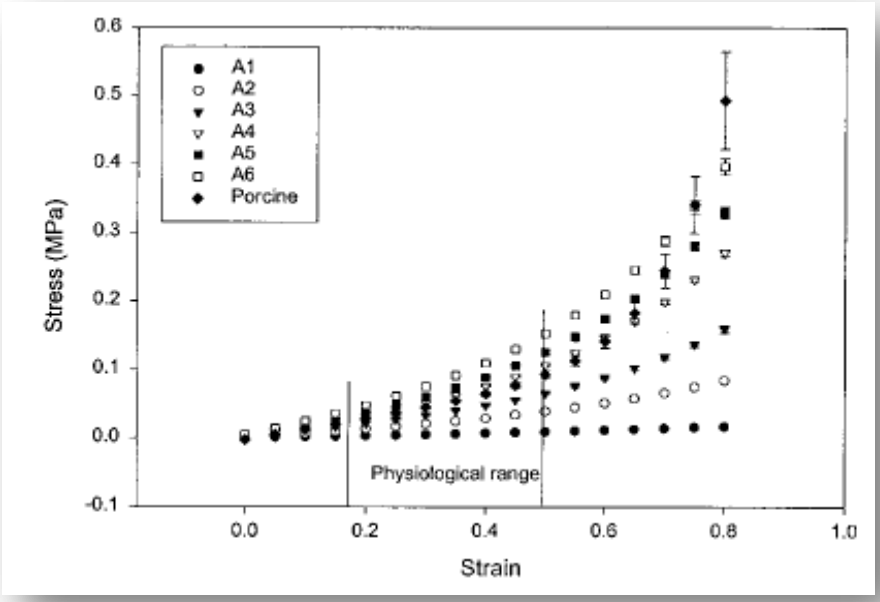

Figure 5.3 Stress/strain curve of 15% PVA cryogels after 1-6 freeze-thaw cycles and its comparison with the behaviour of porcine aortic root. After 4 cycle cryogel behaviour was nearly identical to that of aortic root, especially within the physiological strain of 0.2-0.5(Wan 2002)

5.3 Effect of Additives on Cryogelation and Cell Behaviour

Physical hydrogel formation is a process where non-covalent bonds are prevalent, such as the hydrogen bonds that are formed between the pendant groups of PVA. All the additives used in this study also have the ability to form hydrogen bonds, albeit without the same propensity for crystallisation. Moreover, the efficacy of blending with the PVA chains determines the rate of interference with crystallization and freezing point depression by these biomacromolecules. In the

freezing process hydrogen bonds may also form between PVA and the additives and can provide an additional strength to the final cryogel structure to a certain degree. Such a relationship was previously observed with cryogels produced from PVA and Egg Albumin (Bajpai, Saini 2006) In the same study BSA adsorption for 2:1 w: w ratio hydrogels was reported as 57.8% which was comparable to the results obtained inthe current study.

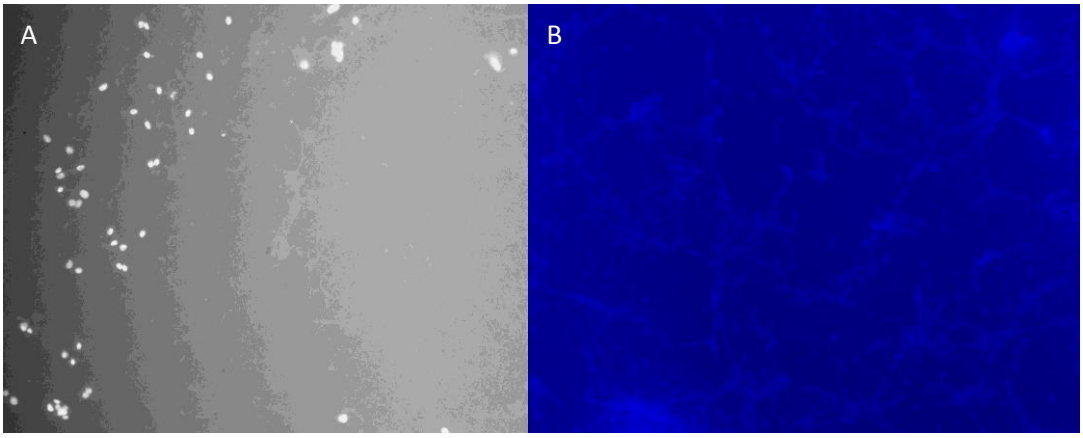

Figure 5.4 Improvement of Smooth Muscle Cell attachment to PVA/Chitosan cryogels after adsorption of fibronectin a)before b) after.

Collagen type I is a protein which is the main constituent of connective tissue, whereas bovine albumin is a serum protein responsible for transport of lipid molecules in blood (Allen 2006); physical differences between the protein molecules together with their different affinity to the surface and different packing led to the difference in adsorption amount. Bovine Serum albumin is a globular protein whereas collagen type I is a fibrous protein. Thus on a surface, due to the restrictions imposed by their shape, packing of bovine serum albumin is much easier compared to the collagen and it would decrease the competition for the

available surface thus increase protein adsorption. The difference in their size would also have an effect. The inability of PVA to effectively absorb proteins was previously demonstrated (Nuttelman et al. 2001), so the ability of the composites to absorb proteins can be attributed to the additive presence.

The presence of the additives also changes the freezing behaviour of the solution and thus would indirectly affect the crystallization of PVA. This might be responsible for the clustering effect observed with the composite hydrogels, especially with PVA/Starch hydrogels, probably due to the highly branched nature of starch as a macromolecule. Several different additives have been studied for their effect on gelation properties and also on the coagulation bath process. For example, PVA/Chitosan blends result in significantly more porous membranes after going through the same coagulation procedure (Chuang et al. 1999). In the same study, from DSC (Differential Scanning Calorimetry) results it was noted that blending caused a decrease in PVA crystallinity too and that there was a phase separation between the components. The phase separation between the composite components necessitated the use of coagulation bath treatment, since the interaction between the components was loose and the additives could easily leach out. Also, ESCA (Electron Spectroscopy for Chemical Analysis) studies in Chuang's study found Nitrogen enrichment of the surface, which showed that the coagulation did not result in total domination of the surface layer with PVA. In another study, two different additives (chitosan and dextran) were compared with respect to their effect on cryogel crystallization, morphology and mechanical properties and it was shown that dextran was better in promoting PVA

crystallization (Cascone et al. 1999). Also different additives cause different degradation rates, similar to the results presented in this study.

The differences in degradation rates before and after coagulation bath treatment can be attributed to the differences of response of each additive to thermal cycles and coagulation. The most responsive biomacromolecule amongst the ones utilized in thermocycling was gelatin (Hellio, Djabourov 2006). As a denatured form of the triple helix structure of collagen, gelatin has the ability to form a assembly of coils below its melting temperature, and, particularly, it forms high water content gels at 30-35 °C (Guo et al. 2003). Since the freezing part of the freeze-thawing cycle is well below the melting temperature, the gelatin part can form a gel on the template of PVA cryostructures which might be partially responsible for it higher strength. PVA/Gelatin cryogels had higher cell proliferation rates, better protein adsorption capacity, a similar degradation rate with the other composites and very good mechanical properties, which justified their selection for further endothelialisation and cell encapsulation studies. However, both chitosan and starch based composites were also able to maintain cell proliferation and can also be used for other target tissues. For example, it has been observed that PVA/Chitosan cryogels were less prone to contamination due to the antimicrobial properties of chitosan (Shi et al. 2006) and the PVA/Starch cryogels formed after one freeze-thaw cycle were significantly stronger than the others.

5.4 Endothelial Cell characteristics on PVA/Gelatin Cryogels

An important aspect of vascular tissue engineering is the investigation of the properties of the endothelial lining. Since the endothelium is not only a structural

part of the vasculature but also has an active role in thrombus formation, inflammation and regulation of smooth muscle cell behaviour, changes in endothelial cells in the presence of biomaterials should be monitored. Endothelial cells, like smooth muscle cells, exist in two extremes of phenotype, quiescent and activated. In quiescent phenotype endothelial cells mainly maintain the tone of the vessel wall, quiescence of smooth muscle cells and steady flow of the blood (Boura et al. 2005). One of the main agents in these functions is NO secretion, which is important for vascular tone and also prevention of platelet adhesion and aggregation. Aside from several secreted factors such as prostocyclin, von Willebrand factor and factor H, several membrane-bound proteins like thrombomodulin, thromboplastin and CD46, cell-cell contact molecules are also tightly regulated in the response of endothelial cells (McGuigan, Sefton 2007). In the present study, the reason for selection of NO and 3 cell-cell contact molecules was the dual nature of their expression and secretion. Their levels are also strongly correlated with the activated or quiescent endothelial phenotype. The level of confluence is effective in determination of thrombogenicity and non-thrombogenicity of the endothelium, where a confluent layer is less thrombogenic. Thus it is worthwhile to monitor whether there is a decrease in pro-thrombogenic factors as the lining formation happens or not. The reason for starting with lower cell densities was that observation of the endothelium formation can be performed for a longer period. The main difference between *in-vivo* conditions and a biomaterial surface is the composition of the underlying substrate which is the basement membrane in arteries, whereas it is a randomly adsorbed layer of serum

proteins on a biomaterial surface, which does not contain any component of the basement membrane.

The first aim was to quantify the proliferation rate on the PVA/Gelatin cryogels compared to TCPS. Tissue culture polystyrene is a stiffer surface with surface plasma treatment that ensures protein adsorption. However, mechanically it is quite different from the natural basement membrane of endothelial cells. The compliant nature of the cryogels is much closer to that of the arteries, but their compliance and relative hydrophilicity caused a decrease in the initial attachment compared to the TCPS. For day 5, the proliferation rates were closer to each other and by day 10 there was no significant difference. Similar lagging of growth rates with respect to TCPS was also observed with other biomaterials (He et al. 2005).

Moreover, the specific interaction between cells and the biomaterial base and its effect on cell behaviour is also an important factor for the final outcome (Coyle et al. 2007). This juncture is critical for PVA based cryogels due to the inherent problem with cell adherence to PVA because of its hydrophilicity. As a composite, PVA/Gelatin contains gelatin on the surface that acts as the first layer of protein adsorption and protein adsorbed from serum will determine the strength of endothelial cell attachment. PVA/Gelatin hydrogels exhibited a good capacity to absorb serum proteins. Previously, high levels of apoptotic cells have been reported for pure gelatine coatings of polystyrene surfaces (Prasad Chennazhy, Krishnan 2005). This might be purely due to the poor strength of coating which can lead to removal during culture which, in turn, would trigger an apoptotic response due to the changes in cell attachment mode.

In a hydrogel surface, gelatin is embedded within the hydrogel architecture and shows low removal rates following coagulation bath immersion. This could explain why a similar dramatic increase in apoptosis was not observed in this study. The problem with the PVA/Gelatin cryogels was that the initial attachment was less effective compared to TCPS surface, since a higher level of necrotic and apoptotic cells in the initial period of culture was observed. This was another reason for the lag in the proliferation rate.

Apoptosis of endothelial cells is a prominent outcome of vascular pathology and apoptotic cells are procoagulant signals as anti-coagulant surface markers are lost systematically during the apoptotic pathway (Irani 2000). Nearly all cardiovascular diseases are accompanied by widespread endothelial apoptosis (Stefanec 2000). This can be triggered by ROS (reactive oxygen species, biochemical signals such as Tumor necrosis factor (TNF), elevated low-density lipoprotein (LDL) concentration, changes in physical environment (hypothermia, hypoxia) and disturbances in blood flow (Stefanec 2000). On the other hand under normal resting conditions *in-vivo* only 0.1% of endothelial cells show apoptotic pro-properties (Davies 2009), where some of the prominent anti-apoptotic factors are NO presence, shear stress and VEGF. So, determination of whether a biomaterial surface can sustain endothelium without triggering apoptosis is important.

Necrosis and apoptosis are two distinctly different modes of cell death. Necrosis is generally induced by toxic or traumatic events, which leads to organelle damage, uncontrolled swelling and rupture of the cells, whereas apoptosis is much more controlled; which minimizes the release of cellular contents and can be

characterized by cell shrinkage, chromosome condensation and fragmentation (Bonfoco et al. 1995). In the current study, there was a significant group of necrotic cells 2 days after seeding onto the PVA/Gelatin cryogels, with a smaller population of apoptotic cells present. A similar trend was observed on TCPS too, although to a lesser extent. This initial necrotic population can be traced to the cells which were unable to attach or have attached only loosely to the surface initially, which can trigger both apoptotic and necrotic pathways. Loose attachment and subsequent spherical shape was shown to induce apoptotic response in endothelial cells previously (Chen et al. 1997). However, in the present study after 5 days and removal of unattached cells, the remaining cells were healthy and the percentage of both apoptotic and necrotic cells diminished as the endothelium was formed. Even up to 20 days, the lining was intact. Thus it can be said that PVA/Gelatin cryogel surface was able to sustain long-term endothelial lining without inducing cellular death, unlike the tendency observed in some other biomaterials (Coyle et al. 2007, Serrano et al. 2006)

When subconfluent, endothelial cells act in a similar way as fibroblasts and migrate and proliferate aggressively (Kudo 2005). However, as confluency is reached, due to contact inhibition and organization of cell-cell contacts, a single well-organized lining is formed. Thus, for endothelial cells homotypic and heterotypic cell contacts and extremely organized cell junctions are crucial for their proper functioning (Figure 5.5). PECAM is a good indicator of endothelial cell junction presence or formation and it is an integral part of the endothelial diffusion barrier function, since it is a cell type specific homophilic adhesion protein (Dejana, Corada &

Lampugnani 1995). It is also implicated as taking a part in control of proliferation and apoptosis, by inhibiting cell division as a part of contact inhibition and suppressing apoptotic pathways, and also having a potential role in mechanotransduction together with VE-cadherins (Dejana 2004). So under static conditions the ideal profile would be a constant increase in PECAM expression over the culture period, which was observed by the PVA/Gelatin cryogels over 10 days. PECAM expression level was only slightly higher for PVA/Gelatin cryogels for the first 5 days, but by day 10 there was an 8 fold increase compared to TCPS, which might indicate that due to the differences in the initial attachment to the surfaces, there may be a higher tendency for PVA/Gelatin cryogels to form cell to cell contacts.

ICAM is a surface protein mainly related to endothelium-leukocyte interactions and during inflammation its expression increases significantly (van der Zjipp 2003). However, even quiescent endothelial cells constitutively express it, and thus it is a good marker of constitutive expression during endothelium formation. On PVA/Gelatin cryogels, the ICAM expression stayed steady and comparable to that of TCPS, consistent with the expectations. This meant that, as the cell proliferation progressed and more cells came into contact with each other, they tended to form cell junctions and a concomitant several fold increase of ICAM expression associated with inflammation did not materialise (Remy et al. 1999). Slight increases in ICAM expression by human umbilical vein endothelial cells (HUVEC) over a 7 day period were previously observed on plasma treated PET and ePTFE,

but the methodology used was trypsinization and subsequent FACS analysis; thus it might have underestimated the overall expression (Pu et al. 2002).

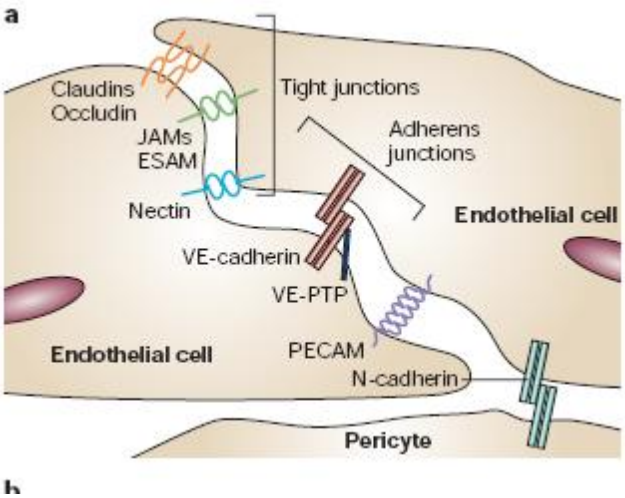

Figure 5.5 Homotypic cell-cell contacts present in arterial endothelial cells; PECAM is an important constituent and an increase in its expression points to an increase in endothelial cell contacts(Dejana 2004)

Selectin-P is a member of the Selectin family of adhesion molecules, which also contains L and E Selectins. It is a cell adhesion molecule expressed on activated endothelial cells and together with Selectin-E modulates platelet, leukocyte and endothelial interactions. Thus it is an important factor and an indicator of inflammatory response. The expected ideal case would be an elevated expression at the onset of the culture and then a decrease in expression as endothelial cells reverted back to their quiescent phenotype. The expression pattern of Selectin-P on PVA/Gelatin cryogels followed this pattern. In the beginning of the culture, there was an upregulation of Selectin-P expression, but, as in the ideal case, expression subsided as the culture progressed and it was only 1.41 fold more than TCPS control by day 10. Upregulation of Selectin-P on biomaterial surfaces has been reported

before (Guidollet et al. 1999), as has the upregulation of Selectin-E (Imbert et al. 2001) but in these studies the experiments were terminated on day 3 at most, so the long term data was not available. For longer term cell cultures (up to 10 days), similar upregulation of PECAM and downregulation of Selectin-P was also reported for porcine endothelial cells on amniotic membrane (Tsai et al. 2007).

5.5Effect of Disturbed Shear stress on Endothelial Cells

Perturbations in wall shear stress are one of the main reasons for acceleration of atherosclerotic plaque formation and are known to affect the neointimal hyperplasia levels in vascular grafts (Liu 1998). In general, the effect of shear stress on cells is magnitude dependent and at high shear rates (≥ 1000 s^{-1}) it can cause membrane damage, lysis and/or subsequent apoptosis (Zoro et al. 2008). However, since endothelial cells are under constant shear stress under *in-vivo* conditions, they are better equipped morphologically and phenotypically to endure shear stresses compared with other cell types, except blood cells (White 2007). For them, the mode of shear stress is a more important factor.

Under normal conditions blood flow is predominantly laminar. However, at bifurcation points or due to plaque formation, conditions for turbulent blood flow can be created (Hahn 2009). There are several distinct responses of endothelial cells to turbulent flow compared to laminar flow induced shear stress. Under turbulent flow conditions, cell proliferation is higher, alignment is poorer, and there is a higher rate of apoptosis and inflammatory gene expression (Figure 5.6). Although, shear stress created by orbital shaker systems has been previously proposed as a model for atherosclerosis advancement or neointimal hyperplasia

(Dardik et al. 2005), exploitation of the turbulent flow for facilitation of endothelium formation or strengthening is not available in the literature. It was our aim to show that such utilization of the turbulent flow is feasible and quantify its effects.

The growth of cells on PVA/Gelatin and TCPS showed a similar pattern except that from apoptosis data, initial attachment was higher on TCPS. With turbulent shear, cells assumed a random orientation, and apoptosis and cell proliferation increased. In an orbital shaker system with a circular cell culture plate, the shear stresses at the centre and at the periphery are not the same, with lower shear stresses expected at the centre. The lower shear area generally shows higher proliferation and apoptosis rates, which might be an additional benefit of ramping. However, it is useful to have the decreased necrosis effect in the beginning of the culture.

The Reynold's number for the flow regime was calculated as (Dardik et al. 2005):

$$R_e = \frac{\omega R^2}{v} \qquad \text{Equation 5.1}$$

Where ω is rotational speed (rps), R is radius of rotation of the orbital shaker (provided by the manufacturer as 2.5 mm) and v is kinematic viscosity and the value of R_e was 1512 with a slight underestimation due to the utilization of measurements made in room temperature. Although no lower limit for turbulent flow has been set in the literature for such a flow system, previously Doppler velocimetry measurements of the flow regime was performed to show oscillatory and disturbed nature of the shear stress (Dardik et al. 2005), which has also been verified by a computational model ((Berson et al. 2007) and personal

communication with Dr. Sharpe) and with the presence of eddy current and high rate of dissipation of energy due to turbulent shear was experimentally measured even for rotational speeds as low as 50 rpm (compared to the rate used 135 rpm in this study)(Kaku et al. 2006). Thus the claim of disturbed shear effect is valid.

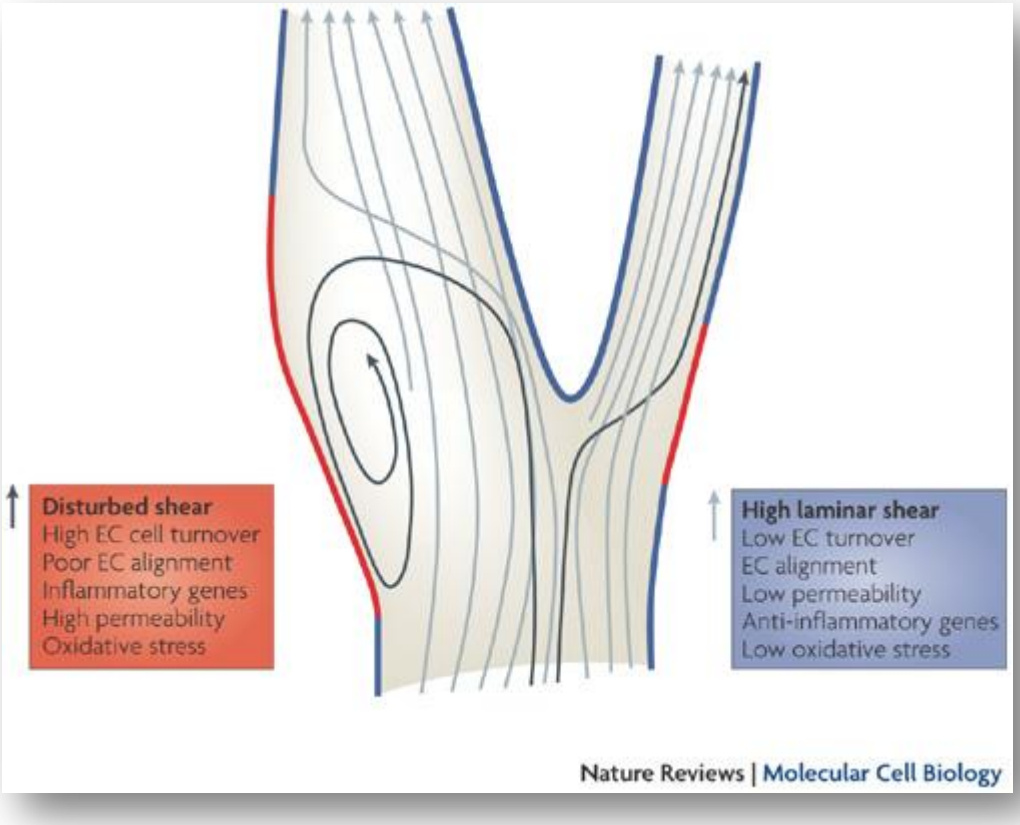

Figure 5.6 Effects of shear stress mode on endothelial cell functions. Even though, disturbed shear conditions triggers inflammatory responses and causes increased permeability they can be used for rapid endothelialisation of graft surfaces due to the increased cell turnover (Hahn 2009)

Moreover, dispersed cellular presence and the resulting change in surface roughness, surface porosity of the base and oscillatory fluid motion would also contribute to the changes in the shear regime.

In the present study, application of a ramped shear stress regime resulted in a dramatic increase in cell proliferation, with a smaller increase in apoptosis which was satisfactorily compensated by the increase in cell number. The lining was intact at physiological level of stress associated with small diameter arteries (Zhang et al. 2008), with only small patches of cell free areas observed by microscopy after 5 days. A similar significant increase in proliferation on TCPS with turbulent flow versus both static and laminar flow was observed previously (Figure 5.7). Coating of grafts with cell adhesive proteins such as fibrin glue has increased the patency rates in the past (Deutsch et al. 2008, Kannan et al. 2005), even though there was still endothelial cell loss following implantation mainly due to shear stress. It has been reported that the attachment of this layer is vulnerable under turbulent shear stress, which exists at the points of implantation (Chien 2008). This was consistent with our observation that direct application of shear stress, similar to the case when the blood flow is re-established *in-vivo,* caused widespread removal of the endothelial cells. Ramping has been used successfully with other grafts (Inoguchi et al. 2007) and it was also beneficial in our model. The increase in the apoptotic cells compared to the static cultures was significant, but negligible when compared to the overall viable cell numbers.

Ramped shear stress related increases in apoptosis were overshadowed by the fact that it caused a dramatic decrease in necrotic cell count, which might be due to the attachment strengthening effect of sub-physiological level of initial shear, which was not strong enough to trigger widespread detachment, but perhaps effective

enough to alter gene expression patterns to reinforce cell attachment (Chien 2008b).

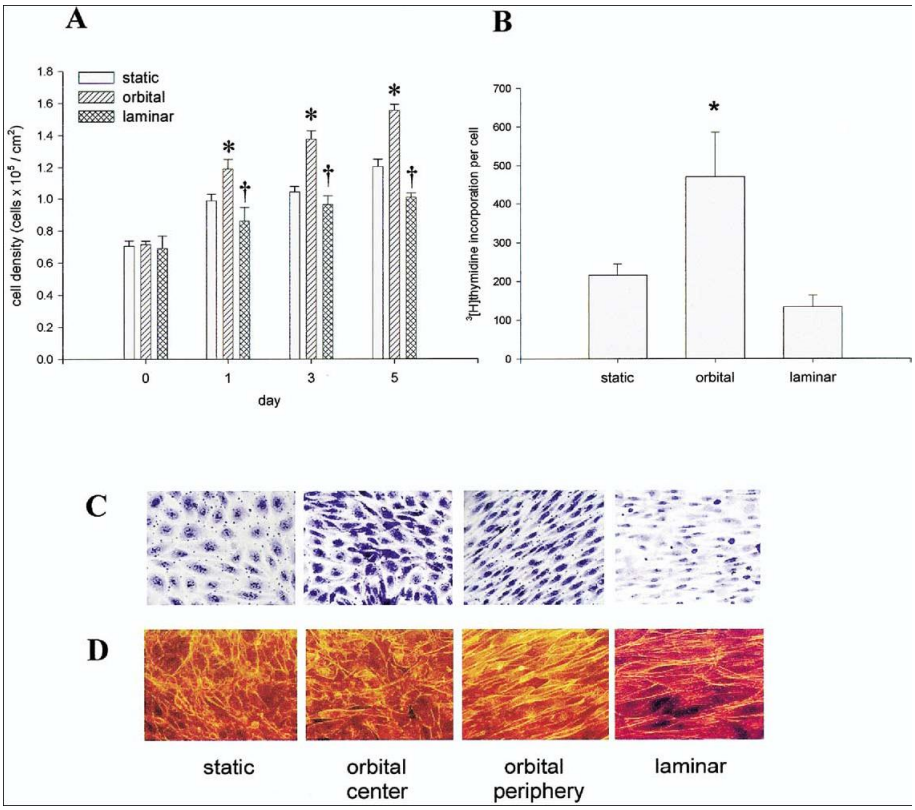

Figure 5.7 Effect of turbulent flow on endothelial cell proliferation on TCPS; the proliferation rate was significantly higher over a 5 day period; whereas laminar flow inhibited cell growth compared to the static controls. (Dardik 2005)

The appeal of PVA-based cryogels is the potential to produce scaffolds with a wide range of mechanical properties similar to soft tissue. However, as demonstrated by the previous results, the attachment behaviour is different compared to TCPS. For endothelial cells, the decrease in surface porosity upon coagulation bath treatment is beneficial in the sense of ensuring that the endothelial cells would stay on the surface of the gel, rather than penetrating inside. The initial increase in surface

roughness after application of coagulation bath might be deemed detrimental in the sense that rougher surfaces trigger more platelet adhesion, if exposed. However, since coagulation improved cell attachment and the surface gets even smoother with the application of shear, this will decrease the possibility of thrombosis in the uncovered areas of the hydrogel even if the endothelial lining is removed. The time constraint is an important problem in tissue engineering, where the long culture periods decrease the feasibility of engineered tissues; due to the related costs and risks such as contamination (Yow et al. 2006).

For cell-cell contact gene expression, the application of shear stress was highly influential, especially when ramping was applied. Endothelial cells responded to turbulent flow in a manner that would trigger an inflammatory response under *in-vivo* conditions; i.e. with a marked increase in Selectin-P expression. In the present study, the presence of shear stress was the dominant factor and it diminished the differences between PVA/Gelatin cryogels and TCPS. Only in ICAM expression, which can be seen as a part of inflammatory response when there is a several fold increase, there was a significant difference (4.59 fold increase). This jump in pro-inflammatory gene expression could be observed both for laminar and turbulent shear conditions (He et al. 2005). The difference between these cases was that if the disturbed shear is maintained then expression would continue steadily, while there would be a decline under laminar flow (Chien 2008a). When comparison was made between static versus shear conditions, there was a significant increase in the expression of all genes. In the current study, the increase in both PECAM and ICAM expression indicated a higher level of cell-cell contact, which is essential for the

barrier function of the endothelium. Similar jumps under disturbed shear were previously observed too (Sorescu et al. 2003). As for quenching the inflammatory response, it has been shown that even in 24 hour laminar shear application; most of the pro-inflammatory and proliferative genes are downregulated (Chen et al. 2001). Microarray analysis further showed that the laminar and disturbed flow conditions were distinctly different mechanical stimuli with significantly different gene expression profiles (cell adhesion molecules, inflammation, proliferation, apoptosis, secretion related genes and receptors) (Brooks, Lelkes & Rubanyi 2002).

Modulation of phenotype must be an important tool in tissue engineering, where cells respond to abnormal conditions in a way that would be beneficial for the long term goal of scaffold maturation. Shear stress created by the turbulent flow can be considered such a condition, where non-physiological effects can be exploited in order to facilitate production of a full endothelial lining. The side effect of invoking inflammatory response can be quenched by application of laminar or static culture conditions following the application of the turbulent flow and acceleration of endothelial formation. This way, the necessary *in-vitro* culturing time can be shortened and endothelial cells would be acclimatised to the conditions of implantation.

A steady release of low levels of NO is crucial *in*-vivo and has been used as a control for endothelial functionality frequently both *in-vitro and in-vivo* (Sessa et al. 1994, Oemar et al. 1998). The mean nitric oxide concentration in blood is reported as 19.6 µM (Fareed et al. 2004) for healthy adults and is known to fluctuate considerably for several pathological and inflammatory conditions. The concentration of NO in

culture medium was 5.26 μM for PVA/Gelatin hydrogels by day 5 under static conditions, where the lining was not complete, which suggests that physiological levels of NO can be achieved once a full lining is in place. In general, shear stress can increase production of NO and also several other genes (Ballermann et al. 1998). The overall NO concentration has increased in culture medium after application of shear, 13.79 μM, and it was much closer to the physiological values. It has been shown that ramping the shear stress has a positive effect on NO synthesis (Noris et al. 1995).Thus, this might explain the difference between direct and ramped shear NO levels (8.17 vs. 13.79 μM). Thus, flow conditions, achieved the expected positive effect on NO synthesis.

Tissue engineering seeks to produce healthy artificial tissues under *in-vitro* conditions. However, this does not necessarily mean that the cells used for tissue maturation need to be exposed to physiological conditions throughout the culture period. Applications of pathological conditions for pre-determined periods of time might facilitate tissue maturation, since cells are apt to react to such conditions in a fast manner (Resnick et al. 2003). The modulation of these conditions might be beneficial for short term gains, such as increased proliferation or increased secretion of proteins. Results of this study has shown that on PVA/Gelatin hydrogels endothelial lining formation can be facilitated and maintained by short term application of shear stress under disturbed flow conditions, to accelerate cell proliferation and cell-cell contact formation.

5.6 Encapsulation via Cryogelation

When an aqueous polymer solution is frozen, polymer chains are pushed together by the growing ice crystals. This packing effect is the main reason behind the hydrogel formation upon freeze-thawing of PVA solutions as packing improves the probability of hydrogen bond formation between the PVA chains (Kirsebom, Mattiasson & Galaev 2009). Cryogelation via freeze-thawing is a process mainly governed by the crystal formation rate. This rate is affected by the concentration of the polymer, freezing and thawing rate and freezing and thawing temperatures. It is known that the main determinant in the PVA gel formation is the thawing rate (Millon et al. 2007). Moreover, the presence of additive and also the composition of the solvent would affect crystallization and subsequently the final gel structure. In this study, a PVA based cryogel was formed in the presence of cells and several other additives without compromising the viability of the encapsulated cells. This was achieved through application of cryopreservation methodologies for cells in conjunction with the cryoprotectant properties of PVA.

Cryopreservation has been developed and perfected for sperm, embryo and oocyte preservation and storage (Chin Heng, Yu & Chye Ng 2004). Even though methodologies developed in these areas are transferable to thicker tissues or microcapsules and sometimes provide benefit such as reduced immune reaction upon implantation (Sotres-Vega et al. 2006), there are additional necessities that have to be covered. For cell encapsulation, the main concerns were the stability of the pH of the solution, the temperature of the initial gel solution, the need for an adhesion promoting component in the gel solution, protection against cryodamage (intracellular and extracellular) and presence of proliferation promoting factors

upon thawing and if necessary an additional crosslinking method with minimal effect on cell viability.

For PVA composites developed in this study, the adhesion promotion was solved by the presence of gelatin, which blends well with PVA and even reinforced the gel structure as the results in the previous sections suggested. Gelatin has been used to improve the encapsulation efficacy of other gel structures used in vascular tissue engineering such as modified dextran that can be photopolymerised (Liu, 2009). However, the gelatin stability was less than the current study and by 3 days nearly twice the amount of gelatin released from cryogels was released from these systems. No such problems occurred with the encapsulation system developed in this study.

To stabilize the pH of the gelation mixture and also to provide necessary nutrients to cells upon thawing, as gel presence may prevent the initial diffusion of the nutrients, a HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffered medium solution(Serum and antibiotic free RPMI 1640 supplemented with HEPES) was used and shown to stabilize pH in 7.20-7.40 range.

Cryodamage can happen either directly (during cooling stage) or indirectly (due to long term storage at subzero temperatures). So, in a storage system, protection against both these forces is essential. This protection against cryodamage is two-fold. The first step is to prevent intracellular ice crystal formation which can be achieved by DMSO or glycerol. Both act by replacing the intracellular water; i.e. dehydration of cytoplasm (Toner, Kocsis 2002). The next step is the prevention of intercellular ice crystal formation which can pierce the cellular membrane and

induce apoptosis or direct necrosis. In particular, replacing the water bound to the membrane surface is crucial. Molecules such as albumin or trehalose have been shown to be effective in reducing membrane damage. The presence of serum is helpful for the prevention of intercellular crystal formation. Also, polymer chains of PVA would provide a better protection with their interactions with the membrane, as this property was previously observed with the freeze-thawing of mouse embryos (Nowshari, Brem 2000). Besides being active in ice crystal formation prevention, the serum will also alter gel formation resulting in the presence of big particles such as serum albumin in its constitution and also by providing cells with necessary growth factors upon thawing and also to a limited extent by providing cell adhesion proteins that will adsorb to the inner surface area of the gel. This would also provide a change in the freezing regime because of the higher osmolarity of the final solution due to presence of serum and media contents, The improved osmolarity has been shown to be beneficial for viability of the encapsulated cells (Villanueva, Bishop & Bryant 2009).

DMSO was chosen in this study as the principal cryoprotectant due to two reasons. Firstly, there is a body of work that suggested DMSO is a superior cryopotectant in complex 3D structures (Pancrazio, Wang & Kelley 2007). Secondly, DMSO has the ability to dissolve PVA and by changing the freezing regime, it was hypothesized that it would form bigger pores during cryogelation. This hypothesis was supported by the results of the current study. Finally, the presence of the cells acted as fillers for reinforcement of the system, and thus alleviated the need for longer thawing regimes. The presence of 2 million cells, acting as beads with a volume of at least 10

μm^3, has also changed the phase separation process resulting in a better porosity (Nicodemus, Bryant 2008). Histology sections demonstrated that cells are present in the big pores in the hydrogel structure. In these pores cells were generally arranged in large groups, which showed the relationship between pore formation and cell presence. Moreover, due to the improved porosity the final cryogel structure was more suitable for migration of the cells, and also for nutrient and oxygen transport, and would be feasible for secondary seeding of cells if the cell number is lower than expected after thawing. Both the presence of PVA and DMSO markedly improved the viability of the encapsulated smooth muscle cells. The presence of serum had a positive effect by itself, but when applied together with DMSO the increase in viability was even higher.

To prevent cryodamage cell suspensions were frozen slowly to prevent water crystal formation and thawed rapidly in order to prevent recrystallisation, which would cause similar damage. Although, the initial step of controlled freezing is beneficial for gel formation, rapid thawing is detrimental. When a PVA solution dissolved in distilled water is thawed in a rapid fashion (30 min, for example), the final product is an extremely weak gel. However, the encapsulation mixture was able to form stable gels in much shorter thawing rates (gel formation was achieved even with 5 minute thawing procedures, by adding the culture medium slowly in minute amounts, which disrupted the cryogel formation less). This response was a direct result of the effect of the mixture components on the final gel structure. As the suspension freezes, formation of water crystals will result in a phase separation between the frozen and non-frozen phases which are affected by the solute

concentration. High solute concentration leads to freezing point depression that directly affects the rate of water crystal formation. Crystallite formation also depends on the ability of the components of the solution to bind water. The increase in pore size can be explained by the difference between the packing of strands of polymer chain by the pushing out effect of crystallisation compared to packing of spherical cells which would lead to less effective packing and result in more space. The viscosity effect mainly depends on the characteristics of the PVA solution, such as polydispersity ratio, or stereoregularity and degree of hydrolysis. The final gel has three distinct parts, as in the conventional cryogel; a solution part, an amorphous layer and crystallites that decrease the movement of amorphous parts. However, the difference is that the solution part is now a suspension of cell rather than a simple aqueous solution, which was the reason behind the dramatic changes in the gel structure. In a way, the primary aim of encapsulating and preserving viable cells (which was achieved as the cells were viable upon thawing and were able to proliferate afterwards) had a positive side-effect of improving the cryogel ultimate tensile strength and porosity.

Current cryopreservation techniques are mainly devised and optimized for cell suspensions and their application to adherent monolayers or thick biological samples are known to be less effective (Malpique et al. 2009). Even though cells remained viable, they tend to go into the apoptosis route and lose the capability to proliferate (Heng et al. 2006). Specifically, vascular tissue cryopreservation can cause prolonged proliferation lag periods (Lehle et al. 2005). It has been shown that the presence of polymers or encapsulation of the cells is beneficial for cell viability

after thawing. This has been achieved by microcarrier systems such as PEG, alginate or matrigel (Itle, Pishko 2005). However, these are pre-formed systems on which freeze-thawing cycle of storage may have some adverse effects; especially on mechanical properties. Thus, PVA offers a unique opportunity to combine these two beneficiary properties with the ability to form cryogels for development of a one-step preservation and scaffold production method. Moreover, such encapsulation techniques would also enable incorporation of growth factors or angiogenic factors. However, the additive content should be carefully monitored since, depending on the solution viscosity of the PVA, below a certain concentration PVA cryogels formed are not stable (Figure 5.8).

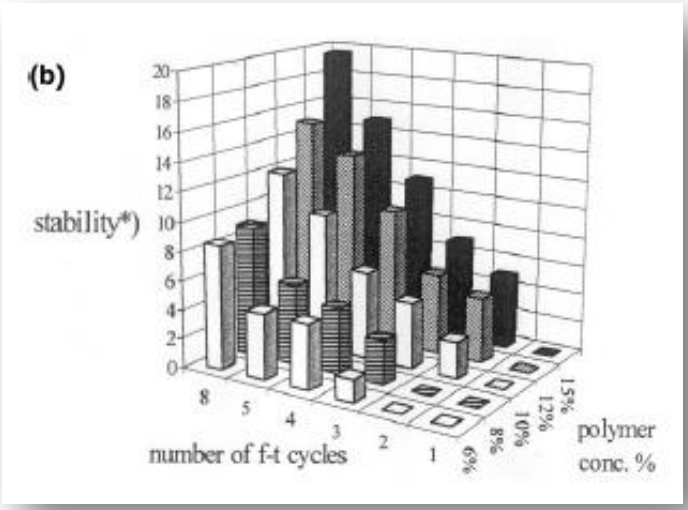

Figure 5.8 Relationship between cryogel stability freeze-thaw cycle number and concentration. The stability improves with freeze-thaw cycles and with concentration. Thus for 1 cycle cryogels, dilution of PVA solution with too much additive can cause gel instability. This was the reason why higher DMSO and Serum concentrations were not used (Szczesna-Antczak 2001)

In the current study, cell viability was not affected by 1 month cryostorage at -80 °C and most probably longer storage times can be achieved in liquid nitrogen, most probably up to a year. Also, the protective capabilities of the cryogel was shown, where monolayers of smooth muscle cells cryopreserved resulted in distinctly less viable cells. A decrease in viability after the storage time frame within a month was observed with cardiomyocytes (Yokomuro et al. 2001) (Figure 5.9). Thus the current results of steady viability over a 1 month storage period were encouraging. Moreover, the mechanical properties of the final cryogels can be easily improved by changing the cell concentration and final thawing rate, resulting in a wide range of cryogels with different mechanical properties since the thawing rate is the determining step in mechanical property development for cryogels, that can be used for different targets in the vascular tree, so the same batch solution might be used for different problems. To our best knowledge, such a system developed with cryogelation as in the current study does not exist in literature. The only similar effort in tissue engineering area was the encapsulation of pancreatic islet cells in PVA cryogels while preserving their functionality (Qi et al. 2004). However, in that study only cellular functionality was reported. Another body of work was done to encapsulate yeast and bacteria cells for chemical engineering application but without any modification of cryogelation procedures (Szczesna-Antczak, Galas 2001). However, these systems have shown similar high porosity and permeability and improved mechanical strength and their successful utilization in bioreactor systems was a proof-of concept for tissue engineering applications (Plieva et al. 2008). Since the encapsulation procedure is affected by several parameters as

discussed above, to clarify their effects in a systematic way, factorial experimental designs were utilized.

Statistical experimental models, or in a more general and sophisticated manner combinatorial methods with high throughput screening are a useful way to deduce several factors. Through them, interactions with several parameter in an experimental system can be determined, while minimizing the number of experiments necessary to reach statistically significant conclusions. Utilization of these methodologies is not widespread in the biomaterials field but certainly combinatorial approaches are very useful tools for biomaterial research (Brocchini 2001), especially when the multifactor nature of most of the processes pertaining to cell/biomaterial interaction is concerned (Kennedy et al. 2006). There might be limiting, inhibiting or compounding effects of several factors which would remain unrevealed with one-factor tests. Moreover some factors would only be able to work synergistically; such as in the case of DMSO and Serum in this study. Optimization of a wide range of parameters on the same experiment settings is particularly important in biomaterials, since the behaviour is so dependent on properties such as cell passage number, batch and the chemicals used. Also, when the necessity of doing long-term experiments in tissue engineering is considered, multi-factor experimental designs can become indispensable tools in tissue engineering (Villani et al. 2008, Chen et al. 2006). In this study, design of experiments methodologies revealed the necessity of using DMSO and Serum together for improving the cell viability in the cryogelation system and also the relationship between the coagulation bath and PVA viscosity while at the same time

providing results about the interactions between PVA viscosity, DMSO and serum.

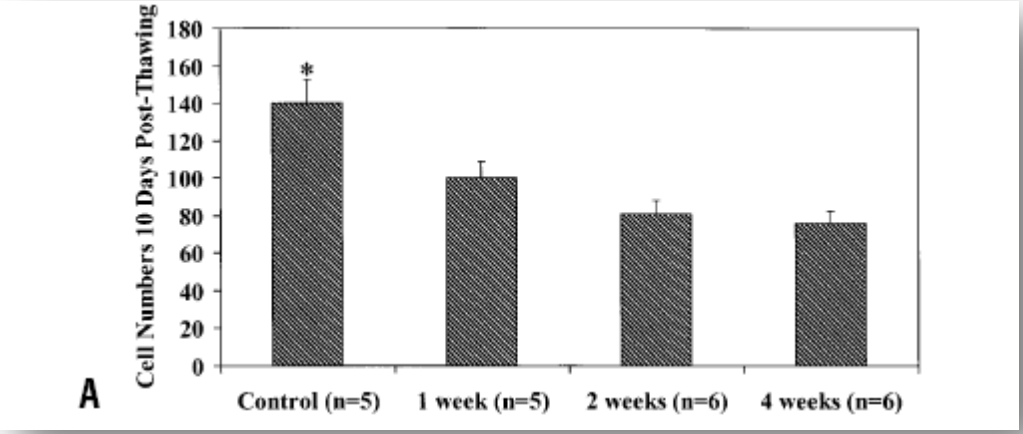

Figure 5.9 Viability of cardiomyocytes with respect to the cryostorage period. Longer storage time caused lower cell viability (Yokomuro 2001)

5.7 Cryogel Mechanical Properties and Effects of Encapsulation

One of the appealing features of using cryogelation aside from non-toxic nature of the process is the improved mechanical properties of the final structure due to extensive crystallite formation which makes it easier for the structure to carry the load applied (Hassan, Peppas 2000b). The unique advantage of PVA cryogels in the area of cardiovascular tissue engineering is their close resemblance of the typical exponential stress/strain curve of arterial samples. For arterial tissue, this behaviour is conventionally explained by the differential behaviour of collagen (high stiffness) and elastin (low stiffness) constituents, which are crudely, imitated by the amorphous and crystalline regions of the PVA cryogels (Millon, Wan 2006). In a way, addition of complex carbohydrates such as chitosan or starch would even further mimic the aortic behaviour, by providing the properties of proteoglycans. It

has been reported that increasing the number of freeze-thaw cycles had an effect on mechanical properties which levels off after several freeze-thaw cycles (Wan et al. 2002). The increase in the cryogel strength upon application of cyclic strain during cryogelation can be due to induction of extensive secondary crystallization triggered by extensive mobility of the sol portion of the hydrogels due to the strain (Millon, Mohammadi & Wan 2006).

Since freezing is equiaxial in the present study, the properties of PVA composite cryogels are isotropic, previously it was shown in our laboratory that the PVA based composite cryogels have similar stress/strain curves to those of the porcine aorta (Mathews et al. 2008). This similarity has been also noted by other works, and it has been suggested that the uniaxial straining of the PVA cryogels during formation can lead to anisotropic mechanical properties, even more similar to that of vasculature (Millon, Mohammadi & Wan 2006).

The improvement in mechanical properties in the presence of the additives can be explained by their effects on PVA structuration and freezing. The initial effect in increase in tensile strength of the system by encapsulation mixture due to the presence of additives may be the depression of the freezing temperature of the mixture and by preventing freezing promoted more crystal nuclei formation. For the cryogel structure, staying in these low temperature conditions for a long time without freezing results in a more crystalline structure with coarser crystallites. Previously, it has been shown that the presence of both hydrophilic and hydrophobic particles in the gelation mixture had a reinforcing effect on the gel structure by improving phase separation and packing of the PVA chains (Lozinsky,

Savina 2002). Thus the effect was dependent on the surface hydrophilicity of the particles, their size and concentration. In this sense, the contributors to the improved cryogelation in the encapsulation system were; cells, DMSO and serum proteins. The biggest contributor is DMSO, which is known to improve the crystallinity and hence the mechanical properties of cryogels (Lozinsky 2002). The ionic strength increase due to the presence of complex salts in the culture medium might have a dissociative effect on hydrogen bonding and such effects were previously reported with simpler salt solutions (such as sodium Chloride)(Nugent et al. 2005b) However, nucleation capacity of the proteins in the serum and cells in particular can easily overcome this barrier. Together with the effect of the different biomacromolecule additives on tensile strength, a wide range of cryogels with notably different mechanical properties can be prepared either with or without cellular presence within the cryogels.

5.8 Effect of Cyclic Strain on Smooth Muscle Cells

The cyclic strain has a regulatory function *in-vivo* in which it ensures contractile phenotype in smooth muscle cells and consequent quiescence (Kurpinski 2006). It is also important for alignment of the smooth muscle cells as it provides the mechanical cues for such alignment (Standley 2002). 2D *in-vitro* studies have demonstrated that the cyclic strain inhibits smooth muscle cell proliferation (Morrow et al. 2008). However, in 3D scaffold conditions an increase in proliferation was also observed (Figure 5.10). This is mainly due to the different mode of transmission of the strain (strain was transferred from all strands of the polymer matrix rather than the substrate adhesion points in the case of 2D

stretching) and also the different shape and attachment of the smooth muscle cells, which also results in different responses under static conditions (Stegemann, Nerem 2003). In the current study, the application of cyclic strain resulted in a slight increase in proliferation, extensive alignment by the cells and comparable viability with respect to the static culture conditions, similar to previous observations (Butcher, Barrett & Nerem 2006). This points to PVA cryogel extensibility being favourable for transmission of mechanical stimuli. One experimental problem would be the overestimation of the cyclic strain levels, as it was shown that the strain function of the Flexercell is not very reliable (Colombo, Cahill & Lally 2008).

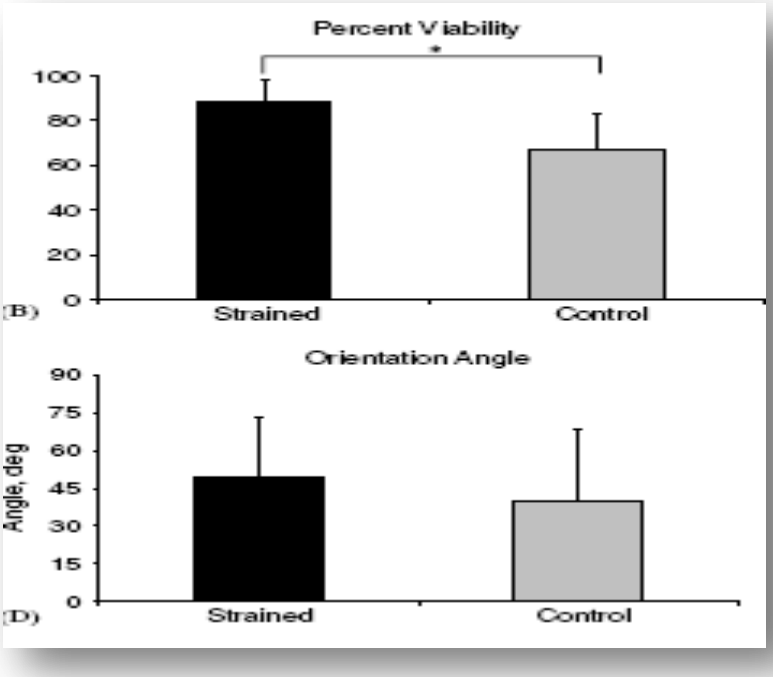

Figure 5.10 Effect of cyclic biaxial strain on smooth muscle cell orientation and viability. Strain cause higher alignment and higher cell numbers (Butcher 2006)

Generally, mechanostimulation together with cell activity leads to gel compaction. Even though PVA cryogels weren't compacted, there was a decrease in their tensile

strength compared to the static controls. This was mainly due to the cryogels not being completely formed, as the observation of the cryogel formation showed that gel formation continues during culture. This disruptive effect wasn't apparent for cryogels which went through cyclic strain without cells; since disrupted connections can be re-established quickly. However with the cells present and with their movement and possible MMP activity, the level of disruption would be more severe, resulting in reduced strength. This can be overcome by increasing initial static culture period. The experiments were conducted as they were so that the tests could be conducted at exactly same time point. It has been shown that the long-term application of cyclic strain (up to 20 weeks) would result in an improvement in mechanical strength (Kim et al. 1999). Also biochemical stimulation with growth factors such as b-FGF or PDGF together with mechanical stimuli could result in faster improvement of mechanical properties (Stegemann, Nerem 2003).

5.9 Endothelial Cell/ Encapsulated SMC Co-culture

Aside from being responsive to mechanical cues, endothelial cells are in contact with the blood cells and also smooth muscle cells via paracrine and endocrine pathways. It has been shown in the past that the co-culture of endothelial cells on smooth muscle encapsulated scaffolds promoted smooth muscle quiescence and prevent hyperplasia depending on the eNOS expression level (Kader et al. 2000). The communication between endothelial cells and smooth muscle cells are two way and both can induce quiescence or proliferation on each other (Leung, Sefton 2007). For these reasons, in this study the effects of three factors on endothelial functionality (via NO synthesis and eNOS presence) were checked. The culture time

indirectly corresponded to the smooth muscle cell number, as our previous results showed they proliferated within the hydrogels for the given time period. Storage time parameter could affect the cryogel stiffness by the effect of period of freezing, while the cell number was shown to be steady in this study. The shear stress has been shown to improve NO synthesis and it was used to see whether there would be a further improvement as a result of interactions with the other factors.

This effect of shear stress was augmented when it was applied to the endothelial cells seeded to the longest cultured cell encapsulated cryogels. Storage time did not have a substantial effect on NO synthesis. The encapsulated smooth muscle cell culture experiments showed that the cryogel was remodelled during the culture time and with the higher cell number and a stronger hydrogel, higher secretion of NO could be triggered. Unresponsiveness to the storage time suggested that the cell number was the same and the cryogel structure did not change much due to the longer storage. Since the main gelation step is thawing as stated above. Previously, it has been shown that seeding of endothelial cells after a period of culture onto the encapsulated smooth muscle cells results in a more contractile phenotype (Williams, Wick 2005). However in Williams and Wick's study there was migration of endothelial cells into the scaffold too. This problem was prevented by addition of a smooth membrane surface for porous collagen scaffolds which was also successfully applied *in-vivo* (Wu et al. 2007). Direct contact of endothelial cells with smooth muscle cells are shown to slow down their spreading ability; thus it is better avoided (Wallace, Champion & Truskey 2007). In the current study, immunofluorescent labelling of eNOS as an endothelial-only marker in co-culture

experiments showed that the endothelial cells remained on the outer surface, as required. Also under co-culture conditions, some of the smooth muscle cells within the porous body of the cryogel were expressing α-smooth muscle actin which denoted a conversion to the contractile phenotype following proliferation.

CHAPTER 6

6 Conclusion and Future Work

The main conclusions of the present study are, presented with reference to the objectives set out in section 1.1 of this thesis:

1. The utilization of a two step physical crosslinking system for production of highly elastic, soft tissue-like composite hydrogels was developed. Effects of this crosslinking system on the physical properties of cryogels in the presence of different additives were elucidated, which contributed to the current literature on cryogel production (Liu et al. 2009). Also there was a gap in the literature as to the effect of successive freeze-thaw cycles on cell behaviour on cryogels and preliminary findings of this study showed that increasing the number of freeze-thaw cycles would be a good route to pursue to improve cell behaviour on top of mechanical benefits (Vrana et al. 2008). It has been concluded that the two-step crosslinking methodology utilized is a versatile method to produce composite cryogels with a wide range of additives , which can resist degradation and have good mechanical properties. Of the systems investigated, PVA/Gelatin had the most desirable properties for the further investigation towards target application, vascular tissue engineering.
2. In the light of the findings on the effects of disturbed shear stress' positive effect on cell proliferation of endothelial cells, the current study

developed a ramp disturbed shearing regime which improved endothelialisation of the cryogel surface substantially with a limited increase in apoptosis and good NO synthesis levels. The upregulation of pro-inflammatory molecules can be dampened by the static culture conditions, as was shown in the results section. Also, to our best knowledge, a comprehensive characterization of endothelial cell behaviour on cryogels was missing in the literature; so the current study has also set the groundwork on that front. In general, a PVA-based composite cryogel surface can be fully endothelialised with functional endothelial cells and this process can be controlled by changing the shear stress conditions. Moreover, endothelial cells can withstand physiological shear stress levels following attachment to the cryogel surface.

3. Freeze-thawing has a good prospect as a potential encapsulation method, with suitable modification of the conventional techniques. In the current study, an encapsulation and storage method based on freeze-thawing regime and PVA/Gelatin composites was developed and implemented for several different cell types. Cryogels were able to sustain cellular viability and proliferation and have shown comparable mechanical properties to the conventional cryogels.
4. The developed cryogel model was also able to sustain a co-culture system. As required, segregation of the cells in the co-culture system (endothelial cells on the surface, while smooth muscle cells within the cryogel structure) was achieved and both cell types were shown to be

healthy. The initial goal of developing a system that uses PVA cryogelation for both scaffold formation and cell cryostorage was achieved. The developed system can be practically used for other soft tissue applications and this study has developed a research route for the future.

These promising results suggested that, PVA based cryogels can be further investigated for their possible applications in biomaterials and tissue engineering fields. Following sections contain planned future work for attaining superior outcomes.

6.1 Future Work

6.1.1 Cryogel formation and Encapsulation via Cryogelation

This study demonstrated that cryogelation is a versatile system that can be used to produce hydrogels with varying mechanical properties and physical properties. The ability of these cryogels to promote cell attachment can be achieved by the development of composites with biomacromolecules and the changes induced by the presence of these biomacromolecules further widens the scope of cryogels that can be produced. These composite cryogels with a wide range of properties can be produced by using a two step non-toxic physical crosslinking method, which is cost-friendly and advantageous over chemical methods in the sense that no remnant chemicals would be present in the gel.

Cryogelation is a physical crosslinking process, which depends on the hydrogen bond formation and crystallization capabilities of PVA. However, as shown in the

results section, PVA needs additives to become suitable as a scaffolding material. Thus, a next step in this field of research would be the design of artificial polypeptides that contain amino acid sequences which would allow abundant hydrogen bonding and crystallization to allow cryogelation and that at the same time that contains sequences for cell and growth factor binding which would result in a more robust and cell-friendly storage and encapsulation system. Recently, custom-made proteins with engineered functions have been tailored and it has been shown that a "from scratch" approach is easier for production of functional artificial proteins (Koder et al. 2009). This would allow a wider control over the final properties of the hydrogel, such as O_2 binding for long term O_2 supply or controlled release triggered upon cell spreading, with minimal dependence on synthetic polymers. In the same manner, tethering of cell-adhesive sequences or introduction of these sequences into the PVA chains during synthesis would be another route; similar to that achieved for biodegradation of modified PEG systems (Bryant et al. 2004, Burdick, Anseth 2002).

Another aspect of cryogelation is using catalysis systems that would work at subzero temperatures which would result in even more robust gels, while minimally compromising cell viability (Kirsebom, Mattiasson & Galaev 2009). Also instead of slowly freezing the system, vitrification (solidification or freezing without any crystal formation) can be used to ensure higher cellular viability since the thawing was the actual gel formation part. Vitrification is known to improve cellular viability after storage and it has also been used for storing tissue engineered scaffolds (Wen et al. 2009). However, the lack of ice crystal formation and high rate of temperature

change in both freezing and thawing in vitrification might also affect crystallite formation by PVA too, so this system should be tried to see the benefits with respect to cell viability and the degree of gel stability. Another possibility would be elimination of DMSO from the mixture with more benign but equally effective cryoprotectants such as polyampholytes becoming available (Matsumura, Hyon, 2009). Finally, a mild chemical coagulation bath was shown to be effective in improving the stability of the cryogels with encapsulated cells, although it caused some morphological problems (for example, clumping of the cells). A biological coagulation bath solution (a saturated carbohydrate, protein, lipid solution; that would cause a similar polymer precipitation) can be used instead and it might be also act as an additional reservoir for nutrients.

Another possible route for improving encapsulation efficiency is to add recombinant morphogens, which are known to affect smooth muscle cell survival and proliferation, such as Sonic Hedgehog (Morrow et al. 2009). Another aspect of cryogelation is its possible protective capabilities for mesenchymal or embryonic stem cells, which are known to be more susceptible to cryodamage or cryodamage-induced apoptosis, and pores in the gel can help them maintain their clump-like structure. Similarly, addition of growth factors, either to the gelation mixture or via micro or nanocapsule formulations to improve their protection, could improve cryogel behaviour (Coleman, Case & Guldberg 2007).

6.1.2 Cryogel Endothelialisation and Shear Stress Conditions

In this study it has been shown that the endothelialisation of a hydrogel surface can be facilitated by application of turbulent flow induced shear, or disturbed shear in

general, to utilize the activating effect of it as a signal. This aspect has only been investigated for pathological conditions, but similar suggestions for the use of non-physiological stimuli are present in the literature. As an example, it has been suggested that the strain and shear stress values should be matched with those of the foetal development, since this phase is known for fast remodelling of cardiovascular system with low levels of MMP activity (Stock, Vacanti 2001). At strains levels of 5%, with a low oxygen tension and a high heart rate, vessel development is optimum (Stock, Vacanti 2001). Alteration of stress and strain conditions to trigger large scale cellular response would be beneficial in this sense. One possible route would be the application of turbulent flow in the beginning of the culture period to facilitate the endothelialisation, then converting back to a laminar flow mode to quench the inflammatory response. Thus, in the end a strong endothelium which expresses minimum amount of inflammation related proteins can be achieved. This can be further improved by surface coating of the cryogels with either single components of basement membrane such as laminin or collagen type IV or a concoction of them. The basement membrane composition together with the effect of shear stress would have a synergistic effect and also can lead to a much stronger endothelium which cannot be removed by the shear conditions *in-vivo*. In a similar way, utilization of nanofibrous thin layers, such as those produced by electrospinning, might provide a more resilient endothelial lining due to the more favourable properties of the surface for cell attachment and focal adhesion formation (He et al. 2006). Hybrids of smooth muscle cell encapsulated cryogels and electrospun composite fibres would be able to match the mechanical properties of cryogels with superior endothelialisation of nanofibres. The effect of

fibre properties on endothelial cells has been widely studied (Ma et al. 2005, Santos et al. 2008) and this route is being investigated in our laboratories.

The current study established the properties of the endothelial lining formed on PVA/Gelatin cryogels. However, blood compatibility is closely related to the blood endothelium interactions; thus a necessary step for determination of the non-thrombogenicity of the system is utilization of full blood for determination of platelet activation and adhesion and also leukocyte endothelium interaction. It has been suggested that endothelial cells do not need to be in their natural luminal location to function as a regulatory unit and perivascular implantation of encapsulated endothelial cells in a 3D collagen matrix can perfectly direct non-thrombogenicity of implanted products while keeping them quiescent (Methe, Edelman 2006/12). Our preliminary results showed that endothelial cell encapsulation was indeed possible via the method developed in this study and such a system would be beneficial as a generic auxiliary, perivascular scaffold for developing non-thrombogenicity for other scaffolds; such as bone tissue engineering scaffolds as the highly dense endothelial presence in scaffold proximity may result in secretion of anti-thrombotic and anti-inflammatory factors.

Another uncharted route is the application of shear stress to encapsulated smooth muscle cells. It has been shown that denudation of endothelium and the presence of turbulence is a strong contributor of neo-intimal hyperplasia formation and this fact can be exploited to improve ECM formation and proliferation of smooth muscle cells (Opitz et al. 2004)

The next step in this research field in our laboratories is the utilization of pulsatile flow to determine tubular PVA scaffold maturation under co-culture conditions. For this end, a CellMax bioreactor system was modified by another research group for utilization of 4mm diameter tubular scaffolds (Figure 6.1).

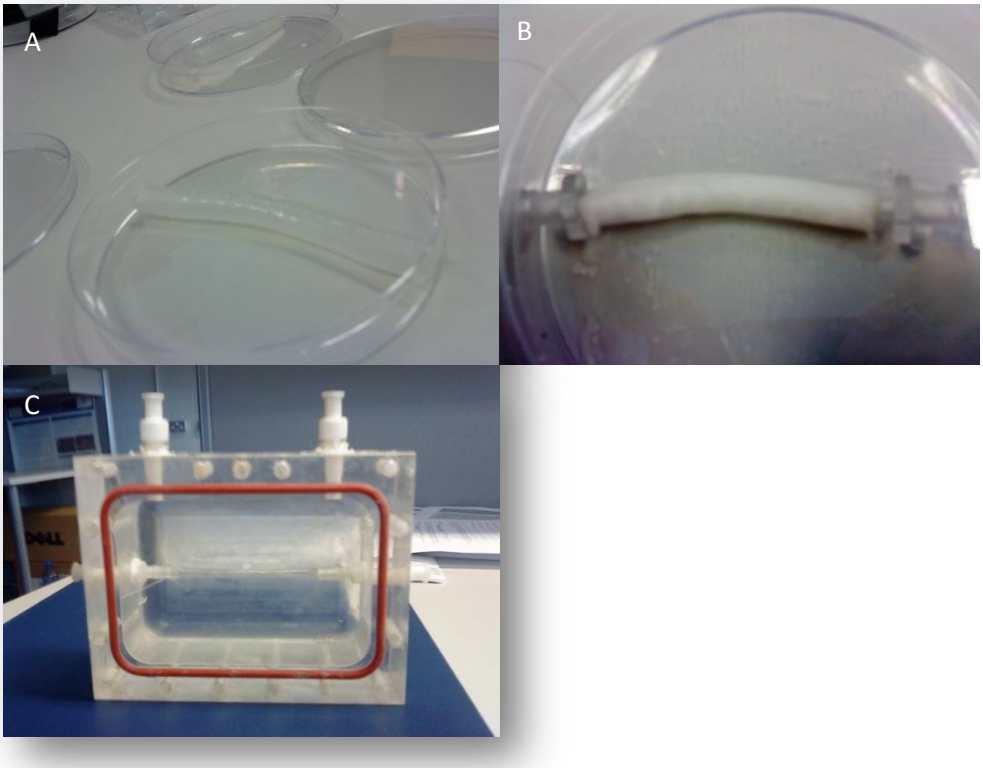

Figure 6.1 Tubular PVA/Gelatin scaffolds for bioreactor experiments a)Tubular PVA/Gelatin cryogels before coagulation bath treatment b) after coagulation bath treatment and sterilization c) PVA Gelatin cryogel within the bioreactor casing; system is connected to CellMAX cartridges which provides the oxygenation of the circulating medium and a pump whoich is capable of applying 6 different modes of pulsatile flow.

Tubular scaffolds from PVA/Gelatin cryogels were manufactured and their diameter and thickness were controlled by using the coagulation bath treatment. These tubes will be used for co-culture under pulsatile flow applications. The important analysis would be characterization of ECM secretion under these conditions, since one of the biggest problems of vascular tissue engineering is elastin production *in-vitro* (Patel et al. 2006, Heydarkhan-Hagvall et al. 2006).

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Appendix A

Mold Design for Cryogel Formation

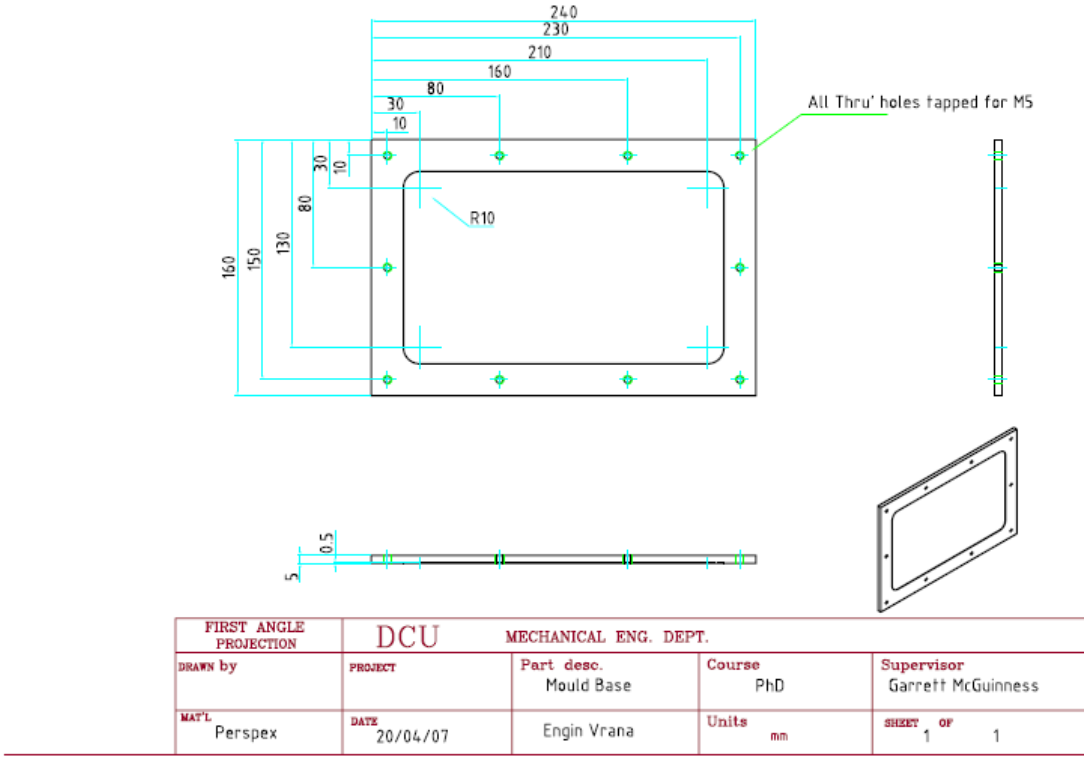
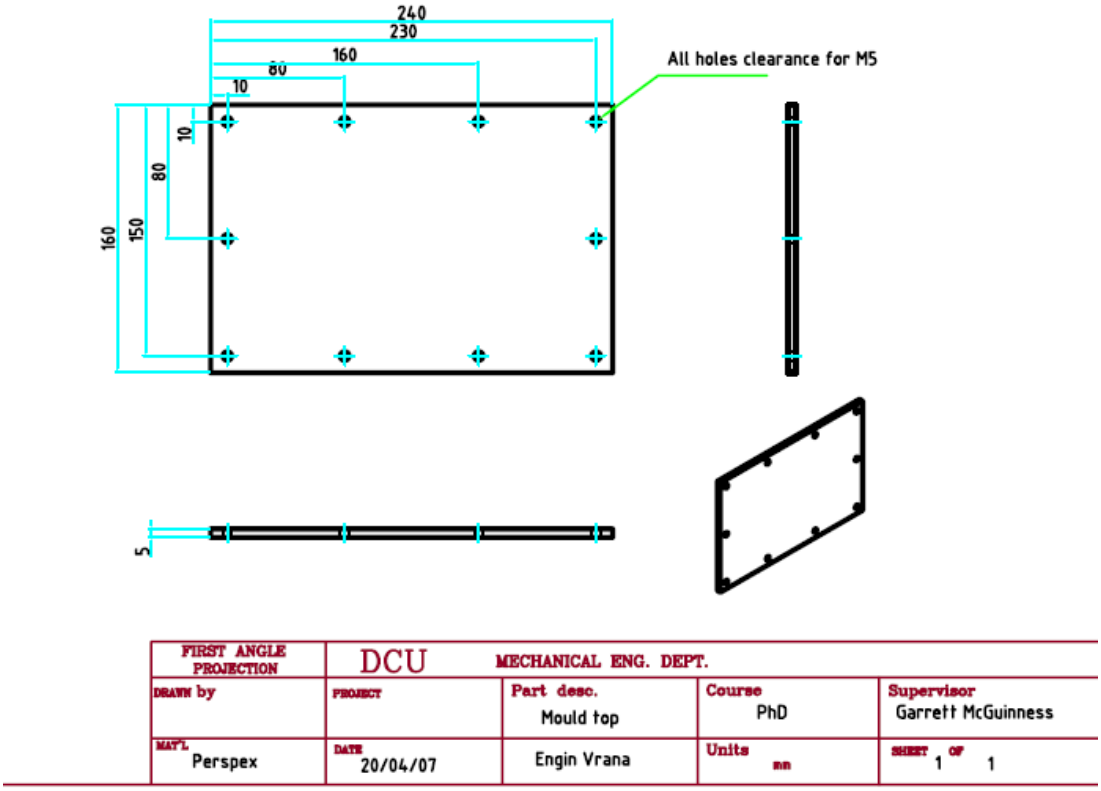


Figure A1. Mold design used in cryogelation experiments, bottom part.



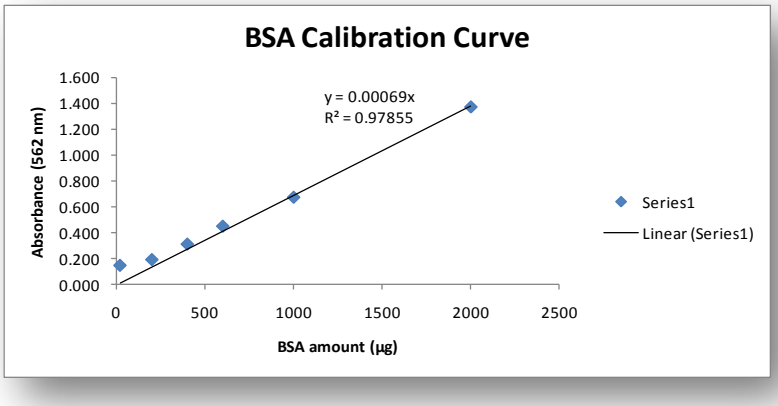
FIRST ANGLE PROJECTION	DCU	MECHANICAL ENG. DEPT.		
DRAWN by	PROJECT	Part desc. Mould top	Course PhD	Supervisor Garrett McGuinness
MAT'L Perspex	DATE 20/04/07	Engin Vrana	Units mm	SHEET 1 OF 1

Figure A.2 Mold'stop part.

Appendix B

Calibration curves for protein adsorption and Nitric Oxide Synthesis

The following graphs are representatives for calibration curves drawn before any set of experiments. For Collagen type I negative control readings were done with 0.2% Acetic acid solution to see its interference. For both BSA and collagen type I the curve was drawn up to 2 mg/ml concentration in order to ensure the reliability at the target range of 1 mg/ml.



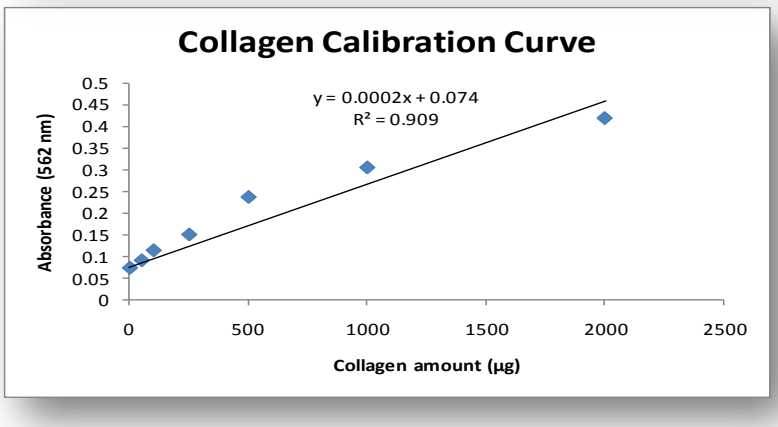


Figure B1. Calibration curves for protein adsoption studies with BCA a) BSA b) Collagen Type I

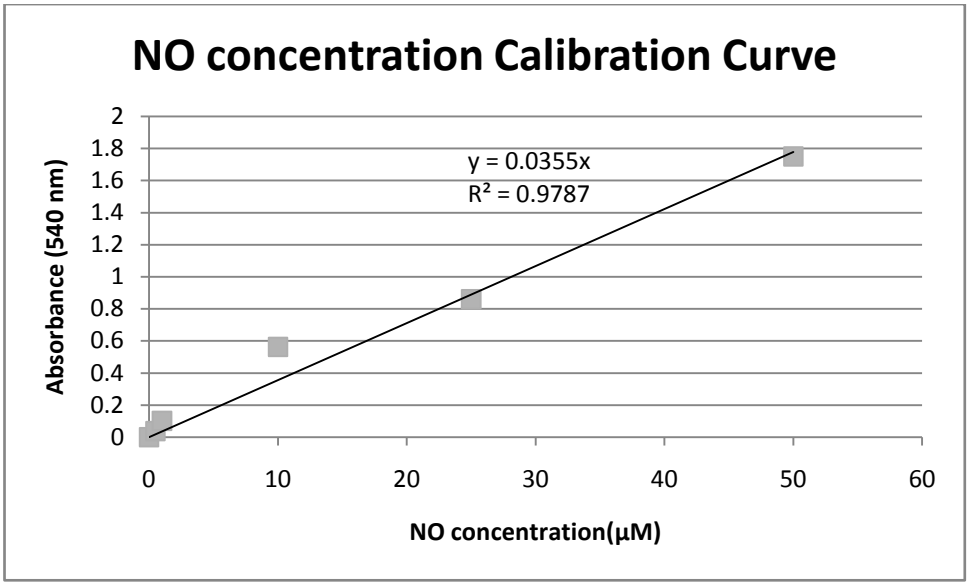


Figure B.2 Representative calibration curve for NO levels

Appendix C

Alamar Blue Percentage of Reduction determination

The percentage reduction of Alamar Blue after 1 hour incubation was calculated using two different formulas, depending on the wavelengths used for absorbance measurements. When 570 nm and 600 nm were used, as recommended by the manufacturer, following formula was used:

$$\%_{reduction} = \frac{(E_{O600} \times A_{570}) - (E_{0570} \times A_{600})}{(E_{R570} \times A_{N600}) - (E_{R600} \times A_{N570})} \times 100 \qquad \text{Equation C.1}$$

Where extinction coefficients (Es) are as in the table below:

Table C.1. Extinction coefficients of Alamar Blue in reduced and Oxidized states

Wavelength (nm)	Reduced (R)	Oxidized(O)
570	155677	80586
600	14652	117216

Where A_{570} and A_{600} refers to readings from the treatment samples and A_{N570} and A_{N600} refers to negative controls, Alamar blue solution only incubated in the same conditions.

When due to the technical difficulties other wavelengths needed to be used, following formulation was used (for 565 nm (Low wavelength, LW) and 592 nm (High wavelength, HW))

AO_{LW}= Absorbance of Alamar Blue in media (RPMI 1640) - Absorbance of Media only

AO_{HW}= Absorbance of Alamar Blue in media (RPMI 1640) - Absorbance of Media only

Correction Factor R_0= AO_{LW}/ AO_{HW}

And Percentage reduction is equal to:

$$\%_{reduction} = \frac{A_{LW} - (A_{HW} \times R_0)}{A_{NLW} - (A_{NHW} \times R_0)} \times 100 \qquad \text{Equation C.2}$$

Where A_{LW} and A_{HW} are readings from the treatment samples(i.e. cells seeded onto the hydrogels) A_{NLW} and A_{NHW} are negative control readings(Alamar Blue solution).

Appendix D

List of primers

Table D.1 List and sequence of primers used in the study

Target Gene	Product size	Left Primer	Right Primer
ICAM	162 bp	AAGAGTGCCTGGCAAAAAGA	GAGGTCAGCGCTAATTCTGG
PECAM	164 bp	CTGGAGTCTTCAGCCACACA	TATAACCCGCTGTCCCACTC
Selectin P	234 bp	GGGCTATCTCTCAGGGAACC	GACATGACTGAGCGACGTGT
GADPH	176 bp	GGGTCATCATCTCTGCACCT	GGTCATAAGTCCCTCCACGA

Appendix E

ANOVA table for 2^4 factorial design

After all experimental runs are completed ANOVa was carried out for the design space to determine the contribution of each factor and their interactions; to fit the overall response in an equation based on equation E.1:

$$Y = b_0 + \sum b_j X_j + \sum b_{ij} X_i X_j + \sum b_{ijk} X_i X_j X_k$$

Table E.1. Full ANOVA table for the model developed for cell viability for 2^4 factorial design. * denotes statistical significance(p≤0.05)

Source	Sum of Squares	df	Mean Square	F-Value	p-Value
Model	157.37	15	10.49	4905.83	< 0.0001*
A-DMSO	*0.17*	*1*	*0.17*	*80.31*	*< 0.0001**
B-Serum	*0.17*	*1*	*0.17*	*80.84*	*< 0.0001**
C-Co.B.	*54.52*	*1*	*54.52*	*25494.49*	*< 0.0001**
D-PVA	*65.30*	*1*	*65.30*	*30534.30*	*< 0.0001**
AB	*3.306E-004*	*1*	*3.306E-004*	*0.15*	*0.6994*
AC	*0.051*	*1*	*0.051*	*23.70*	*0.0002**
AD	*0.046*	*1*	*0.046*	*21.66*	*0.0003**
BC	*0.010*	*1*	*0.010*	*4.75*	*0.0447**
BD	*0.13*	*1*	*0.13*	*59.76*	*< 0.0001**
CD	*36.83*	*1*	*36.83*	*17223.70*	*< 0.0001**
ABC	*0.045*	*1*	*0.045*	*21.22*	*0.0003**
ABD	*3.627E-003*	*1*	*3.627E-003*	*1.70*	*0.2113*
ACD	*6.706E-004*	*1*	*6.706E-004*	*0.31*	*0.5833*
BCD	*1.803E-003*	*1*	*1.803E-003*	*0.84*	*0.3722*
ABCD	*0.085*	*1*	*0.085*	*39.72*	*< 0.0001**
Pure Error	0.034	16	2.138E-003		

Std. Dev.	0.046	R-Squared	0.9998
Mean	3.81	Adj R-Squared	0.9996
C.V%	1.21	Pred R-Squared	0.9991
PRESS	0.14	Adeq Precision	173.966

The Model F-value of 4905.83 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise.

Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case A, B, C, D, AC, AD, BC, BD, CD, ABC, ABCD are significant model terms. Values greater than 0.1000 indicate the model terms are not significant.If there are many insignificant model terms (not counting those required to support hierarchy), their removal may improve your model.

The "Pred R-Squared" of 0.9991 is in reasonable agreement with the "Adj R-Squared" of 0.9996.

"Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Ratio of 173.966 indicates an adequate signal. This model can be used to navigate the design space.

Appendix F

Statistical Analysis

The following examples cover how the statistical analysis were made and elaborate some cases where gving the statistical analysis within the main text was not feasible.

Collagen adsorption

The p value obtained (0.004) was low enough to claim that there is a significant difference between the test groups; and with Tukey's pairwise multiple comparison method; it can be shown that PVA/Gelatin surface adsorbed significantly higher amounts of collagen compared to PVA/Chitosan and PVA/Starch cryogel surfaces.

Table F.1. Tukey's test results for Collagen adsorption experimentsq value changes with the number of the samples and when it is significant it is denoted with a "Yes" remark.

Comparison	Difference of Ranks	q value	p≤0.05
Gel vs. Chi	101.000	4.242	Yes
Gel vs Sta	94.000	3.948	Yes
Chi vs Sta	7.000	0.294	No

Smooth Muscle Cell Proliferation with respect to Freeze/Thaw Cycle number and Collagen Coating

The p value obtained (0.005) was low enough to claim that there is a significant difference between the test groups; and with Tukey's pairwise multiple comparison method; it can be shown that there was a significant difference between 3^{rd} and 1^{st} cycles; both when collagen coating was used (q= 4.51) or not used (q=3.88)

Endothelial Cell Proliferation on Cryogels with Different Additives Day 10 and Day 15

For day 10 the p value was slightly higher than the significance level set for this study ($p= 0.08$ where significance level was set as $p\leq 0.05$), however by day 15, $p=0.011$ and there is a statistically significant difference between PVA/Gelatin vs. PVA/Chitosan and PVA/Starch ($q=3.00$ and 3.58, respectively)

When required, 2 or 3 way ANOVA tests were carried out such as in the case of change in water content with respect to, additive, coagulation bath treatment and cycle number. Afterwards, the pairwise comparison tests were done with Holm-Sidak method.

Table F.2 Three-way ANOVA analysis of water content of cryogels

Variation Source	DF	SS	MS	F	p
Additive	3	1.431	0.477	20.059	<0.001
CB treatment	1	3.322	3.322	139.730	<0.001
Cycle	1	2.992	2.992	125.846	<0.001
Additive x CB treatment	3	0.193	0.0642	2.702	0.051
Additive x Cycle	3	0.620	0.207	8.690	<0.001
CB treatment x Cycle	1	0.183	0.183	7.691	0.007
Additive x CB treatment x Cycle	3	0.214	0.0714	3.002	0.035
Residual	80	1.902	0.0238		
Total	95	11.057	0.116		