Biomimetic hydrogels based on natural dextran and gelatin for vascular tissue engineering application

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**BIOMIMETIC HYDROGELS BASED ON NATURAL
DEXTRAN AND GELATIN FOR VASCULAR TISSUE
ENGINEERING APPLICATION**

LIU YUNXIAO

SCHOOL OF CHEMICAL AND BIOMEDICAL ENGINEERING

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Summary

Hydrogels are crosslinked polymer networks with high water content. They have the capability to mimic native ECM and thus are highly desirable as 3D scaffolds for cell encapsulation. Synthetic hydrogels for cell encapsulation have hitherto been based on poly(ethylene glycol), which is non-natural, non-biodegradable, and only terminal-functionalizable. Dextran is highly hydrophilic but also biodegradable and pendant-functionalizable. More importantly, it resembles the native glycosaminoglycans.

This study aims to fabricate hydrogels based on natural dextran and gelatin, which could promote 3D SMC spreading and proliferation. Two series of hydrogels were fabricated. The first hydrogel series is based on the interpenetrating polymer network (IPN) of gelatin and dextran bifunctionalized with methacrylate (MA) and aldehyde (AD) (Dex-MA-AD). These IPN hydrogels not only supported endothelial cell (EC) adhesion and spreading on the surface, but also allowed encapsulated SMCs to proliferate and spread in the bulk interior of the hydrogel; however, the Schiff base reaction was not easily controllable and while SMC spreading within the hydrogel did occur, it was rather limited.

In order to improve SMC spreading, dextran was functionalized with

methacrylate and lysine (Dex-MA-Ly). A second hydrogel system based on Dex-MA-Ly and methacrylamide modified gelatin (Gel-MA) was developed. The behaviors of SMCs encapsulated within these hydrogels were influenced by the mechanical stiffness. Rapid cell spreading, high SMC proliferation, and extensive cellular network formation occurred within softer hydrogels; while in stiffer hydrogels SMCs maintained a round morphology and their viability declined during culture. In a softer hydrogel, the encapsulated SMCs appear to be relatively more contractile in the initial culture compared to those on tissue culture polystyrene dish due to physical constraint imposed by the hydrogels; but they become more synthetic with time, possibly due to the inability of the cells to reach confluence during the culture period inside these cell-mediated degradable hydrogels.

The impact of endothelial cells (ECs) on SMCs was investigated using an EC/SMC co-culture model in which SMCs were encapsulated in Dex-MA-Ly/Gel-MA hydrogels and exposed to a monolayer of sub-confluent ECs during culture. Without EC co-culture, we found that when the seeding density was higher, a softer Dex-MA-Ly/Gel-MA hydrogel increased the contractility of the SMCs somewhat as shown by increased expression of several contractile marker genes (*i.e.* α -actin, calponin and smooth muscle-myosin heavy chain (SM-MHC)). Co-cultured with ECs, SMC growth

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Due to the impressive cellular proliferation and network formation, these new hydrogels combining polysaccharide and protein derivatives appear to be excellent candidates for further development as bioactive scaffolds for vascular tissue engineering. A functional vascular media layer may be developed through the use of these biomimetic hydrogels in combination with EC stimulation.

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Table of abbreviations

2D	two-dimensional
3D	three-dimensional
ADCs	anchorage-dependent cells
α -SMA	α -smooth muscle actin
ECM	extracellular matrix
ECs	endothelial cells
ESCs	embryonic stem cells
FBS	fetal bovine serum
FGF	fibroblast growth factor
GAGs	glycosaminoglycans
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HA	hyaluronic acid
hEGF	human epidermal growth factor
IEL	internal elastic lamina
iPSCs	induced pluripotent stem cells
LO	lysyl oxidase

MMP	matrix metalloproteinase
MSCs	mesenchymal stem cells
PCL	polycaprolactone
PDGF	platelet-derived growth factor
PEG	poly(ethylene glycol)
PEGDM	poly(ethylene glycol) dimethacrylate
PET	polyethylene terephthalate
PGA	polyglycolic acid
PLA	polylactic acid
PLGA	poly(lactic-co-glycolic acid)
PTFE	polytetrafluoroethylene
RGD	Arg-Gly-Asp
SIS	small intestinal submucosa
SMCs	smooth muscle cells
SMemb	nonmuscle-myosin heavy chain B
SM-MHC	smooth muscle-myosin heavy chain
TCPS	tissue culture polystyrene
TEBVs	tissue-engineered blood vessels
TGF	transforming growth factor
VEGF	vascular endothelial growth factor

UV	ultra-violet
VICs	valvular interstitial cells

Chapter 1 – Introduction

1.1 Background

Vascular diseases, particularly coronary and peripheral vascular atherosclerosis, continue to be the leading causes of mortality and morbidity in developed countries today ¹. Synthetic grafts made of polyethylene terephthalate or polytetrafluoroethylene have performed well when used clinically to replace large-diameter (~10 mm or more) blood vessels. However, they have failed *in vivo* in replacing small-diameter (< 6 mm) vessels for a variety of reasons, including thrombosis, compliance mismatch, and neointima formation ^{2,3}. It is believed that grafts capable of dilation and contraction and responsive to vasoactive agents are needed for the replacement of vessels with smaller diameters. Tissue engineering has been proposed as a promising approach to generate biologically functional small-diameter vascular grafts. Significant progress has been made in this field over the past two decades. However, despite this, current tissue engineering approaches are inadequate to yield functional tissue-engineered blood vessels (TEBVs) with both the structural and functional complexity comparable to native blood vessels.

A blood vessel typically has three concentric layers, *i.e.* tunica intima,

media and adventitia. The media is made up of circumferentially oriented smooth muscle cells (SMCs) embedded in extracellular matrix (ECM). It contributes significantly to the vessel's tensile strength and compliance. SMCs in the media respond to vasoactive stimulants and contribute to the contraction and dilation of a blood vessel. In attempting to re-create a native-like biological substitute, many tissue engineering approaches have focused on the regeneration of the blood vessel media layer⁴⁻⁶.

SMCs are an important cellular component of small-diameter blood vessels. However, their mere presence in TEBVs is not sufficient for generating a functional graft. Controlling the phenotype of SMCs is critical for creating a successful blood vessel substitute. The SMC phenotype is plastic and spans a continuum between the two ends of synthetic and contractile with genetic indicia^{7, 8}. This phenotypic plasticity and diversity allow SMCs to perform multiple functions during normal development and under pathological conditions. For the purpose of tissue regeneration, both synthetic and contractile phenotypes of SMCs are needed. The synthetic SMC phenotype is required for the cellular expansion and deposition of ECM components, such as collagen and elastin, to replace the scaffold as it degrades. Before implantation into the body, a transition to a contractile SMC phenotype is required for the engineered vessel to achieve vasoactivity.

In tissue engineering approaches, a scaffold is typically needed for organizing the cells before they create their own ECM. The scaffold should provide initial mechanical support and also supply biophysical and biochemical cues to regulate and manipulate cell behaviors, such as spreading, proliferation, and differentiation⁹. Hydrogels, which are highly hydrated crosslinked polymer networks, are thought to resemble native tissues and are thus increasingly used as scaffolds for cell encapsulation in tissue engineering^{10, 11}.

However, few cell types have been successfully encapsulated inside hydrogels. Chondrocytes, which do not require extensive cell spreading, have been encapsulated within various hydrogels while still showing good viability¹²⁻¹⁷. Anchorage-dependent cells (ADCs), such as fibroblasts and SMCs, require extensive cell spreading in order to avoid apoptosis and to maintain the natural phenotype¹⁸. However, the initial nanometer-scale mesh size of hydrogels, typically smaller than the cell size^{19, 20}, has been shown to promote a round cell morphology and to inhibit cell spreading²¹. Thus it is desirable that the hydrogel degrades during culture to make the space needed for ADCs to spread and proliferate.

Most of the degradable synthetic hydrogels used for cell encapsulation are based on poly(ethylene glycol) (PEG)^{19, 22-41}. For example, hydrogels prepared from copolymers of poly(lactic acid) and PEG are hydrolytically degradable²².

Compared with non-degradable PEG hydrogels, these hydrolytically degradable hydrogels improved the spreading, differentiation, as well as ECM synthesis and spatial distribution of the encapsulated cells^{22, 23, 27}. However, it is difficult to synchronize the rate of hydrolytic degradation with cellular growth and matrix accumulation to match tissue evolution^{24, 26}.

Enzymatically labile hydrogels that are degraded by cell-secreted or exogenously applied enzymes constitute an alternative family of degradable hydrogels which have been shown to support three-dimensional (3D) cell spreading^{19, 25, 28-41}. Many of these synthetic hydrogels are also based on PEG. One such series of proteolytically degradable hydrogel is based on photopolymerization of acrylated PEG derivatives containing peptide substrates (for plasmin or matrix metalloproteinases) in their backbone^{29, 32}. Another PEG-derived protease-degradable hydrogel is based on the Michael addition between reactive thiol groups on cysteine residues of a peptide sequence and vinyl sulfone terminals of end-functionalized PEG^{19, 30, 31, 33}. Fibroblasts, SMCs and mesenchymal stem cells (MSCs) have been shown to spread, migrate, and establish cell-cell contacts within such hydrogels^{19, 39}. Proteins have also been modified to make protease-degradable hydrogels. Seliktar's group^{25, 28, 36, 40, 41} has reported their findings concerning photopolymerizable hydrogels made from PEGylated collagen and fibrinogen. These biosynthetic hydrogels have

been shown to support the outgrowth of dorsal root ganglion cells ⁴⁰, and promote bone formation in a rat segmental bone defect ³⁶. An advantage of a proteolytically degradable hydrogel is the pericellularly localized degradation profile, which enables the tuning of the hydrogel degradation rate to closely match tissue formation rate ³⁵. However, recreating the complexity of native ECM using PEG-based hydrogels is still a challenge as synthetic PEG is inherently bioinert and dissimilar in structure and function to native ECM components.

Dextran, a natural biodegradable polysaccharide with abundant hydroxyl groups, is highly hydrophilic and can be modified to make hydrogels. It typically has a high molecular weight so that relatively good mechanical properties can be expected from its hydrogel. More importantly, dextran is chemically similar to glycosaminoglycans (GAGs) which are important constituents of ECM.

In this thesis hydrogels based on natural dextran and gelatin were developed for SMC encapsulation. Gelatin is capable of promoting cell adhesion and proteolytic degradation and is relatively inexpensive compared to collagen or fibrinogen that has been used by others ⁴². We postulate that the combination of polysaccharide and protein in a composite hydrogel should more closely mimic the natural structure and function of ECM, particularly

proteoglycan, than do PEG-based systems. Detailed studies of the effect of mechanical stiffness of hydrogels on SMC spreading, proliferation, and 3D cellular network formation were carried out. The phenotype of SMCs within the 3D hydrogel environment and in the presence of endothelial cells (ECs) was also investigated.

1.2 Objective and Scope

The overall aim of the study was to design and synthesize novel biomimetic hydrogels based on dextran and gelatin, which were to be capable of supporting extensive spreading, proliferation, and phenotype regulation of SMCs encapsulated within them. Specifically, the objectives of this study were as follows:

(1) Design functionalized dextran and gelatin to produce ultra-violet (UV) photo-crosslinkable hydrogels which can support extensive spreading and proliferation of SMCs encapsulated inside the 3D environment.

(2) Fabricate hydrogels with tunable mechanical stiffness and examine the influence of the mechanical stiffness of the hydrogel on SMC spreading, proliferation and 3D cellular network formation.

(3) Investigate the phenotype change of SMCs within 3D hydrogels as the cells reached maximum density and compared with SMCs on two-dimensional

(2D) culture.

(4) Modulate the spreading, proliferation, and phenotype of SMCs in 3D hydrogels via co-culture with ECs.

1.3 Outline

This thesis has 7 chapters. In the first chapter, the background, scientific aim, and objectives of this PhD project are described. Chapter 2 is the literature review. In Chapter 3 methacrylate- and aldehyde-bifunctionalized dextran (Dex-MA-AD) was synthesized. The capacity of hydrogels based on Dex-MA-AD and gelatin to support 2D EC attachment and promote 3D SMC spreading and proliferation was investigated. In Chapter 4, the followings are described: the synthesis of methacrylated dextran-*graft*-lysine (Dex-MA-Ly) and methacrylamide modified gelatin (Gel-MA), and the influence of mechanical stiffness (specifically shear storage modulus which varies from 898 to 6075 Pa) of hydrogels based on Dex-MA-Ly and Gel-MA on SMC spreading, proliferation and 3D cellular network formation. In Chapter 5 the softer hydrogels from Chapter 4 were used for SMC encapsulation. The cellular phenotype was investigated via relevant gene expressions, and compared with those of SMCs cultured on 2D tissue culture polystyrene (TCPS). In Chapter 6, the impact of ECs on SMCs spreading, proliferation and differentiation in 3D

hydrogels was investigated. The conclusions and recommendations for future works are offered in Chapter 7.

Chapter 2 – Literature review

2.1 Tissue engineering

2.1.1 Overview of tissue engineering

The term “Tissue engineering” was coined by Professor Y. C. Fung of the University of Californian at San Diego in 1985 ⁴³. Later, a review paper in Science on the great medical potential of tissue engineering by Professors R. Langer and J. Vacanti, accelerated the momentum in this field ⁴⁴. Tissue engineering was defined as the re-creation of new tissue or organ by applying engineering principles in combination with biological sciences and technologies.

Tissue engineering has been driven by the need to find replacements for damaged or failed tissue/organs. Currently, organ transplantation or autologous graft from another part of the patient’s own body is applied to replace damaged tissue/organ. However, donor shortage and the risk of complications have limited these procedures. Tissue engineering involves the expansion of harvested cells in temporary scaffolds in the presence of proteins (such as growth factors and adhesive proteins) and other stimulants (such as mechanical stress) to mimic the native microenvironment so as to produce native-like tissue

substitutes. It offers an alternative solution for creating biological tissue or organ substitutes so that the long waiting time or rejection associated with organ transplant can be eliminated. Early tissue engineering efforts included attempts by various pioneers to regenerate the skin, blood vessel, cartilage and liver ^{43,}

45-50

Two major approaches are being used in tissue engineering for cell recruitment. The *in vitro* approach involves seeding cells onto scaffolds outside the human body. Cellular proliferation, phenotype, and spatial organization are controlled so that the cell count increases and extracellular matrix (ECM) production is stimulated to produce a functional graft suitable for transplantation. The other approach is *in situ* tissue regeneration. Scaffolds, combined with growth factors, drugs, or DNA, are implanted with the capacity to recruit local cells *in vivo* upon implantation. In both approaches, the interaction and integration of cells with the scaffolds are critical to the success of the engineered tissue.

2.1.2 Cell source in tissue engineering

Cells are an important component in tissue engineering. Through cellular proliferation, differentiation, and secretion of ECM, biodegradable scaffolds are remodeled to generate new functional tissues. Cells used for tissue engineering

can be autologous, allogeneic, or xenogeneic. For any engineered tissue, it is important to identify the relevant cell types and to obtain sufficient cell numbers. Some cells such as endothelial cells, fibroblasts, chondrocytes, and smooth muscle cells are highly proliferative⁵¹. Isolation of these types of cells from native tissues and expansion *in vitro* are feasible for the regeneration of related tissues. However, maintaining the differentiated cell phenotype in line with the original during *in vitro* expansion is generally difficult. For example, repeated expansion of chondrocytes results in the downregulation of proteoglycan synthesis indicating the reversion to a dedifferentiated phenotype^{45, 52}. Aortic smooth muscle cells undergo phenotypic change and become fibroblast-like cells upon passaging⁵³. For other cells, such as adult cardiomyocytes, neurons, and hepatocytes, proliferation is slow or stagnant^{45, 51}, to the degree that obtaining large numbers of these cells is not easy.

Due to the limited accessibility of cell sources or the dedifferentiation of mature cells, pluripotent stem cells have been investigated as an alternative cell source for tissue engineering. These include embryonic stem cells (ESCs)⁵⁴ and somatic stem cells such as mesenchymal stem cells (MSCs) derived from bone marrow (BM-MSCs)⁵¹ or from umbilical cord (UC-MSCs)^{51, 55}. Stem cells have the capacity for replication as undifferentiated cells and differentiation into various cell types. For example, BM-MSCs can differentiate into adipocytes,

osteoblasts, chondrocytes, smooth muscle cells, and skeletal muscle cells⁵⁵⁻⁵⁷.

Somatic stem cells are more attractive for tissue engineering since there are no ethical and legal concerns such as those associated with the use of ESCs. For stem cells, the purification and regulation of differentiation to the desired cell types are critical in the approaches of tissue engineering.

Differentiated somatic cells have been reprogrammed back to totipotency via transfer of nuclear contents into oocytes⁵⁸ or fusion with ESCs^{59,60}. This indicates that oocytes or ESCs contain certain factors that could induce this reprogramming. The factors essential for the maintenance of ESC identity have been identified^{61,62}. This knowledge contributes to the development of induced pluripotent stem cells (iPSCs). iPSCs are a type of pluripotent stem cell artificially derived by inducing the expression of certain stem cell-associated genes in a non-pluripotent cell, typically an adult somatic cell^{61, 63-66}. For example, iPSCs have been created from mouse fibroblasts^{61, 63, 64}, adult human fibroblasts^{65,66}, and mouse hepatocytes and gastric epithelial cells⁶⁷. iPSCs are important in research and therapeutic applications due to its potential to create patient- and disease-specific stem cells without needing to deal with ethical concerns. However, most of the systems used viral vectors to insert the genes into the host cells, thus increasing the risk of tumorigenicity. Therefore, it is necessary to develop new safe gene delivery methods, such as those using

plasmid⁶⁸.

2.1.3 Scaffolds in tissue engineering

Scaffolds are another important building block used in tissue engineering. They provide mechanical support and guide the 3D organization of the newly forming tissue. Scaffolds can be fabricated from naturally derived materials such as collagen, fibrin, hyaluronic acid (HA) and hydroxyapatite, or from synthetic polymers such as polylactic acid (PLA), polyglycolic acid (PGA) and polycaprolactone (PCL). The chemical, structural, and mechanical properties of scaffolds influence cellular behavior. An ideal 3D scaffold should be biocompatible and capable of being gradually replaced by newly formed tissue after implantation. High porosity and high interconnectivities of scaffolds are required to facilitate cell ingrowth and nutrient diffusion. Porous scaffolds can be fabricated via a variety of techniques such as phase separation, solvent casting combined with porogen leaching, gas foaming, emulsion freeze drying, electrospinning, and 3D micro-printing^{55, 69}.

Scaffolds are fashioned from biomaterials; the use of these has enabled tissue engineering to progress⁷⁰. To date, biomaterials have gone through three generations of development⁷¹. The first generation of biomaterials was developed during the 1960s and 1970s. The physical properties of these

biomaterials were designed to match the replaced tissue, and they were designed to be as inert as possible to minimize degradation or harm to the host⁷¹. The second generation of biomaterials was designed with bioactive components to initiate controlled reactions under physiological conditions, or be resorbable to obtain better integration with the host tissue. Examples of these biomaterials include synthetic hydroxyapatite ceramics to produce bioactive fixation⁷², and biodegradable suture composed of PLA and PGA⁷³.

The third generation of biomaterials has been designed to stimulate specific cellular response at the molecular level by combining bioactive and resorbable properties. The incorporation of cell-specific recognition factors, such as RGD (Arg-Gly-Asp) peptide and fibronectin protein, has been attempted to mimic the ECM to encourage cell attachment and induce cell migration^{74, 75}. Biomaterials have been designed with controlled release of growth factors via either diffusion or degradation of materials in an attempt to mimic natural development or healing⁷⁶⁻⁷⁸. Intelligent growth factor release, such as release based on cellular demand accomplished through cell-mediated proteolytic degradation of the matrix, better emulates the mechanism of growth factor release in tissue repair^{79, 80}. Synthetic materials susceptible to proteases, including plasmin and matrix metalloproteinases (MMPs), have been synthesized to mimic the proteolytic degradation of natural ECM^{32, 81-85}. These

materials are still much simpler than natural ECM. They lack the essential temporal and spatial complexity of natural ECM. However, they have the advantage of promoting 3D cell migration, which is fundamental to the morphogenesis of tissue or organ ⁸⁶.

Biomaterials for tissue engineering are developing rapidly. The current trend is towards designing smart biomaterials which can provide signals for guiding cellular growth and thus tissue regeneration. Further understanding of biology, particularly of tissue morphogenesis and healing, will direct the development of intelligent biomaterials.

2.2 Vascular tissue engineering

2.2.1 Blood vessel structure

Blood vessels have a concentric layered structure (Fig. 2.1A) ^{87, 88}. The innermost layer is called the tunica intima. It has an endothelium on the internal elastic lamina (IEL). The endothelial layer serves as a tight non-thrombogenic barrier inhibiting platelet activation and preventing thrombosis through the secretion of specific molecules like nitric oxide ^{89, 90}. Endothelial cells (ECs) also provide vasoactive molecular signals, such as prostacyclin and endothelin, to the underlying vascular smooth muscle cells (SMCs), and regulate vascular tone ⁹¹. IEL, composed of elastic fibers, is convoluted so that with each dilation

and contraction, the confluent EC layer is still intact and not torn. Hence, the convoluted IEL structure plays an important role in keeping the integrity of a confluent monolayer of ECs under physiological conditions ⁹².

The tunica media, the muscular layer of artery, is separated from the tunica intima by the IEL. A typical tunica media consists of concentrically oriented SMCs densely packed within a network of ECM such as collagen, elastin and proteoglycans. In elastic arteries, the media is subdivided into layered lamellar units (composed of the elastic lamella and adjacent SMCs) (Fig. 2.1B) ^{88, 93, 94}. The larger vessels which experience more stress have the greater number of lamellar units ⁹⁴. SMCs in the media response to stimulating signals secreted by ECs or directly from the nervous system to contract or dilate, which leads to the constriction or dilation of the vessel (*i.e.* vasoactivity) ⁹³.

The external layer is the tunica adventitia, consisting of fibroblasts embedded in a loosely packed collagen matrix. The tunica adventitia helps the anchorage of a vessel to its neighboring tissues, and also provides additional structural support to the blood vessel.

Fig. 2.1 (A) The layered structure of blood vessels. (B) Elastin distribution within the vessel wall. Left is a muscular artery; right is an elastic artery. These pictures are adopted from references ^{87, 88} with permission.

The ECMs surrounding the vascular cells contain primarily collagen, elastin, and some proteoglycans and glycoproteins. The collagen types in blood vessels are mostly fibrillar collagen Type I and III ⁹⁴. They bear most of the stress forces during the dilation and contraction of blood vessels due to their

high tensile strength and elastic modulus. Elastin with a low tensile strength serves mainly as an elastic reservoir and helps distributing stress evenly throughout the vessel wall ^{88, 94, 95}. In the media of muscular arteries (with medium diameters), such as coronary and below-knee arteries, elastin molecules are assembled into fibers. In the elastic arteries (with large diameters), such as the aorta and its immediate major branches, the assembled elastin fibers are further arranged into concentrically organized sheets of elastic lamellae around the media (Fig. 2.1B) ^{88, 96}. Proteoglycans, which are highly hydrated, contribute to vessel compressibility, and together with collagen and elastin maintain the structural framework of the vessel under pulsatile blood flow ². In addition to the above structural matrix, there are also matrix macromolecules which provide regulatory signals to influence cell attachment, polarization, migration, and function. Adhesive glycoproteins, such as fibronectin, laminin, and fibulins, are one type of these matrix molecules.

2.2.2 Vascular smooth muscle cells

SMCs are an essential cellular component in blood vessels. They provide mechanical stability, and regulate the balance of production and degradation of ECM. They also mediate vasoactivity by cellular contraction and relaxation. All these functions are important in vascular development, maintenance, and

remodeling^{7, 8, 97}.

The different functions of SMCs are associated with a diversity of phenotypes. SMCs have been shown to possess the capacity to shuttle between multiple phenotypes, ranging from contractile to synthetic^{7, 8}, which is referred to as phenotype plasticity. In normal blood vessels, SMCs are in the quiescent contractile phenotype. They are characterized by spindle-shaped morphology, low rate of proliferation, low activity of ECM production, and expression of contractile protein, ion channels, and signaling molecules required for contraction⁹⁸. However, in response to blood vessel disease or injury, such as damage to the IEL, SMCs convert to a synthetic phenotype. This results in cobblestone morphology, vigorous proliferation and migration, increased ECM production, and loss of contractile apparatus^{7, 8, 98}. A similar phenotype shift occurs when SMCs are isolated from native vessels and cultured *in vitro*^{99, 100}.

A variety of SMC-specific genes and proteins can be used as markers for SMC phenotype state^{7, 8}. These include the contractile markers α -smooth muscle actin (α -SMA), smooth muscle-myosin heavy chain (SM-MHC), smoothelin, and calponin; many of which are involved in SMC contraction, either as a cytoskeletal component or as a regulator of contraction^{8, 19, 98, 101-103}. When SMCs convert towards a more synthetic phenotype, these contractile proteins have been shown to decrease the expression levels⁸. However,

virtually all these contractile protein markers, possibly except SM-MHC and smoothelin, have been detected in non-SMCs^{7, 8, 104, 105}. For example, the most abundant protein α -SMA in differentiated SMCs is also detected in fibroblasts, endothelial cells, and cardiomyocytes under certain conditions⁷. Nonmuscle-myosin heavy chain B (SMemb) is a suitable synthetic marker whose expression is quickly upregulated in proliferating SMC¹⁰⁶. However, it is also detected in neuronal lines¹⁰⁷.

In vivo, the SMC phenotype is dependent on a variety of biochemical and/or biomechanical signals, such as ECM components, growth factors, cell-cell interaction, and mechanical stress^{6-8, 108}. The degree of phenotypic plasticity of a specific SMC cell line is influenced by the cell origins¹⁰⁹.

2.2.3 Vascular disease and replacement

A variety of events and processes, such as inflammation, injury, or disease, can weaken the blood vessel wall and result in aneurysm and dissection (Aneurysm refers to a localized balloon-like bulge of vessels which increases the risk of vessel rupture; dissection refers to a tear in the wall of a blood vessel.). But occlusion caused by atherosclerosis, a chronic inflammatory response in the arterial wall, is the most common cause of blood vessel failure. Atherosclerosis is characterized by endothelial dysfunction, vascular

inflammation, and the buildup of lipids and cellular debris – plaque – at the intima of the vessel wall. This results in decreased blood vessel luminal space and consequent decreased flow of blood to downstream tissues. It can lead to serious problems including heart attacks, strokes, gangrene, or even death. The prevalence of atherosclerosis is increasing in an ageing society¹¹⁰; cardiac and peripheral atherosclerosis are, today, the leading causes of death in the Western World.

Functional vascular grafts are required to replace disease-affected peripheral or coronary vessels. Allogeneic grafts have demonstrated a long-term potency but their clinical use is hampered by their high immunorejection and the consequent need for patients to take immunosuppressant drugs. Autografts, predominantly from saphenous veins or radial arteries, are currently the most widely used benchmarks for bypass grafting^{3, 111}. However, the limited availability of suitable vessels and the undesirable side effects arising from the removal of healthy vessels from their normal locations have hindered its application. Synthetic conduits made from expanded polytetrafluoroethylene (ePTFE, also called Teflon) and polyethylene terephthalate (PET, with the tradename Dacron) have been widely used with great success for larger-diameter (>6 mm) vessel replacements¹¹²⁻¹¹⁴. However, the use of synthetic grafts suffers from a high failure rate due to thrombus and plaque

formation when applied to small-diameter (<6 mm) vascular replacements.

Initial attempts were made to solve the high failure rates associated with small-diameter synthetic grafts. These focused on endothelialization (that is seeding ECs onto the lumen wall of synthetic grafts), and the use of biomaterials which improve blood compatibility and inhibit thrombosis¹¹⁵⁻¹¹⁷. The success of endothelialization has been limited due to weak attachment and poor retention of human ECs on the surface of grafts. In addition, compliance mismatch between synthetic grafts and native vessels has led to anastomotic hyperplasia (unusual SMC proliferation), which increases the likelihood of thrombotic stenosis or occlusion¹¹⁸. These limitations have led to the development of tissue-engineered vascular grafts which offer the possibility of overcoming the limitations associated with recent and current options.

2.2.4 Tissue engineering of small-diameter vascular grafts

Due to substantial clinical needs and the disappointing use of synthetic grafts, tissue engineering has been utilized to produce biological grafts to replace damaged or diseased small-diameter blood vessels^{119, 120}. By using autologous cells with or without a scaffold, tissue engineering introduces the possibility of creating a substitute with both structural and functional similarities to native vessels. Much progress has been made over the last two

decades^{1, 3, 93, 118, 121-123}. Some approaches have been pursued which can be grouped into two categories: (1) scaffold-based tissue engineering; and (2) non-scaffold-based “tissue self-assembly”.

2.2.4.1 The ideal tissue-engineered vascular grafts

An ideal tissue-engineered blood vessel (TEBV) should meet several requirements^{6, 112, 121, 123-126}. It must be nonthrombogenic which is associated with a confluent endothelial lining⁹³. It must have the capability for inducing healing upon implantation and then lead to the integration of the implant into native tissues. It must have the appropriate mechanical properties to sustain hemodynamic stress without rupture. An arterial graft is typically required to withstand normal blood flow in pressures ranging from 80 to 120 mmHg. It should possess a burst strength of more than 1700 mmHg (similar to that of saphenous vein), and a suture retention strength of 3N^{127, 128}. The compliance (the capacity of a vessel to distend and increase volume with increasing transmural pressure) of a TEBV is also an important parameter. This is because mismatching between the native vessel and a graft has been reported to induce initial hyperplasia and cause graft failure¹²⁹. For males aged 40-80 years at 150 mmHg, the compliance is in the range of 0.24-0.46 MPa⁻¹⁶.

Additionally, a TEBV must exhibit vasoactivity (that is vasoconstriction/

dilation) upon implantation. Contractile SMCs within a TEBV are required for vasoactivity. However, in vascular tissue engineering, it is required that initially SMCs proliferate to obtain a large population and enough ECM components to build up the tissue. Therefore, the modulation of SMC phenotype is paramount in vascular tissue engineering.

Finally, the manufacture of the grafts must be done in a relatively short time and the cost must be not too high. Although tissue engineering of blood vessels has been an active focus of research for many years, currently there is no TEBV that meets all the requirements. In the following sections, the approaches applied towards developing biological vascular grafts will be highlighted, together with the causes of failure.

2.2.4.2 Scaffold-based tissue-engineered vascular grafts

In a typical vascular tissue engineering approach, cells are combined with 3D scaffolds which initially serve as templates that finally degrade, resulting in a neovessel¹³⁰. Scaffolds can be made of either naturally derived or synthetic materials. Natural materials have the advantage of bearing biological features. Synthetic materials, however, eliminate the immunogenic characteristics associated with natural materials and offer a great degree of control over the scaffold properties. Designing scaffolds with the capacity to guide vessel

regeneration is a challenge in vascular tissue engineering.

Protein-based hydrogel scaffolds

Fibrillar structural proteins such as collagen and elastin are abundant and important in blood vessels. The reconstruction of protein matrices with embedded cells was one of the earliest approaches that have been applied in vascular tissue engineering^{46, 93}. Living cells were suspended in a protein solution and the suspension was poured into a mold. The microenvironment conditions, such as the pH level and temperature, could then be modulated and a protein matrix with evenly dispersed cells was reconstructed. Cells can then attach to the proteins, migrate through the matrix and/or remodel the scaffold.

Collagen type I is often used in tissue engineering due to its abundance in native tissues and easy process of isolation and reconstruction. In the case of blood vessels, the pioneering work was done by Weinberg and Bell⁴⁶. A multilayer tubular structure was made, composed of collagen embedding SMCs and fibroblasts as the medial and adventitial layers respectively. An EC layer seeded on the luminal surface was created that resembled the structure of a native blood vessel. Certain features, the healthy and well differentiated SMCs and functional ECs, made this model attractive. However, the lumen did not have a convoluted elastic surface; it could not, even in principle, maintain a competent and perfectly intact endothelium under biological pressure and

diameter variations^{92, 131}. Imperfect endothelialization was also because of the thrombogenicity of the ECM proteins of the medial layer. Further, this model had poor mechanical properties. Dilation and rupture were observed at less than 10 mmHg of intraluminal pressure. Embedding a Dacron mesh into the wall did increase the burst pressure to 40~70 mmHg. The burst pressure was further reinforced to 120~180 mmHg when two Dacron meshes were embedded separately in the collagen matrix⁴⁶. However, the improved burst pressure was still significantly lower than that of human saphenous vein (1680 mmHg) or native arteries (>2000 mmHg)^{3, 124, 132}.

In order to improve the mechanical properties of such constructs, a variety of methods have been used¹³³⁻¹³⁹. Crosslinking of the protein matrix is one way but this should be done in a way that avoids damage to the cellular components. Non-enzymatic crosslinking of proteins by reducing sugars^{140, 141}, and enzymatic crosslinking using lysyl oxidase (LO)¹⁴² have been explored. Although the results of these studies were encouraging, crosslinking alone has not resulted in the production of protein hydrogels with mechanical properties adequate for implantation. The possible reasons for low mechanical properties of collagen-based TEBVs are suppressed ECM production (especially elastin) and lower SMC density due to poor proliferation^{88, 121, 143-145}.

Fibrin has also been explored for the fabrication of hydrogels in vascular

tissue engineering. Fibrinogen is the precursor of fibrin. The enzyme thrombin when added cleaves fibrinogen, leading to insoluble fibrin which assembles into protein fibers with diameters ranging from 40 to 400 nm¹²¹. The technique for creating fibrin-based hydrogel constructs is similar to creating collagen-based hydrogel, except that the fibrinogen solution with SMCs suspended is mixed with thrombin solution before injection into the mold^{4, 146}. The gel structure can be manipulated by adjusting the concentrations of thrombin¹⁴⁷ and calcium ion¹⁴⁸, as well as the ionic strength. The degradation of fibrin gels can be controlled through the use of fibrinolytic inhibitors such as aprotinin or ϵ -aminocaproic acid¹⁴⁹. Fibrin binds a variety of ECM proteins (such as fibronectin) and growth factors (for example the fibroblast growth factor (FGF) and the vascular endothelial growth factor (VEGF))¹²¹. It has been reported that fibrin stimulates collagen and elastin biosynthesis of SMCs¹⁴⁵. Finally, it is possible to get fibrin from a patient's own blood. All these make fibrin an attractive scaffold material for vascular tissue engineering.

Decellularized native tissues

Decellularized native tissues have been applied to tissue engineering both experimentally and clinically¹⁵⁰⁻¹⁵². They are made up of natively organized structural and functional ECM proteins obtained by the removal of cells from a native tissue. Decellularized tissues are typically implanted into the body

without cells, and are assumed to be remodeled by in-migrating host cells following implantation due to their natural compositions.

For the tissue engineering of blood vessels, decellularized tissues of either vascular or nonvascular origin can be used. Arteries (for example the carotid and aorta) and veins (such as the jugular) from porcine and canine species are used widely. This has the benefit of maintaining the natural composition and structure of blood vessels. However, the ultimate tensile strength and compliance may be reduced following the process of decellularization ¹⁵³. Additionally, the use of xenografts resulted in infection, thrombosis, and aneurysm formation ^{112, 154}.

Small intestinal submucosa (SIS) is another widely used decellularized tissue for vessel substitutes. SIS contains about 90% collagen (primary type I), fibronectin, glycosaminoglycans, proteoglycans, and glycoproteins ¹⁵⁵, as well as various growth factors ¹⁵⁶. SIS has been implanted in sheep, and showed progressive remodeling with moderate and regressive neointimal formation and moderately stable endothelialization ¹⁵⁷. However, poor understanding of the thrombogenic properties and immune response to exogenic collagen are problems associated with SIS ¹⁵⁸.

Biodegradable polymer scaffold

Several biodegradable polymer scaffolds have been applied to vascular

tissue engineering. Many properties, including molecular weight, microstructure, mechanical properties, and degradation of the synthetic scaffolds, can be tailored to enhance tissue ingrowth and remodeling ¹⁵⁹. However, toxic monomers or catalyst and degradation byproducts may alter the local cellular microenvironment and cellular functions ^{160, 161}. Typically, cell entrapment during scaffold formation is not feasible, since the scaffold fabrication conditions are too harsh for cell survival. Cellularization is generally accomplished by subsequent cell-seeding *in vitro* or cell-invasion *in vivo* which may result in suboptimal cell distribution ¹⁶².

Polyglycolic acid (PGA) is the most widely investigated synthetic scaffold material ¹⁶³⁻¹⁶⁵. PGA can be easily fabricated into different shapes. However, its degradation is very rapid which may lead to the breakdown of the scaffold before cells have produced a structurally competent tissue. Furthermore, the accumulation of acidic degradation products would lead to cell toxicity ¹⁶⁶. Several approaches have been taken in attempt to improve the mechanical properties as well as the biocompatibility of PGA. These include the copolymerization of glycolic acid (GA) with L-lactic acid (LA) to make poly(lactic-co-glycolic acid) (PLGA) ¹⁶⁷ and the blending of PGA with other polymers ^{168, 169}. However, these synthetic grafts are relatively noncompliant compared with native vessels. This may result in intimal hyperplasia and

decrease the patency of the grafts in the long-term¹⁷⁰.

2.2.4.3 Cell sheet-based tissue-engineered vascular grafts

In 1998, L'Heureux *et al*¹⁷¹ reported a novel approach to develop vascular graft that was exclusively based on the use of cultured human cells. By culturing human vascular SMCs with ascorbic acid (in order to decrease collagen production), a cellular sheet was produced. Wrapping this sheet around a tubular support resulted in a concentric vascular media layer. In the same manner, human fibroblasts were grown to form a sheet which was placed around the constructed media layer to create the adventitia layer. After maturation, a single cohesive layer was formed through fusion of the SMCs and fibroblasts layers. Finally, the tubular support was removed and the luminal surface was seeded with ECs. This sheet-based blood vessel construct had a three-layered structure which was well organized. Numerous ECM proteins, including elastin, were detected within this construct. The complete vessel had burst strength of more than 2000 mmHg which is comparable to human vessels. Furthermore, these grafts were biologically functional and responded to vascular agonists^{172, 173}. When implanted in a canine model, the graft showed good handling and suturability. However, blood infiltrated intramurally in the wall of the vessel; and a low patency rate (50%, 7 days after implantation) was

observed¹⁷¹. This model has the same problem as all constructs with a smooth and unconvoluted luminal surface. This is that pressure variation-induced luminal expansion pulls the ECs away from each other, which can expose the thrombogenic ECM proteins to the humoral clotting cascade.

Sheet-based vascular tissue engineering has been improved by eliminating the need for SMCs¹⁷⁴. This tissue-engineered blood vessel consisted of a living adventitia, a decellularized internal membrane, and an endothelium. Both the adventitia and the internal membrane were obtained from autologous fibroblasts sheets. The mechanical properties were comparable to human vessels. Complete tissue integration, formation of vasa vasorum, and regeneration of vascular media were obtained *in vivo*. However, the time required to produce this autologous tissue-engineered blood vessel (about 28 weeks) was too long for urgent clinical use. In addition, the long-term patency and vasoactivity of this TEBV have yet to be investigated.

2.2.4.4 Summary

Currently, there is no tissue engineering approach that has yielded vascular grafts meeting all the requirement of an ideal TEBV. For example, high burst strength and compliance match seem to be two conflicting characteristics: compliant grafts often lack high burst strength¹⁴¹ whereas grafts with high burst

strength are often noncompliant¹⁷¹. In native blood vessels, the main determinant of mechanical properties is the composition and orientation of ECM and cells in the vessel wall (mainly the media). The SMCs in the media are responsible for the synthesis of large amount of collagen (which offers tensile stiffness to the vessel⁹⁴) and elastin (which provides compliance⁹⁴). However, in current vascular tissue engineering approaches, SMC proliferation and secretion of ECM were unsatisfactory¹²¹.

The biological functions of TEBVs are concerns that are beyond mechanical properties. In order for a TEBV to perform vasoactivity, SMCs must be of the contractile phenotype. Therefore, the phenotype modulation of SMCs in vascular tissue engineering is vital. Initially, SMCs should be highly proliferative and capable of synthesizing large amounts of ECM to build up the vessel wall. However, proliferative SMCs do not carry out vasoactivity. They must be induced to possess a contractile phenotype before implantation in order to avoid intimal thickening and to allow the TEBV to function^{6, 175}. Modulation of SMC phenotype switch to carry out all the required SMC functions at the appropriate stage has not yet been realized in current tissue engineering approaches.

SMCs phenotype and function are influenced by the local chemical and mechanical environment, such as growth factors, ECM components, and

cell-cell interactions. For example, collagen type I, collagen type IV and laminin promote a more contractile phenotype of SMCs, whereas fibronectin has been shown to promote a more synthetic SMC phenotype^{8, 176, 177}. Compared with collagen gel, fibrin gel promoted elastogenesis¹⁴⁵. Creating a microenvironment which could modulate SMC phenotype and function may lead to the success of TEBVs.

2.3 Hydrogels for cell encapsulation

Tissue engineering has benefited greatly from the use of three-dimensional (3D) scaffolds which provide a template for cell adhesion, proliferation, migration, and differentiation. Designing of scaffolds with the capacity to recapitulate the native cellular microenvironment is a current trend¹⁷⁸. Hydrogels created from a variety of hydrophilic polymers, either naturally derived or synthetic, are the most appealing candidates as scaffolds for tissue engineering. They have many advantages: high water content which promotes facile mass transport; tunable gel chemistry; and, most importantly, tissue-like environment suitable for cell survival. All of these features result in their increased use in tissue engineering^{11, 179-181}.

Hydrogels can be created via crosslinking, either physically or chemically, in the presence of living cells, so that direct cell encapsulation within the 3D

environment is realized. In a typical cell encapsulation strategy, cells are suspended in a precursor solution which is then gelled to entrap cells within the hydrogel matrix. There are several requirements that one should consider when using hydrogels for cell encapsulation. Firstly, the precursor solution must be non-toxic to the cells. Secondly, the gelation process must be mild and not harmful to the cells. Thirdly, the physical and chemical structure of hydrogels must be suitable for cell survival. In the following sections, important considerations for the rational design of hydrogels for cell encapsulation will be described.

2.3.1 Gelation mechanism of hydrogels

Hydrogels can be formed through various mechanisms. For example, through ionic interaction, hydrophobic interaction, hydrogen bonds or covalent bonds¹⁸². For cell encapsulation, gelation should be conducted under mild conditions so that living cells can be present without suffering damage.

Ionic interaction

Extracted from seaweeds, alginate consists of guluronic (G) and mannuronic (M) acids organized in various sequences and relative percentages. When divalent ions (for example Ca^{2+}) are present, the interaction with G blocks will lead to the formation of hydrogels¹⁸³. It can also be crosslinked by

positively charged molecules, such as chitosan^{184,185} and poly-L-lysine¹⁸⁵. Because of the gentle gelation, alginate hydrogels have been used for cell encapsulation¹⁸⁴⁻¹⁸⁶.

Molecular self-assembly

Certain proteins and peptides can self-assemble to form stable hydrogels¹⁸⁷⁻¹⁹¹. This self-assembly is driven by non-covalent bonding, which includes four commonly mentioned interactions: ionic bonds, hydrogen bonds, van der Waals forces, and hydrophobic interactions¹⁹². Collagen is one such protein and was applied earliest in cell encapsulation^{26,93}. Collagen molecules form a triple helix structure with three helically wound proline-rich polypeptide strands. In each of the three chains, the amino acid sequence Gly-X-Y is repeated multiple times, where X and Y are often proline and hydroxylproline respectively. Glycine amino forms a hydrogen bond with the X-position amide carbonyl on an adjacent strand. The triple helix is stabilized by stereoelectronic effects and water-bridged hydrogen bonds¹⁹³. Individual collagen molecules self-assemble to generate fibrils, which are further strengthened by disulfide links between chains¹⁹⁴.

As alternatives to natural proteins, synthetic peptides have been created with the capacity to form hydrogels through self-assembly under suitable conditions^{187-192, 195, 196}. Some self-assembling peptides have unique amino acid

sequences with hydrophobic and hydrophilic side groups located alternatively. The hydrophilic groups include alternating positively and negatively charged ones. In aqueous solutions, they form the β -sheet structure with one hydrophilic and one hydrophobic surface. Exposure of the self-assembly peptides to a salt solution or physiological media typically accelerates the formation of interweaving nanofiber structures. The diameter of the self-assembled fibers is typically around 10 nm, and pores of the resulting scaffolds range from 5 to 200 nm in diameter^{187, 192}. The self-assembling peptide scaffolds have a high water content (>99.5%) and, more importantly, possess a structure similar to native ECM. In addition, specific cell-matrix interactions can be accomplished through the designing of peptide sequences^{196, 197}. These hydrogel scaffolds are potentially useful in 3D cell culture. Bovine chondrocytes encapsulated in peptide hydrogels have shown a differentiated phenotype and abundant ECM production (Fig. 2.2)¹⁸⁷.

Fig. 2.2 (A) Model structure of a self-assembling peptide molecule. Hydrophobic and hydrophilic residues locate alternatively on the backbone. The peptide promotes β -sheet formation. K: hydrophilic positively charged lysines; D: hydrophilic negatively charged aspartic acids; L: hydrophobic Leucines. (B) A chondrocyte-encapsulated peptide hydrogel construct. (C) Image of chondrocytes encapsulated in the peptide hydrogel. These pictures are adopted from reference ¹⁸⁸ with permission.

Radical chain polymerization

Radical chain polymerization is the most widely used crosslinking method for the fabrication of hydrogels for cell encapsulation. In this strategy, functional monomers with two or more vinyl groups are crosslinked in the

presence of initiators and suitable initiating signals, such as light or heat. Upon exposure to the initiating signals, radicals are generated by the reaction of the initiator, which initiate the kinetic chain propagation. Initiators are generally small molecules with high reactivity but may be cytotoxic. Chain transfer to the membrane proteins or molecules of cells during polymerization can cause deleterious effects ¹⁹⁸. By controlling the chemistry and concentration of initiators, as well as the intensity of initiating signal, polymerization can be modulated to result in a fast gelation (seconds to several minutes). This could minimize damage to living cells ¹⁸⁰. There are further requirements for the monomers. They must be water soluble. Their molecular weight should not be too low (> 2-3 kD), as molecules with lower molecular weight are more liable to cellular internalization which may lead to cell damage ^{11, 180}.

Several initiators are used for cell encapsulation. These include: 2, 2-demethoxy-1-phenylacetophenone (DMPA or Irgacure 651) ^{32, 198}, 2-hydroxy-1-[4-(hydroxy ethoxy)phenyl]-2-methyl-1-propanone (Irgacure 2959) ^{198, 199}, eosin-Y/triethanolamine/N-vinyl-2-pyrrolidinone (NVP) ^{20, 200}, and ammonium persulfate (APS)/N,N,N',N'-tetramethylethylenediamine (TEMEDA) ^{13, 201}. Irgacure 2959 is the most widely used UV-induced photoinitiator. It is tolerated comfortably by many cell types during cell encapsulation ^{198, 202}. The toxicity of Irgacure 2959 towards cells is dependent

on its concentration and UV exposure time¹⁹⁸. Besides, cells vary in sensitivity to photoinitiators due to their different expressions of antioxidant enzymes¹⁹⁸. DMPA was reported to be more toxic than Irgacure 2959^{198, 202}, though successful encapsulation of rat aortic SMCs has been reported using DMPA in NVP with a short UV exposure (20 s)³².

Instead of UV irradiation, visible light has also been used to initiate crosslinking. The eosin-Y/triethanolamine/NVP system is the commonly used visible light initiating system. APS/TEMEDA is a redox-initiating system. APS is an oxidizing reagent while TEMEDA is a reducing reagent. When the two are mixed, active radicals are formed to initiate polymerization. This system has been explored for the encapsulation of a variety of cells, including chondrocytes¹³ and marrow stromal stem cells²⁰³. The cytotoxicity was dependent on initiator concentrations²⁰³. Choosing an appropriate combination of initiating system and reaction condition is the first step towards successful cell encapsulation involving radical chain polymerization.

Mixed mode polymerization

As an alternative to chain polymerization, mixed mode polymerization, combining radical mediated chain growth and step-growth polymerization mechanisms, has been employed to form hydrogel networks^{204, 205}. This gelation is based on the copolymerization of multifunctional macromers

containing thiols and (meth)acrylate functionalities. Two competing reactions are involved: acrylate radical homopolymerization and thiol-acrylate step growth reaction. Since propagation and chain transfer occur simultaneously, the resulting kinetic chains are shorter than in homopolymerization, which is important in tuning the degradation process and the ultimate degradation products (Fig. 2.3)^{180, 205}. Further, by changing the stoichiometric ratio of thiol to acrylate, the network structure could be easily modified. More importantly, thiol-containing peptides can be introduced into the networks of hydrogels either in a pendant fashion or as crosslinkers, so that biological functionalization of the networks can be realized^{204, 206-208}. In addition to the advantages in manipulating network structures, thiol-acrylate mixed mode polymerization could occur without photoinitiators, which resolves the drawbacks associated with photoinitiators, such as toxicity²⁰⁹.

Fig.2.3 Schematic representations of hydrogel networks and their degradation products formed through (a) chain-growth polymerization, (b) step-growth polymerization, and (c) mixed-mode polymerization. These pictures are adopted from reference²⁰⁵ with permission.

MSCs have been successfully encapsulated within hydrogels through the mixed mode polymerization of PEG diacrylate and cysteine-containing peptide^{204, 207, 208}. However, when a higher peptide concentration (15 mM) was used for crosslinking, dramatic cell death was observed. This may be due to the effect of excess thiols on the pH of the system²⁰⁴. Control of the stoichiometric ratios of thiol to acrylate has been shown to be necessary to create a healthy environment for cells.

Chemical reaction

Chemical reactions have been employed to fabricate hydrogels for cell encapsulation. For example, N-carboxyethyl chitosan has been crosslinked using oxidized dextran through Schiff base formation at physiological pH and body temperatures²¹⁰. Gelation time which could be as short as 80 seconds was dependent on the degree of oxidation of dextran. Mouse fibroblasts encapsulated in these hydrogels showed high cellular viability for at least 3 days of culture. Alternatively, a disulfide crosslinking strategy has been explored for preparing hydrogels²¹¹⁻²¹³. Hyaluronic acid was modified with thiol groups, which could be oxidized in air to form disulfide linkages²¹¹. The disulfide crosslinked hydrogel can be formed under physiological conditions without additional crosslinking agents, and no by products were produced during crosslinking. Murine fibroblasts have been encapsulated within

disulfide-crosslinked hydrogels but the gelation time required was rather long (about 30 minutes). Cells remained viable and proliferated during the 3 days of culture though they maintained a round shape which is different from the normal spindle morphology²¹¹.

Hubbell's group has explored the Michael-type addition reaction for cell encapsulation^{30, 84, 214}. This is a mild and highly self-selective chemical reaction between a thiol and an unsaturated ester. In fact, the deprotonated thiols (thiolates) rather than the thiols are the reactive species, so that gelation process can be modulated by pH and pKa of the thiol group. Both of these are made adjustable by the presence of differently charged amino acid residues in close proximity to the thiol^{30, 214}. Typically, a decrease in the pKa value accelerates the gelation. Thiol-containing peptides, proteins or polysaccharides and vinyl sulfone or acrylate terminated PEGs have been used as macromers for cell encapsulation^{30, 33, 215}.

Click chemistry has recently been explored for direct cell encapsulation²¹⁶. Click chemistry refers to a group of reactions with high selectivity and orthogonality (Fig. 2.4A)²¹⁷, such as the 1,3-dipolar Huisgen cycloaddition between azides and alkynes²¹⁸. Click chemistry has been widely used in drug discovery and material science²¹⁷. However, the synthetic schemes are often toxic. For example, the Huisgen cycloaddition between azides and alkynes

requires the use of a copper catalyst which is cytotoxic. The development of an activated alkyne containing a difluorinated cyclooctyne moiety (Fig. 2.4B) has overcome this drawback. The ring strain and electron-withdrawing fluorine enable the reaction with azides to occur under Cu-free conditions ²¹⁹. A difluorinated cyclooctyne-containing peptide has been reacted with four-arm PEG tetra-azide to form hydrogels under physiological conditions ²¹⁶. Gelation occurred in less than 5 minutes, and 3T3 fibroblasts have been successfully encapsulated in the hydrogels and shown a spreading morphology.

A

B

Fig. 2.4 (A) A selection of reactions which meet the requirements of click chemistry. This picture is adopted from reference ²¹⁷ with permission. (B) Click reaction between an azide and a difluorinated cyclooctyne moiety without Cu catalyst. This picture is adopted from reference ²¹⁶ with permission.

2.3.2 Structure and chemistry of hydrogels

Hydrogel-based cell encapsulation involves the entrapment of cells within the 3D networks of hydrogels. The structure and chemistry of hydrogels will largely dictate cell behaviors, such as spreading, proliferation, migration, differentiation and ECM production ^{11, 35, 181}. As one example, the ECM production and distribution of chondrocytes embedded in PEG hydrogels were found to vary, depending on the crosslinking density ²²⁰. An increase in collagen type II production was observed in intermediately crosslinked hydrogels. Only a lower crosslinking density facilitated the homogeneous distribution of cell-secreted glycosaminoglycans (GAGs).

Hydrogels should have suitable mechanical stiffness to support cell migration, proliferation, and differentiation ²²¹. The initial mechanical

properties and mesh size are determined by crosslinking density. The mesh size, which is a measure of the distance between crosslinking points, affects the diffusion of nutrients, biological signaling molecules, and cell-secreted molecules. An increased crosslinking density results in increased mechanical stiffness but decreased swelling and mesh size. Higher mechanical stiffness is advantageous in bearing mechanical loads *in vivo*, but the associated lower swelling and diffusion capabilities may impair cell viability and tissue growth¹⁹. Therefore, the design of hydrogels requires a balance: sufficient mechanical stiffness is needed, but cell viability and macroscopic tissue development should not be impaired.

In addition to physical properties, chemical properties are also important design parameters regarding hydrogels for regulating cell functions. Most hydrogels resist protein adsorption due to the hydrophilic nature of the building blocks. In addition, these building blocks are generally bio-inert without cell adhesion receptors, leading to a non-adhesive environment in which cells are constrained to a round morphology²⁶. This would be advantageous for anchorage-independent cells, such as chondrocytes, in that a spherical morphology is the native state and the outstretching may lead to dedifferentiation⁵². Furthermore, spherical cellular morphology in hydrogels is beneficial to the chondrogenic differentiation of stem cells²²².

However, many cells are anchorage-dependent. They must attach to a substrate and spread in order to survive and maintain their normal phenotype^{223, 224}. For example, although agarose hydrogels have been reported to promote chondrogenic differentiation²²², the proliferation as well as the expression of bone markers of osteoblasts in such gels was inhibited²²⁵. hMSCs encapsulated in PEG hydrogels decreased their viability over time²⁰⁷. In order to overcome this impediment, proteins or peptide sequences containing binding sites for cellular receptors have been incorporated into hydrogels^{28, 30, 35, 214}. For example, tethered RGD peptide was incorporated into PEG hydrogels which had sustained a high level of hMSCs survival^{207, 208}. Other biological cues, such as growth factors, have also been added into hydrogels in attempt to control cell function and tissue morphogenesis^{226, 227}.

2.3.3 Degradation of hydrogels

Generally, the mesh size of hydrogels is in the range of several to hundreds of nanometers, which is much smaller than a typical cell¹¹. For anchorage-dependent cells encapsulated inside hydrogels, cell spreading not only requires adhesion molecules, but also calls for enough space. The degradation of hydrogels will result in increased mesh size, providing space to facilitate cell spreading, migration and ECM diffusion. The degradability and

degradation rate influences cell behaviors. For example, MSCs adopted a spherical morphology when encapsulated in PEG hydrogels that present limited degradation. With sufficient degradation, however, migration, formation of cell-cell junction, and more osteoblastic-like phenotype were obtained ²²⁸. Understanding and manipulating the degradation profiles of hydrogels are important. The degradation of hydrogels should be tuned to match the rate at which the encapsulated cells are producing ECM. If degradation is too rapid, hydrogels may dissolve before macroscopic tissue formation takes place. On the other hand, slow degradation would delay cell spreading and proliferation, which would be problematic in many clinical applications. Furthermore, slow degradation would limit the diffusion of cell-secreted ECM to pericellular regions, a situation that may also influence cell function ²²⁹.

Hydrogels formed through physical crosslinking may undergo dissolution reversing the process of gelation due to changes of the environment. For example, new tissue formation can result in the substitution of monovalent cations for divalent cations, causing the breakdown of alginate gels ¹¹. Hydrogels can also be rendered degradable either hydrolytically or enzymatically with the incorporation of functional blocks. For example, PEG has been used to initiate copolymerization with lactide, glycolide and caprolactone to form triblock copolymers. These are then end-capped with

polymerizable groups so as to form hydrolytically degradable hydrogels^{22, 24, 201, 230, 231}. The degradation rate is controllable through manipulating the structure and chemistry of the block copolymer, such as increasing the percentage of ester bond^{22, 23, 27}. The degradation rate was found to influence the differentiation of osteoblasts²², as well as the distribution of chondrocyte-synthesized ECM in neocartilaginous tissue²³. For hydrolytically degradable hydrogels, degradation occurs immediately after exposure to aqueous solution at a rate predetermined by the macromonomer chemistry. It is difficult to synchronize the degradation rate with cellular growth and ECM deposition, since predicting the exact degradation rate required for a specific cell line is not currently possible. The fact that different cells produce ECM at different rates makes the situation more complicated. Moreover, the released acidic degradation products of hydrogels with ester bonds may influence cell functions.

A more robust degradation scheme of hydrogels has been achieved using exogenously added enzymes²³². One example is the hydrogel with exogenous enzyme-triggered degradability that is formed through polymerization of dimethacrylated polycaprolactone-*b*-poly(ethylene glycol)-*b*-polycaprolactone. A lipase enzyme can catalyze the cleavage of caprolactone ester bonds²³³. Manipulation of the degradation rate to match the rate of ECM deposition by

the exogenous delivery of lipase is feasible. However there are limitations, such as the poor diffusion of lipase in hydrogels with higher crosslinking density.

Hydrogels sensitive to cell-secreted or cell-activated enzymes (such as MMPs, serine protease, and hyaluronidase) make up an alternative family of degradable hydrogels, leading to pericellularly localized degradation^{19, 25, 28-39, 41, 234}. In these hydrogels, the localized degradation profile, imitating the proteolytic recognition of natural ECM, dynamically matches the ECM deposition pattern³⁵. This approach is beneficial since the mechanical integrity of hydrogel is sustained during the process of cell-triggered remodeling. These hydrogels are created from natural materials (for example hyaluronic acid which is degradable by cell-secreted hyaluronidase) or synthetic polymers containing blocks susceptible to cell-secreted protease (such as protein or peptide sequence that is cleavable by MMPs)^{19, 36, 39, 234}.

Recently, photolytically degradable hydrogels with dynamically tunable physical and chemical properties have been developed by Anseth²³⁵. By incorporating a photolabile moiety into PEG macromer, photodegradable hydrogel was formed, as shown in Fig. 2.5. Upon exposure to light, photolabile backbone was cleaved resulting in a decrease of the network crosslinking density. The stiffness, water content, and diffusivity of this hydrogel were changed. All this induced a significant morphology change of encapsulated

hMSCs from spherical to spreading (Fig. 2.5F and G). These photodegradable hydrogels enable the dynamic and external control over cell-material interactions.

Fig. 2.5 Synthesis and degradation of photodegradable hydrogel. (A) The base photodegradable monomer. (B) The photodegradable crosslinking macromer obtained by attaching carboxylic acid of monomer A to poly(ethylene

glycol)-bis-amine. (C) Photolytic cleavage of hydrogel upon irradiation. (D) The degradation of hydrogel can be controlled by irradiation intensity and wavelength. (a) 365 nm at 20 mW/cm², (b) 365 nm at 10 mW/cm², (c) 405 nm at 23 mW/cm². (E) Continuous (a) or periodic (b) irradiation modulates hydrogel degradation. (F) hMSCs exhibit a spherical morphology encapsulated within hydrogel. (G) hMSCs spreading was promoted through irradiation (480 s, 365 nm at 10 mW/cm²). These pictures are adopted from reference ²³⁵ with permission.

In addition to the degradation profile, degradation products also influence cell function, especially for hydrogels based on biopolymers, such as hyaluronic acid (HA) and chondroitin sulfate. HA is a polysaccharide involved in a variety of biological processes *in vivo*. During wound healing, HA influences cellular differentiation, proliferation, and migration ²³⁶. It also promotes angiogenesis through its degradation products ²³⁷. Certain cells like the valvular interstitial cell (VIC) have receptors to internalize HA (that is CD44 and receptor for HA-mediated motility) ²³⁸. In one study, the degraded fragments of HA, as well as methacrylate group modified HA (HA-MA), were found stimulating the growth of VICs in a molecular weight-dependent fashion ²³⁸. Lower molecular weight HA fragments (< 27 kDa) induced greater stimulation of VIC proliferation and synthesis of elastin than did higher molecular weight HA fragments. HA-MA was used to encapsulate VICs through photopolymerization.

The crosslinking density of the hydrogel is tunable, which influences the molecular weight of the degradation products (Fig. 2.6)¹⁸⁰. A high crosslinking density creates fragments with lower molecular weight than does a low crosslinking density. Therefore, it is possible to control the ECM production of encapsulated VICs through the modulation of the crosslinking structure of HA-MA hydrogel.

Fig. 2.6 HA-MA hydrogels with (a) high crosslinking density or (b) low crosslinking density have different structures and degradation products. These pictures are adopted from reference¹⁸⁰ with permission.

2.3.4 Rational design of biomimetic hydrogels

Hydrogels can be made of either synthetic or natural molecules. For tissue engineering applications, hydrogels formed of non-natural synthetic molecules, such as PEG, are highly controllable in both the gelation process and the resulting network structure. But they lack biological cues to promote active cell behaviors, thus act mainly as passive templates (Fig. 2.7A)^{178, 228}. Biologically derived materials (for example purified protein components, such as collagen and fibrinogen) are advantageous due to their inherent biological functions (Fig. 2.7B)¹⁷⁸. In fact the earliest works in cell encapsulation started with hydrogels prepared from natural collagen or fibrin^{11, 93}. However, the control of the gelation process, mechanical properties, and degradation of these hydrogels is difficult. To improve the plasticity of the physical and chemical characteristics of hydrogels to enhance *de novo* tissue formation, many recent studies have focused on recapitulation of the key characteristics of natural ECM in synthetic hydrogels.

Fig. 2.7 (A) Hydrogels formed of synthetic polymers simply function as templates for cells; they lack the capacity of activating cell surface integrins (1) and other receptors (2), through which multiple cell behaviors will be initiated. (B) Hydrogels formed of biologically derived polymers provide multiple biological cues, such as integrin-binding sites (3) and growth factors (4), to direct cell behavior. These pictures are adopted from reference ¹⁷⁸ with permission.

2.3.4.1 Native ECM

The native ECM may be considered to be complex and bioactive hydrogels. These are composed of various insoluble fibrous proteins (such as elastin, collagen, and laminin) and soluble growth factors and cytokines within a hydrated network of glycosaminoglycans (GAGs). The ECM in combination with interstitial fluid provides a structural support with the fibrous proteins resisting tensile stresses and the hydrated GAGs resisting compressive stresses. It also provides signals (for example adhesion ligands, soluble growth factors and cytokines) to the surrounded cells. These signals direct cellular behaviors,

such as adhesion, proliferation, migration, differentiation, and ECM production. At the same time cells dynamically remodel the ECM microenvironment by cleaving the matrix through their secretion of enzymes (such as MMPs) and production of new ECM components. Thus the information flow between ECM and cells *in vivo* is highly bidirectional.

2.3.4.2 Hydrogels that present adhesion ligands

Native ECM presents adhesion ligands such as collagen, fibronectin, vitronectin and laminin. Cell surface integrins bind to these adhesion ligands, through which a cell's cytoskeletons are connected with its surrounding microenvironment. As a result, specific signal pathways are activated, which regulate cell functions^{239, 240}. These binding ligands can be simply incorporated into hydrogels by physical entrapment of ECM proteins into the network²⁴¹. However, the effects may be confounded by denaturing, aggregation, or the heterogeneous distribution of ECM proteins within hydrogels. With the development of molecular biology, adhesive peptide sequences, such as RGD and laminin-derived IKVAV, have been identified from native proteins. Several synthetic schemes are available for tethering the peptide sequences to hydrogel networks either in a pendant fashion or as crosslinkers without compromising their bioactivity^{30, 204, 207, 208, 242}. For example, peptide was conjugated with

PEG acrylate by the reaction of free amino groups on peptide with acryloyl-PEG-N-hydroxysuccinimide, and through polymerization it was incorporated into PEG hydrogels ²⁴². Thiol containing peptides have been incorporated into hydrogel networks through Michael-type addition with vinyl groups ^{30, 214}, as well as photoinitiated thiol-acrylate and thiol-ene chemistries ^{204, 207, 208}. The incorporation of RGD into hydrogels has been shown to promote adhesion and to increase the viability of the encapsulated cells ^{19, 207, 216}.

2.3.4.3 Hydrogels that present enzymatic degradability

Native tissue is in a highly dynamic state which is regulated temporally and spatially by the coordination of cell-matrix and cell-cell interactions. For example, native ECM is cleaved by proteases secreted or activated by cells, such as MMPs and serine protease, through which the remodeling of the cellular microenvironment is accomplished. At the same time, the proteolytic degradation of ECM stimulates 3D cell migration and invasion ³⁵. Synthetic hydrogels have been developed which imitate the proteolytic recognition of natural ECM. These have been made through the incorporation of thiol-containing MMP-cleavable peptide sequences or protein building blocks by Michael-type addition and photoinitiated thiol-acrylate reactions ^{32, 81-85}. The MMPs produced by cells allow cells to migrate, deposit their own ECM, and

remodel the hydrogel environment as it is *in vivo*. These hydrogel systems enable a dynamic feedback between the surrounding environment and cells.

2.3.4.4 Hydrogels that dynamically present ECM effectors

In native tissues, ECM effectors, such as binding ligands and growth factors, are regulated to be present spatially and temporally. For example, early in chondrogenesis, differentiating hMSCs upregulate fibronectin that contains the RGD binding domain for cell adhesion²⁴³. However, fibronectin is thereafter downregulated through the upregulation of cell-secreted metalloproteinase 13 (MMP-13) which cleaves fibronectin^{244, 245}. It has been shown that RGD initiates the chondrogenic differentiation of hMSCs²⁰⁴ but the persistence of RGD impairs this effect²⁴⁶. A hydrogel containing binding domains that were temporarily controllable was made by the incorporation of MMP-13 cleavable RGD peptide sequences into PEG networks²⁰⁸. This method of cleavable and releasable delivery of RGD initiated extensive chondrogenic differentiation of the encapsulated hMSCs whilst maintaining a high level of cellular survival.

Natural ECM has the capacity to bind chemokines, store and release these effectors upon demand. Since very tiny amounts of a growth factor will elicit a biological response, controlling the local concentration of growth factors is a

major focus in the design of hydrogels that can present growth factors. Several strategies to create hydrogels with controlled delivery of growth factors have been attempted. These include physical entrapment in which release is dependent on diffusion and degradation²⁴⁷, and incorporation of heparin which harbors growth factor *in vivo*²⁴⁸. Growth factors have been bound to proteolytically degradable matrices (for example, hydrogels incorporating MMP-cleavable sequences or ECM proteins) and released in a cell-demanded fashion. This imitates the *in vivo* tissue repair situation in which invading cells release growth factors from their storage^{79, 80, 249}.

In vivo, multiple ECM cues work synergistically or antagonistically to direct tissue development, homeostasis and regeneration. In order to induce tissue regeneration, biomimetic hydrogel networks should not only present multifunctional biomimetic characteristics including ligands for cell adhesion, localized matrix dissolution, and morphogenetic signals, but also be able to control the presentation and organization of these cues dynamically and spatially. The simultaneous achievement of all these is a major ongoing challenge.

Chapter 3 – Hydrogel based on methacrylate- and aldehyde- bifunctionalized dextran and gelatin for 2D endothelial cell and 3D smooth muscle cell cultures

The objective of this chapter is to develop biomimetic hydrogels for vascular tissue engineering. Specifically, hydrogels which allow endothelial cells (ECs) to attach and proliferate on the two-dimensional (2D) surface and smooth muscle cells (SMCs) encapsulated within the three-dimensional (3D) environment to spread and proliferate were developed.

3.1 Introduction

Encapsulation of anchorage-dependent cells (ADCs) within hydrogels, whilst preserving their viability and controlling their proliferation and phenotype, is a challenge. In vitro, spreading of ADCs is required in order to avoid apoptosis and to maintain the natural phenotype¹⁸. In 2D, cell spreading can be promoted by cell adhesion molecules, such as RGD, collagen and fibronectin. In 3D hydrogel systems, on the other hand, the extent of cell spreading may be hampered even in the presence of adhesion molecules due to the physical obstruction posed by the dense matrix²¹.

So far, ADCs such as smooth muscle cells, mesenchymal stem cells

(MSCs) and fibroblasts have been successfully encapsulated within PEG-peptide/protein hybrid hydrogels with spreading^{19, 25, 28, 34, 37, 39, 250}. These PEG-based hybrid hydrogels, including PEG-conjugated fibrinogen hydrogel^{25, 28, 34, 37, 250} and PEG-derived hydrogel crosslinked with matrix metalloproteinase (MMP) degradable peptides^{19, 39}, provide both cell adhesion and proteolytic degradation-generated space that enable ADCs to spread within the 3D microenvironment. However, synthetic hydrogels that can successfully encapsulate proliferating and spreading ADCs have hitherto been based on PEGylated proteins. Recreation of the complexity of native ECM is still a challenge since synthetic PEG is inherently bioinert without structural or functional similarity to native ECM components. Also, the fabrication of hydrogels using peptides or natural ECM protein has thus far been restricted to small scale.

On the other hand, dextran, a natural hydrophilic degradable polysaccharide with high water solubility, is an alternative to PEG for hydrogel formation²⁵¹⁻²⁵⁹. Although it is resistant to protein adsorption and cell adhesion, dextran possesses abundant pendant hydroxyl functional groups making it easily amenable to chemical modification for adding specific cell-recognizing sites^{257, 260}. Due to its biocompatibility and susceptibility to enzymatic degradation, dextran has been used as 2D or 3D porous scaffolds in tissue

engineering and in drug delivery ²⁶¹. It has also been used in 3D cell encapsulation by Langer's group ²⁵⁵, in which dextran was modified with RGD for encapsulation of aggregates (rather than individual) of human embryonic stem cells (hESCs). Although enhanced vascular differentiation was reported, cellular proliferation was not observed in this work. Rather than using adhesive peptide, gelatin has been blended with dextran to form hydrogels ^{252, 253, 262}. Gelatin has good biodegradability and low level of immunogenicity and cytotoxicity, and has also been FDA approved as a clotting agent and exudate-absorbing construct ⁴². Suitably modified dextran and gelatin can be made into hydrogels which are proteolytically degradable but more biomimetic than PEG-based hydrogels since both dextran and gelatin are naturally derived.

This chapter reports the synthesis of a methacrylate- and aldehyde-bifunctionalized dextran (Dex-MA-AD) (Fig. 3.1) and the formation of a new cell-encapsulating hydrogel family based on the interpenetrating networks of Dex-MA-AD and gelatin (Fig. 3.2). The hydrogel is formed by ultraviolet (UV)-crosslinking between pendant methacrylate groups on Dex-MA-AD and Schiff base reaction between Dex-MA-AD and gelatin (Fig. 3.2). The synthesized Dex-MA-AD was characterized by ¹H-NMR. The physical properties (*i.e.* sol content, swelling ratio and compressive mechanical properties) of the hydrogels were also measured. Adhesion and spreading of

ECs on 2D culture, and spreading and proliferation SMCs in 3D culture were observed with these hydrogels. Also, this hydrogel possesses relatively high elastic modulus (on the order of 10^4 Pa) making it desirable for vascular tissue engineering.

Fig. 3.1 Synthesis of Dex-MA-AD.

Fig. 3.2 IPNs hydrogel networks formed by photopolymerization of Dex-MA-AD with low (a) and high (b) amounts of gelatin.

3.2 Materials and methods

3.2.1 Materials

Dextran (from *leuconostoc mesenteroides*, average mol. wt. 400,000~500,000 Dalton, M_n), gelatin type B (from bovine skin), dimethyl sulfoxide (DMSO), 4-dimethylaminopyridine (DMAP), deuterium oxide, glycidyl methacrylate (GMA), phosphate buffered saline (PBS) and sodium periodate were obtained from Sigma-Aldrich. 4-(2-hydroxyethoxy) phenyl-(2-hydroxy-2-propyl) ketone (Irgacure 2959) was purchased from Ciba (Singapore). All reagents were used as received. Deionized (DI) water was produced with a Millipore Milli-Q Plus ultra-pure water system.

Human umbilical artery endothelial cells (HUAECs, in EGMTM MV, cryopreserved) and human umbilical artery smooth muscle cells (HUASMCs, in SmGM®-2, cryopreserved) were purchased from Cambrex Bio ScienceWalkersville Inc. (USA). HUAECs were maintained in 75 cm² tissue culture polystyrene flasks in Microvascular Endothelial Cell Medium Bulletkit® (Cambrex Bio ScienceWalkersville Inc. (USA), Endothelial Basal Medium supplemented with 2.5% fetal bovine serum (FBS), Bovine Brain Extract, hydrocortisone, human epidermal growth factor (hEGF), and gentamicin/amphotericin-B (GA)). HUASMCs were cultured in Smooth

Muscle Cell Medium Bulletkit® (Cambrex Bio Science Walkersville Inc., USA).

The medium contains FBS (5%), hEGF, insulin, human fibroblastic growth factor-basic (hFGF-B), and GA. Cells were passaged by trypsinization with 0.25% (w/v) Trypsin-0.03% (w/v) EDTA solution before reaching confluence. Cells within passage 7 were used in this work.

3.2.2 Synthesis of methacrylated dextran (Dex-MA)

Dex-MA was synthesized by modifying dextran with GMA (Fig. 3.1) as described in the literature²⁶³⁻²⁶⁵. Briefly, 2 g dextran was dissolved in 20 ml anhydrous DMSO under nitrogen, followed by addition of 0.4 g of DMAP. GMA (300 μ l) was added to the solution. The mixture was stirred at room temperature for 48 h under nitrogen. Then DMAP was neutralized by adding an equimolar amount of concentrated HCl solution. The product was precipitated with a large excess of acetone and redissolved in water. The Dex-MA solution was dialyzed against DI water at 4°C, lyophilized and stored at -20°C. The degree of substitution (*i.e.* DS, the number of functional groups per 100 glucopyranose residues) of methacrylate was confirmed by proton nuclear magnetic resonance (¹H-NMR) according to the literature²⁶⁴ using a Bruker Avance 300 MHz instrument with D₂O as the solvent.

3.2.3 Synthesis of methacrylated dextran with aldehyde groups (Dex-MA-AD)

Dex-MA was further oxidized with sodium periodate to introduce aldehyde groups. Briefly, 500 mg Dex-MA was dissolved in 10 ml DI water. 60 mg sodium periodate was added to the solution. The mixture was stirred at room temperature in the dark for 4 hours. An equimolar ethylene glycol was added to quench the reaction. The resulting Dex-MA-AD solution was dialyzed against DI water, lyophilized and stored at -20°C. The aldehyde substitution degree was determined using the hydroxylamine hydrochloride titration assay

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3.2.4 Hydrogel preparation

Hydrogels were fabricated by photopolymerization of precursor solutions. Two mold geometries were used: a cylindrical metal mold (12 mm diameter and 10 mm deep) and a flat glass mold with a 0.25 mm-thick spacer frame. The precursor solutions were prepared with 6 wt% Dex-MA-AD and a range (2, 4, and 6 wt%) of gelatin concentrations in PBS, as outlined in Table 3.1. The solution was vigorously stirred in 40°C water bath for 10 min followed by the addition of Irgacure 2959 (I-2959) solution (in PBS); the final concentration of I-2959 was kept at 0.1 wt%. After homogenizing, the solution was injected into

the mold, and exposed to UV light ($\lambda=365$ nm) under argon atmosphere using a UV exposure system (Honle UV technology) equipped with a mercury lamp. For endothelial cell culture studies, hydrogels fabricated by photopolymerization of 6 wt% Dex-MA solution in PBS without gelatin (labeled as D-M, Table 3.1) were also used.

Table 3.1 Compositions of hydrogels.

Sample	Concentration of (mg/ml)			
	Dex-MA	Dex-MA-AD	Gelatin	Total
D-M	60	0	0	60
D-A	0	60	0	60
D-G-2	0	60	20	80
D-G-4	0	60	40	100
D-G-6	0	60	60	120

3.2.5 Characterization of hydrogel

Sol content: Hydrogels disks (12 mm in diameter and about 1 mm in thickness) were placed in PBS at 37°C for 24 h immediately after gelation. The swelling medium was changed every 12 h. This was followed by freeze-drying for 24 h. The sol content, S , was determined by $S = (W_1 - W_2)/W_1$, with W_1 the

initial solid weight of the sample, and W_2 the weight of the sample after freeze-drying.

Equilibrium swelling ratio: The sol-removed dry gels (weight W_2) were immersed in PBS at 37°C in a CERTOMAT® H incubator (Sartorius, B. Braun Biotech International) with constant shake. The swollen hydrogel disks were withdrawn on a filter paper after certain time intervals. After removal of the excess superficial water, the weight of the samples in the swollen state (W_s) was measured. After W_s reached its equilibrium value $W_{s,eq}$, the equilibrium swelling ratio was determined by $(W_{s,eq}-W_2)/W_2$. All experiments were conducted in triplicate.

Dynamic mechanical analysis (DMA): A RSA III Rheometrics System Analyzer (TA instruments) in parallel-plate configuration was used for DMA. Before testing, the samples (typically, of diameter of about 12 mm and thickness about 3 mm) were incubated in PBS overnight. Mechanical spectrometry was carried out using dynamic frequency sweep with frequencies ranging from 0.1 Hz up to 10 Hz at 37°C and with strain amplitude of 5%, which was in the linear region of viscoelasticity. Both auto-tension and auto-strain adjustment were applied. Force ramped from 0.001 N to 0.2 N (depending on the gel strength), and the maximum allowed strain was set at 10%. The storage modulus (E') and loss modulus (E'') of the samples were

measured. The phase angle delta ($\tan_{\delta}$) was computed from $\tan_{\delta} = E''/E'$.

Static compression test: The mechanical properties of the hydrogels were also characterized by compressive stress-strain measurements which were performed on swollen gels using an Instron 5543 Single Column Testing System. The cylindrical gel sample, 12 mm in diameter and 3 mm thick, was put on the lower plate and compressed by the upper plate, which was connected to a load cell, at a strain rate of 0.1 mm/min. The elastic modulus was determined by the average slope of the stress-strain curve over the strain range 0-20%. The fracture stress and fracture strain were also reported. Four parallel samples per measurement were performed, and the obtained values were averaged.

Gelatin release test: The release of gelatin from hydrogels was tested using BCA assay (Bicinchoninic Acid Kit for Protein Determination, Sigma-Aldrich). 120 μ l of precursor solution was used to fabricate a hydrogel film with dimensions of 1.5 cm $\times$ 1.3 cm $\times$ 140 μ m. The hydrogel film was immersed into 1 ml PBS supplemented with 0.05% sodium azide, and incubated at 37°C under constant agitation. At various time points, 100 μ l supernatant was withdrawn and replaced with 100 μ l fresh buffer. The amount of gelatin in the supernatants was determined by BCA assay, and cumulative

release of gelatin was computed. The standard curve was obtained using gelatin solution in PBS with 0.05% sodium azide with concentrations of 200, 400, 600, 800 and 1000 $\mu\text{g/ml}$.

3.2.6 Endothelial cell culture

Hydrogel films were cut into small circular pieces and rinsed with PBS solution for 4 days at 37°C and then transferred to 96-well tissue culture polystyrene (TCPS). HUAECs were seeded on the hydrogel surfaces to measure gel cytocompatibility. TCPS well was used as a positive control. Cell attachment and spreading on the surface of the hydrogels were monitored after seeding using an Axiovert 200 Motorized Inverted Microscope System (Carl Zeiss Vision GmbH) and cell morphology was recorded with a digital CCD camera using Axiovision 4.0 software. Cells were also stained by LIVE/DEAD[®] Viability/Cytotoxicity Assay Kit (Molecular Probe), containing calcein AM and ethidium homodimer (EthD-1). Nonfluorescent cell-permeant calcein AM will be enzymatically converted to intense green fluorescent calcein in live cells, while EthD-1 enters cells with damaged membranes, binding to nucleic acids and producing a bright red fluorescence in dead cells. After cell seeding for certain times, the medium was discharged and the culture was washed with PBS, then “Live/Dead” solution containing 2 μM calcein AM and

4 mM EthD-1 was added and incubated at 37°C for about 20 min. The stained cultures were viewed using fluorescent microscope.

The Cell Proliferation Reagent WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate, Roche Diagnostics) was used for spectrophotometric quantification of cell viability. Following incubation for various periods, 20 µl of WST-1 reagent was added to each well of the 96-well TCPS with 200 µl of fresh culture medium without FBS, and the plates were again incubated for an additional 4 h at 37°C in a humidified, 5% CO₂ atmosphere. During this period, viable cells could cleave WST-1 to water-soluble formazan dye. The absorbance of the formazan dye solution in culture medium at 440 nm was recorded using a 96-well microplate reader (Biorad). Results of the absorbance values were corrected by subtracting the background reading of the media and WST-1 reagents alone in the corresponding trial. 4 parallels were averaged for each hydrogel sample.

3.2.7 Smooth muscle cell encapsulation within hydrogels

Hydrogels for SMC encapsulation were formulated from Dex-MA-AD macromer solution in PBS, gelatin stock solution (10% w/v in SMC culture medium) and I-2959 photoinitiator solution (0.25% w/v in PBS). The final concentration of Dex-MA-AD was kept at 6% w/v, I-2959 at 0.1% w/v, while

gelatin concentration varied from 2% to 6% w/v. Cultured monolayers of SMCs were trypsinized, centrifuged and then re-suspended in hydrogels precursor solutions to a final concentration of about 4×10^6 cells/ml. 50 μ l of cell-laden precursor solution was injected into each well of a 96-well TCPS, and subjected to low intensity UV illumination (365 nm, 20 mW/cm²) for 5 min under argon atmosphere. The encapsulated cell/hydrogel constructs were supplied with 200 μ l SMC culture medium. The medium was refreshed every day.

The viability of the encapsulated cells was determined after 1 day, 7 days and 14 days of incubation. The medium was discarded and the constructs were washed twice with PBS. Live and dead cells were determined by the LIVE/DEAD[®] Viability/Cytotoxicity Assay Kit (Molecular Probe). Hydrogel constructs were incubated in “Live/Dead” solution containing 2 μ M calcein AM and 4 mM EthD-1 for about 45 min at 37°C. Fluorescent microscopy was performed using a fluorescein optical filter and a rhodamine optical filter. SMC proliferation was also tested using the Cell Proliferation Reagent WST-1 after 7 and 14 days of incubation, as described above. Typically, after refreshing the cell-laden hydrogels with 200 μ l medium without FBS, 20 μ l of WST-1 reagent was added. After incubating for an additional 4 h, hydrogels were smashed to ensure complete dissolving of the formazan dye, and then the absorbance of the medium at 440 nm was recorded.

3.3 Results

3.3.1 Synthesis of bifunctional dextran

Dex-MA-AD was synthesized by coupling GMA to dextran followed by oxidation of Dex-MA with sodium periodate, as shown in Fig. 3.1. The ^1H -NMR spectrum of dextran (Fig. 3.3a) confirms its structure^{264, 265, 267}. The ^1H -NMR spectrum for Dex-MA-AD (Fig. 3.3b) shows, in addition to the distinctive resonance peaks of pristine dextran, the methyl protons from the methacryloyl group (position 7) at about δ 1.85 ppm and the protons from the double bond (position 8) at δ 5.65 ppm and δ 6.14 ppm; there is no detectable aldehyde protons peak (position 9, expected at about δ 8.3 ppm¹²), and this may be due to the low extent of oxidation. However, when grafting L-lysine onto Dex-MA-AD by reaction with aldehyde groups, peaks assigned to lysine was detected in the ^1H -NMR spectrum (see Fig. 4.2), confirming the existence of aldehyde groups in Dex-MA-AD molecules. Using the integrated areas of the relevant chemical shifts in Fig. 3.3b, the DS of methacrylate in Dex-MA-AD was determined to be 14, and using an alternative technique of hydroxylamine hydrochloride titration assay^{12, 266}, the DS of aldehyde was determined to be 13.9 ± 1.3 , this corresponds to $(7.8 \pm 0.7) \times 10^{-4}$ mole aldehyde groups per 1 g of Dex-MA-AD molecules.

Fig. 3.3a (Cont'd)

Fig. 3.3b

Fig. 3.3 ^1H -NMR spectra of (a) dextran, and (b) Dex-MA-AD dissolved in D_2O .

3.3.2 Hydrogel sol content

The precise compositions of the UV photopolymerized hydrogels are outlined in Table 3.1. Fig 3.4a shows that for all the hydrogels, the sol content decreased with increasing crosslinking time up to the initial 5 min and plateaued at longer times. Sol content increased monotonically with gelatin concentration; this is attributed to increased viscosity and steric hindrance to methacrylate crosslinking²⁶⁸. Fig. 3.4b shows the influence of UV intensity on sol content for a 5-min crosslinking time. As expected, for all the hydrogels, the sol contents decreased as the UV intensity increased. With 5 min exposure at 20 mW/cm², the D-A hydrogel reached a sol content of 4.6 ± 3 %; the D-G-2, D-G-4 and D-G-6 hydrogels had sol contents of 9.1 ± 2.2 %, 13 ± 2.8 % and 15.7 ± 2.6 %, respectively.

Fig. 3.4 Influence of (a) crosslinking time (at 20 mW/cm²) and (b) UV intensity (with 5 min irradiation) on sol content of hydrogels.

3.3.3 Hydrogel water content

All four hydrogels contained significant amounts of water, as reflected in their equilibrium swelling ratios of ~ 5 to 6 (Fig. 3.5). The D-A gel had an equilibrium swelling ratio of 5.56 ± 0.09 . The ratio was significantly decreased to 4.91 ± 0.03 in the D-G-2 hydrogel (20 mg/ml gelatin). With further increase of the gelatin concentration to 40 mg/ml and 60 mg/ml (D-G-4 and D-G-6 hydrogels), the equilibrium swelling ratio increased to 5.36 ± 0.45 and 5.83 ± 0.07 respectively.

The equilibrium swelling ratio is generally influenced by the crosslinking density, gel composition, charge density, *etc* ²⁶⁹. In PBS, the ionic charges on gelatin molecules are shielded by buffer salts, so we postulate that the charge effect on equilibrium swelling ratio can be neglected here. Of the three IPN hydrogels, D-G-6 with the highest sol content due to methacrylate crosslinking steric hindrance also had the highest equilibrium swelling ratio. D-G-2 had higher sol content than D-A, but had lower equilibrium swelling ratio; this may be due to increased crosslinking density in D-G-2, compared to D-A, contributed by the Schiff base reaction with the additional interpenetrating gelatin added.

Fig. 3.5 Equilibrium swelling ratios of hydrogels (with 5 min irradiation at 20 mW/cm²). Comparisons were made with pairs between D-A and IPN hydrogels using Student's *t*-test: * denotes significant difference in equilibrium swelling ratio at the level of $P < 0.05$. (n=3)

3.3.4 Characterization of hydrogel mechanical properties

3.3.4.1 Dynamic mechanical properties

Fig. 3.6 shows the dynamic storage (E') and loss (E'') moduli of the hydrogels. All the hydrogel samples have $E' > E''$ over the entire frequency range (0.1 to 10 Hz) with E' on the order of 10^4 Pa, indicating an elastic solid behavior²⁷⁰. Also, the E' and E'' generally increased as frequency increased, indicative of

network structures relaxation at higher frequencies, which is the case for classical hydrogels ²⁷¹. The D-A hydrogel had the lowest E' and highest E'' among all the hydrogels. The $\tan_{\delta}$ for the D-A hydrogel was much larger than those of IPN hydrogels and increased dramatically with frequency. The IPN hydrogels showed no obvious change of $\tan_{\delta}$ versus frequency and all three compositions had nearly the same $\tan_{\delta}$.

The different rheological behaviors between D-A and the IPN hydrogels are due to their different network structures. The D-A hydrogel crosslinking structure was formed only by methacrylate polymerization, while in the IPN hydrogels, both the methacrylate polymerization and Schiff base reaction contribute to the network formation. Our IPNs hydrogels' storage moduli values are higher than the reported values of less than 10^4 Pa for Dex-MA hydrogels with similar concentration and methacrylate DS ^{263, 272}; this may be due to the incorporation of gelatin in our hydrogels. Generally, the dynamic mechanical properties of a hydrogel depends on the density of polymer chains, the friction between the chains, and the homogeneity of hydrogel intrinsic structure ^{273, 274}. Incorporation of gelatin in hydrogels not only increases the density of polymer chains, but also increases the friction between chains due to Schiff base formation, thus leading to higher storage moduli of IPN hydrogels than the D-A hydrogel.

Fig. 3.6 Dynamic mechanical analysis of hydrogels. (a) Storage modulus (E' , solid symbols) and loss modulus (E'' , hollow symbols) versus frequency at 37°C. (b) $\tan_{\delta}$ versus frequency.

3.3.4.2 Static compressive properties

Fig. 3.7 shows typical compressive stress-strain curves of the hydrogels. The elastic modulus, fracture stress and fracture strain are summarized in Table 3.2. The D-A hydrogel had an elastic modulus of about 15.41 ± 3.00 kPa, an ultimate compressive stress of about 21.91 ± 3.36 kPa and an ultimate strain of about 62.57 ± 6.15 %. The addition of gelatin into the hydrogel increased the elastic moduli to 2.4-3.4 fold (*i.e.* to 37.42 ± 5.04 kPa and 51.94 ± 0.12 kPa), but decreased the fracture strains to about 0.7 fold of the original value. The low-gelatin-content hydrogel (20 mg/ml) had a fracture stress not significantly different ($P>0.05$) from that of D-A (21.91 ± 3.36 kPa). The D-G-4 and D-G-6 hydrogels, with gelatin concentrations of 40mg/ml and 60mg/ml had higher fracture stresses (36.78 ± 0.16 kPa and 31.11 ± 0.55 kPa, respectively).

Fig. 3.7 Typical stress-strain curves of D-A, D-G-2, D-G-4, and D-G-6 (a, b, c, and d, respectively) hydrogels at room temperature.

Table 3.2 Compression properties of hydrogels at room temperature.

Samples	Modulus (kPa)	Fracture stress (kPa)	Fracture strain (%)
D-A	15.41 ± 3.00	21.91 ± 3.36	62.57 ± 6.15
D-G-2	37.42 ± 5.04 *	25.99 ± 1.87	45.27 ± 1.64 *
D-G-4	51.94 ± 0.12 *	36.78 ± 0.16 *, #	46.63 ± 0.36 *
D-G-6	51.37 ± 8.8 *	31.11 ± 0.55 *, #, &	46.99 ± 2.54 *

Comparisons were made with pairs with statistical analysis by the Student's *t*-test: at the level of $P < 0.05$, * significantly different from that of D-A hydrogel; # significantly different from that of D-G-2 hydrogel; & significantly different from that of D-G-4 hydrogel. (n=4)

3.3.5 Characterization of gelatin release

As shown in Fig.3.8, most of the gelatin release occurred during the first two days and by the third day the release rate had declined. The amount of gelatin released increased monotonically with the gelatin content in the hydrogel. After 3-day immersion, $13.8 \pm 1.6\%$, $17.2 \pm 1.4\%$ and $20.5 \pm 2.0\%$ of gelatin originally in the formulation were released from D-G-2, D-G-4 and D-G-6 hydrogels, respectively. In the more gelatin-rich formulations, the declining fraction of initial gelatin retained can be understood in term of lower methacrylate crosslinking efficiency as well as insufficient reaction between aldehyde and amino groups. The released gelatin during the observed time period (3 days) was believed to be the unbonded one instead of hydrolytically cleaved one from the networks, since Schiff base has been reported to be stable in water at pH 7 at 40°C for at least 2 days²⁷⁵.

Fig. 3.8 Gelatin release at 37°C. The cumulative released amount of gelatin was normalized to initial gelatin content.

3.3.6 EC growth on the surface of hydrogels

The adhesion and proliferation of vascular endothelial cells on the IPN hydrogels are studied by culturing the cells on thin (approximately 140 μm thick) hydrogel films. TCPS and D-M hydrogel (hydrogels fabricated from 6 wt% Dex-MA solution) served as positive and negative controls respectively. The results are shown in Fig. 3.9. After 4 h of culture, ECs did not spread on D-M hydrogel (Fig. 3.9A). Most of the cells remained round and cell

aggregations were formed. In contrast, spindle shaped cells and cell spreading could be seen on all IPN hydrogels (Fig. 3.9B-D) and on TCPS (Fig. 3.9E) after 4 h culture.

Fig. 3.10 shows ECs stained using Live/Dead assay (live cells are stained green, dead cells red). The ECs spread quite well on all IPN hydrogels (Fig. 3.10B-D), with clearly visible pseudopods (indicated by arrows). There appear to be more cells adhering on the surfaces of the IPN hydrogels than on the TCPS control (Fig. 3.10E).

Fig. 3.11 shows the results of a WST-1 assay, after one day of culture. The cells grew much better on IPN hydrogels than on D-M hydrogel. Cells cultured on TCPS appear to have somewhat lower viability than on the IPN hydrogels, but the differences are not statistically significant. There is also a hint of declining viability with increase in gelatin concentration among the IPN hydrogels, but this trend is too weak to be significant given the measurement uncertainties. The difference in cell viability between the IPN hydrogels and the negative control, D-M, is highly significant and establishes that incorporation of gelatin into the hydrogel networks promotes EC adhesion and spreading.

Fig. 3.9 Phase contrast microscopic images of endothelial cells growing on surface of D-M (A), D-G-2 (B), D-G-4 (C), D-G-6 (D) hydrogels and on TCPS (E) 4 h after seeding. Scale bar represents 100 μm . Cell seeding density: 3×10^4 cells/well.

Fig. 3.10 Fluorescence micrographs of endothelial cells growing on surface of D-M (A), D-G-2 (B), D-G-4 (C), D-G-6 (D) hydrogels, and on TCPS (E) 1 day after seeding. Live cells were stained green, while dead cells red. Scar bar represents 100 μm . Cell seeding density: 3×10^4 cells/well.

Fig. 3.11 Viability of the endothelial cells cultured on the surfaces of hydrogels after 1 day. All samples and TCPS control exhibit improved viability compared with D-M hydrogel (at the significance level $P < 0.05$ by Student's t -test as indicated by *).

3.3.7 SMC encapsulation within hydrogels

The hydrogel constructs with embedded cells were imaged with optical phase contrast microscope at various durations (4 h, 1 d, 3 d and 6 d) of culture.

After 4 h, cells inside D-G-2 and D-G-4 hydrogels were almost round (Fig. 3.12A and Fig. 3.12B), while inside D-G-6 hydrogels, some of the cells had started to form extensions (Fig. 3.12C), indicating that the cells were starting to spread. After one day, cells inside D-G-2 hydrogels were also starting to spread, indicated by the protruding pseudopods (Fig. 3.12A), whereas evidence of cell spreading inside D-G-4 hydrogel was not apparent until 3 days (Fig. 3.12B). Moreover, after 3 days, direct cell-cell contact between spreading cells could be seen clearly in the D-G-2 and D-G-6 hydrogels, as indicated by the circles in Fig. 3.12A and Fig. 3.12C. The cell-cell contact became obvious after 6 days in D-G-4 hydrogel (Fig. 3.12B, highlighted by circles). Spindle shaped cells were seen within all hydrogels, indicating that the hydrogels support SMC spreading.

Fig. 3.12 A (Cont'd)

Fig. 3.12 B (Cont'd)

Fig. 3.12 C

Fig. 3.12 Phase contrast macroscopic images of smooth muscle cells encapsulated in D-G-2, D-G-4, and D-G-6 hydrogels (A, B, and C, respectively) after 4 h, 1 day, 3 days, and 6 days. The magnified inset in image C (4 h) is a close-up of the cell highlighted with the circle and arrows. Scale bar represents 50 μm . Cell seeding density: 4×10^6 cells/ml.

To ascertain whether the encapsulated SMCs were alive, the hydrogel/cell constructs were tested with the Live/Dead assay. After 1-day encapsulation, (Fig. 3.13 (A1, B1 and C1)), most of the cells inside the hydrogels were stained green (live) while only a few were stained red (dead). Most of the live cells were round in shape. The large preponderance of live cells indicates that the hydrogels are not cytotoxic and that the UV crosslinking process caused little immediate damage to the encapsulated cells. Cell viability after longer time periods culture was also verified. After 7-day culture, many of cells in the D-G-2 and D-G-6 hydrogels had developed elongated spindle shapes (Fig. 3.13 (A2 and C2)), while in D-G-4 hydrogel (Fig. 3.13B2), most of cells were still round and only a few had assumed spindle shape. There were few dead cells in all the hydrogels after 7-day 3D culture. By 14-day culture, the number of spreading live cells in the D-G-4 hydrogel had visually increased (Fig. 3.13B3) and many live cells persisted in the D-G-2 and D-G-6 constructs at this stage of culture.

Fig. 3.13 Fluorescence micrographs of SMCs encapsulated into D-G-2 (A1, A2, and A3), D-G-4 (B1, B2, and B3), and D-G-6 (C1, C2, and C3) hydrogels after 1 day (A1, B1, and C1), 7 days (A2, B2, and C2), and 14 days (A3, B3, and C3) using Live/Dead assay. The living cells were stained green, and the dead cells red. Scale bar represents 100 μm . Cell seeding density: 4×10^6 cells/ml.

Cell viability was also quantified spectrophotometrically using Cell Proliferation Reagent WST-1 (Fig. 3.14). After 7-day *in vitro* culture, cells in D-G-6 hydrogel had the highest viability. D-G-2 and D-G-4 hydrogels had significantly lower viability than D-G-6 but were statistically indistinguishable from each other. After 14 days of *in vitro* culture, cell viability in D-G-2 and D-G-4 hydrogels increased. In these two hydrogels with lower gelatin content, cell viability increased significantly during the 7 to 14 days period, while the increase during the same period for D-G-6 hydrogel was not significant. After the longer-term (14-day) culture, there was no significant difference in cell viability between D-G-2 and D-G-6 hydrogels (*n.b.*; this may be due to the large error bar in the D-G-2 measurement), but D-G-4 hydrogel had statistically lower cell viability than D-G-6 hydrogel. The lower 14-day WST-1 absorbance (*i.e.* proliferation) in D-G-4 also corroborates with the observation of fewer live cells using fluorescent microscope in Fig. 3.13 B3. Although there are variations in the detailed performance of the IPN hydrogel, all three promote cell proliferation in 7 to 14 days of culture.

During the course of the cell culture, mechanical strength loss was also observed, with the most obvious in the D-G-6 hydrogel (data not shown)); this may be a consequence of hydrogel degradation.

Fig. 3.14 Viability of the encapsulated smooth muscle cells in hydrogels after 7 days and 14 days. Comparisons were made with pairs with statistical analysis by the Student's *t*-test: at the level of $P < 0.05$, * significantly different from that of D-G-6 hydrogel after 7 days; # significantly different from that of D-G-6 hydrogel after 14 days. Cell seeding density: 4×10^6 cells/ml. ($n=3$)

3.4 Discussion

We have synthesized and characterized an IPN hydrogel series based on methacrylate- and aldehyde-bifunctionalized dextran (Dex-MA-AD) and

gelatin. The methacrylate groups on Dex-MA-AD were used for UV crosslinking and the aldehyde groups enabled the incorporation of chemically linked gelatin. We chose a low extent of dextran oxidation – DS of aldehyde of 13.9 ± 1.3 – since increasing the density of aldehyde groups on dextran decreases its solubility but hastens the formation of Schiff base, making the thorough mixing of the two biomacromolecules and hence formation of homogeneous hydrogel network difficult.

An optimum time of more than 5 minutes at the higher UV intensity of 20 mW/cm² appear to be feasible for cell viability and acceptable sol content²⁶³ and mechanical properties^{263, 272}. As illustrated in Fig. 3.2, two different kinds of networks contribute to the covalent crosslinking in our system, namely the polymethacrylate kinetic chains produced by carbon-carbon double bond polymerization and Schiff base obtained by reaction between Dex-MA-AD aldehyde and gelatin amino groups. The unique crosslinking mechanism confers superior mechanical properties to the IPN hydrogels. The measured elastic moduli are on the order of 10⁴ Pa, which are considerably higher than the reported 10² Pa value for PEG-based hydrogels which promote SMC spreading^{19, 28, 34}. A hydrogel that is mechanically robust would be highly desirable as a scaffold for tissue engineering of blood vessels.

Cell spreading is highly important for obtaining cell-cell contacts, and

hence the outcome of tissue. In 2D systems, cell spreading can be promoted by cell adhesion molecules, such as RGD, collagen, and fibronectin. Due to the incorporation of gelatin, ECs cultured on the surface of our IPN hydrogels spread after seeding for as little as 4 h. In 3D hydrogel systems, cell spreading may be hampered even in the presence of adhesion molecules, due to the physical obstruction posed by the dense matrix²¹. It has been proposed that the inclusion of degradable crosslinkers sensitive to either hydrolysis^{22, 27} or cell-mediated proteolysis^{19, 30, 31, 33, 35, 39} will permit formation of 3D cell extensions, since degradation will generate space for cells to make their way through the matrix, spread, migrate and establish cell-cell contacts. In our study, the incorporation of gelatin serves the dual purpose of providing cell adhesion sequences and enzymatic degradability. IPN hydrogels promoted 3D SMC spreading and proliferation. Cells acquired an elongated, spindle-shaped morphology as they spread within the hydrogels.

The time course of spreading and proliferation was related to gelatin concentration (Fig. 3.12 and Fig. 3.14), with D-G-6 hydrogels promoting the quickest spreading and proliferation, followed by D-G-2 and then D-G-4 hydrogels. It has been proposed that cell spreading within hydrogels is influenced by both cell adhesion ligand density and matrix stiffness^{19, 21, 34}; high ligand density and soft matrix are expected to promote cell spreading. In our IPN

hydrogels, although higher gelatin concentration (60 mg/ml) increased the stiffness of the hydrogels, the high ligand density enabled the quickly spreading of encapsulated cells.

Our hydrogel marries the unique properties of polysaccharides and proteins to mimic ECM. Gelatin, like other ECM proteins such as collagen and fibrin, is capable of promoting cell adhesion and proteolytic degradability via its biological domains. However, a major drawback in using these proteins solely is the limited control of the physical and degradation properties that can lead to premature matrix breakdown due to the release of cell-secreted matrix metalloproteinases. Polysaccharides have high hydration capability and are also non-antigenic, non-immunogenic²⁷⁶ and feasible for easy modification with photochemically or thermally crosslinkable groups to produce hydrogels. The combination of the two in a single hydrogel produces a material with an excellent combination of mechanical and biological properties. However, in a very small percentage of human population, dextran is able to induce anaphylactoid reactions when binding to endogenous dextran-reactive immunoglobulin (Ig) G antibodies²⁷⁷, and the chemical modification may also lead to immunological reactions. All these should be taken into account before putting dextran into clinical use.

3.5 Conclusion

We have demonstrated a new class of gelatin-bonded dextran-based hydrogel with relatively high modulus that is also suitable for 3D encapsulation of SMCs and 2D culture of ECs. Using bifunctional dextran modified with acrylate and aldehyde groups mixed with gelatin, UV crosslinked hydrogels encapsulating vascular SMCs were fabricated. The Dex-MA-AD component imparted to the hydrogels elastic properties that are superior to commonly reported PEG-based hydrogels. The incorporation of gelatin into the hydrogels provided cell adhesive and enzymatically degradable properties and also significantly increased the compressive modulus and strength through the Schiff base contribution to the crosslinking density. We have shown that these hydrogels promoted adhesion of vascular endothelial cells in 2D culture and supported spreading and proliferation of vascular smooth muscle cells in 3D culture for up to 14 days. The attractive mechanical properties of this new class of hydrogel coupled with 2D and 3D biocompatibility with vascular cells make this a promising material for 3D scaffolds for vascular tissue engineering and regeneration.

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- ✧ **Liu YX**, Chan-Park MB. Hydrogel based on interpenetrating polymer networks of dextran and gelatin for vascular tissue engineering. *Biomaterials* 30: 196-207, 2009.

Chapter 4 – Biomimetic hydrogel based on methacrylated dextran-*graft*-lysine and gelatin for 3D smooth muscle cell culture

4.1 Introduction

In the previous chapter, we reported the initial work on hydrogels based on natural gelatin and dextran bifunctionalized with methacrylate and aldehyde. That series of hydrogels allowed adhesion of vascular endothelial cells on 2D culture and supported spreading and proliferation of vascular smooth muscle cells in 3D culture. However, the Schiff base reaction between dextran and gelatin was not easily controllable and while SMC spreading within the hydrogel occurred, it was rather limited.

In the current chapter, a novel methacrylate and lysine functionalized dextran (Dex-MA-Ly) was designed and synthesized (Fig. 4.1A). A series of hydrogels based on Dex-MA-Ly and methacrylamide modified gelatin (Gel-MA) (Fig. 4.1B) with varying mechanical stiffness was developed by ultraviolet (UV)-crosslinking. The swelling, sol contents and mechanical properties of these hydrogels were characterized. The viability, proliferation and spreading of human umbilical artery SMCs inside these hydrogels were tracked.

The crosslinking of this new series of hydrogels based on Dex-MA-Ly and Gel-MA is much more controllable. Further, we found this series to allow the encapsulated SMCs to spread more extensively compared to the Dex-MA-AD series in Chapter 3.

Fig. 4.1 Synthesis of Dex-MA-Ly (A) and Gel-MA (B).

4.2 Materials and methods

4.2.1 Materials

The chemicals, reagents and cells described in Section 3.2.1 were used in this chapter. Besides, 2,4,6-trinitrobenzene-sulfonic acid (TNBS), methacrylic anhydride, and L-lysine obtained from Sigma-Aldrich were all used as received.

4.2.2 Synthesis of dextran derivatives

Dextran was first modified to introduce methacrylate and aldehyde groups (Dex-MA-AD) (Fig. 4.1A) as described in Section 3.2.2 and 3.2.3. Briefly, dextran was dissolved in anhydrous DMSO under nitrogen, followed by addition of DMAP. GMA was added to the solution. The mixture was stirred for a certain time under nitrogen to produce dextran with methacrylate groups (Dex-MA). Dex-MA was precipitated with a large excess of acetone, purified by dialysis and freeze-dried. By varying the amount of GMA and reaction time, Dex-MAs with different degrees of substitution (*i.e.* DS, the number of functional groups per 100 glucopyranose residues) of methacrylate were obtained. The Dex-MAs were further oxidized by reaction with sodium periodate at room temperature in the dark for 4 h to introduce aldehyde groups (Dex-MA-AD). The Dex-MA-AD solutions (2 wt %) were added dropwise to

L-lysine solution (in 0.1 M NaHCO₃, 2-fold molar excess over aldehyde groups) over 5 h. The reactions were continued overnight. Dextran with methacrylate and lysine functionalities (Dex-MA-Ly) was collected by dialysis and freeze-drying.

The degree of substitution of methacrylate was confirmed by proton nuclear magnetic resonance (¹H-NMR) spectroscopy using a Bruker Avance 300 MHz instrument with D₂O as the solvent. The concentration of free primary amino groups, essentially the amino groups of lysine, was determined by the Habeeb method²⁷⁸.

4.2.3 Synthesis of gelatin derivatives

Gelatin methacrylamide (Gel-MA) was prepared by reaction with methacrylic anhydride (Fig. 4.1B) following the procedure of reference²⁷⁹. Briefly, after gelatin was dissolved in phosphate buffer saline (PBS) at 50°C, methacrylic anhydride was added. The mixture was stirred under nitrogen atmosphere for 1 h, and then dialyzed for 24 h against DI water at 40°C. The product was collected by freeze-drying. The degree of substitution was defined, using the Habeeb method, as the percentage of ε-amino groups that had been modified²⁷⁸. By changing the amount of methacrylic anhydride, Gel-MAs with a range of degrees of substitution were obtained.

4.2.4 Hydrogel formation and characterization

Hydrogels were fabricated by exposure of precursor solutions to UV light ($\lambda=365$ nm) at 20 mW/cm^2 for 5 min under argon atmosphere using a UV exposure system (Honle UV technology). The compositions of the precursor solutions are outlined in Table 4.1. Before UV crosslinking, the precursor solutions (in PBS) with 0.1 wt% of Irgacure 2959 (I-2959) pre-dissolved in PBS (10 mg I-2959 in 6 ml PBS) were vigorously stirred at 37°C for 30 min.

Table 4.1 Compositions of hydrogels.

Sample	Concentration of (mg/ml)			Degree of methacrylate substitution
	Dex-MA-Ly	Gel-MA	Total	
L-G-1A	80	40	120	Dex-MA(9)-Ly Gel-MA(16)
L-G-1B	80	60	140	
L-G-1C	80	80	160	
L-G-2A	80	40	120	Dex-MA(9)-Ly Gel-MA(51)
L-G-2B	80	60	140	
L-G-3A	80	40	120	Dex-MA(30)-Ly Gel-MA(16)
L-G-3B	80	60	140	

Dex-MA(9)-Ly and Dex-MA(30)-Ly: with 9 and 30 methacrylate groups per 100 glucopyranose residues respectively; Gel-MA(16) and Gel-MA(51): with 16% and 51% of ϵ -amino groups been modified.

For sol content and mass swelling ratio measurements, 250 μ l of precursor solution was crosslinked in the well of a 24-well tissue culture polystyrene plate (TCPS). Immediately after gelation, the obtained hydrogel disks were placed in PBS at 37°C for 24 h with swelling medium changed every 12 h, followed by freeze-drying for 24 h. The sol content, S , was determined by $S = (W_1 - W_2)/W_1$, with W_1 the initial solid weight of the sample, and W_2 the weight of the sample after freeze-drying.

The sol-removed dry gels (weight W_2) were re-immersed in PBS at 37°C in a CERTOMAT® H incubator (Sartorius, B. Braun Biotech International). At discrete time intervals the swollen hydrogel disks were blotted on a filter paper. After removal of the excess superficial water, the weight of the samples in the swollen state (W_s) was measured. After W_s reached its equilibrium value $W_{s,eq}$, the equilibrium mass swelling ratio, q , was determined by $q = (W_{s,eq} - W_2)/W_2$. The volumetric swelling ratio, Q , was calculated based as $Q = 1 + q * \rho_1 / \rho_2$ ²⁸⁰, while ρ_1 is the density of dry polymer (mixture of Dex-MA-Ly and Gel-MA, obtained by calculation based on the weight ratio) and ρ_2 is the density of the solvent (buffer solution, 1.09 g/ml). All experiments were conducted in triplicate.

Shear storage modulus was measured using a Thermo Haake Rheostress 600 rheometer in conjunction with a UV spot curing system (LUMATEC®)

SUV-DC-P). Dynamic time sweep in plate-plate geometry was performed at room temperature, at a constant stress of 100 Pa and a constant frequency of 1 Hz. Briefly, 150 μ l of precursor solution was deposited onto the lower plate, and the 2 cm diameter upper plate was moved down to obtain a gap of 0.5 mm. After 60 s the solution was exposed to UV light. The storage (G') moduli were recorded after 5 min of UV exposure. Three parallel samples for each formulation were measured, and the obtained values were averaged.

4.2.5 SMC encapsulation

Cultured monolayers of SMCs were trypsinized after reaching confluence. Hydrogels precursor solutions (in Smooth Muscle Cell Medium Bulletkit®) were added to re-suspend the SMCs. 40 μ l of cell-laden precursor solution was deposited onto the surface of polytetrafluoroethylene (PTFE) film, and subjected to low intensity UV illumination (365 nm, 20 mW/cm²) for 5 min under argon atmosphere. The cell/hydrogel constructs were transferred into 24-well Ultralow attachment plates (Corning, Fisher) and supplied with Dulbecco's Modified Eagle's Medium (DMEM) containing 10% FBS. The medium was refreshed every two days.

SMCs within hydrogels were monitored during culture using an Axiovert 200 Motorized Inverted Microscope System. F-actin of SMCs was stained

using Alexa Fluor[®] 488 phalloidin (Invitrogen) solution (1:200 dilute in 1% BSA/PBS buffer), and viewed using an LSM 510 confocal microscope (Carl Zeiss, Germany), after hydrogel constructs were fixed (using 4% paraformaldehyde), permeabilized (using 0.1% Triton X-100) and blocked (using 1% BSA/PBS solution).

4.2.6 Cell viability

The viability of the encapsulated cells was assessed by LIVE/DEAD[®] Viability/ Cytotoxicity Assay Kit (Invitrogen). The medium was removed and the constructs were washed twice in PBS and incubated in “Live/Dead” solution containing 2 μ M calcein AM and 4 mM EthD-1 for about 45 min at 37°C. Cells were imaged using a Zeiss inverted microscope with fluorescein and rhodamine optical filters.

4.2.7 Cell proliferation

SMC proliferation was quantified using Cell Proliferation Reagent WST-1 (Roche). Typically, after refreshing the cell-laden hydrogels with 200 μ l medium without FBS, 20 μ l of WST-1 reagent was added. After 4 hours of further incubation, the hydrogels were smashed to ensure complete dissolution of the formazan dye after which the absorbance of the medium at 440 nm was

recorded.

5-bromo-2'-deoxy-uridine (BrdU, Invitrogen) was used to visualize proliferative cells. After discrete periods of culture, BrdU was added to the culture medium. After BrdU exposure overnight at 37°C, cells were fixed (4% paraformaldehyde), permeabilized (0.1% Triton-X 100) and treated with HCl solution to break the DNA structure. Alexa Fluor[®] 488 anti-BrdU antibody (Invitrogen) was applied to visualize the proliferative cell nuclei. Cell images were taken using an LSM 510 confocal microscope.

4.2.8 Statistical analysis

Statistical analysis using Student's t-test was performed where appropriate. Data are represented as mean $\pm$ standard deviation of at least three measurements. $P < 0.05$ was considered significantly different.

4.3 Results

4.3.1 Synthesis of dextran and gelatin derivatives

Dextran was modified to incorporate both methacrylate and lysine functionalities (Fig. 4.1A). The structure of Dex-MA-Ly was confirmed by ¹H-NMR spectroscopy (Fig. 4.2). In addition to the peaks of pristine dextran (between δ 4.0 and 3.3 ppm due to protons at positions 2, 3, 4, 5 and 6 of the

glucose unit, at δ 4.8 ppm due to the anomeric proton (position 1) and at δ 5.1 ppm due to the α -1,3 linkages), additional peaks appear at about δ 1.8 ppm (position 7) due to methyl protons from the methacryloyl group, and at about δ 5.6 ppm and δ 6.1 ppm (position 8) due to the double bond protons. Additional signals due to protons of lysine also appear between δ 1.0 ppm and δ 1.7 ppm for γ , δ , β -protons (positions 11, 10, and 12), and at δ 2.9 ppm for ϵ -proton (position 9). The peak of α -protons (position 13) overlaps with that of the position 4 proton of the glucose unit. Two versions of Dex-MA-Ly with different degrees of methacrylate substitution (DS) of 9 and 30 were synthesized; these are hereafter denoted Dex-MA(9)-Ly and Dex-MA(30)-Ly. The amino group concentration measured using TNBS was 4.88×10^{-4} mmole/mg for Dex-MA(9)-Ly, and 4.81×10^{-4} mmole/mg for Dex-MA(30)-Ly. The measured zeta potentials of Dex-MA-Lys, which are zwitterionic, were negative: -23.21 ± 1.99 mV for Dex-MA(9)-Ly and -18.18 ± 1.98 mV for Dex-MA(30)-Ly.

Gelatin was modified to introduce methacrylamide functionality (Fig. 4.1B). By changing the amount of methacrylic anhydride, Gel-MA(s) with a range of degrees of substitution (s, where s is 16, 51, 72, or 78) were prepared. As shown in Fig. 4.3A, the absorbance at both 340 nm and 420 nm decreases with increasing degree of substitution measured using the Habeeb method. Due

to the incorporation of charged carboxyl and amino groups, Dex-MA-Ly has better compatibility with Gel-MA than Dex-MA has. As shown in Fig. 4.3B, the solution of Dex-MA(9)-Ly and Gel-MA(16) is more transparent than that of Dex-MA(9) and Gel-MA(16).

Fig. 4.2 ^1H -NMR spectra of Dex-MA-Ly dissolved in D_2O .

Fig. 4.3 A

Fig. 4.3 B

Fig. 4.3 (A) UV spectrum of gelatin and its methacrylamide derivatives reacting with TNBS. (B) Gross view of Dex-MA(9)/Gel-MA(16) (left) and Dex-MA(9)-Ly/Gel-MA(16) (right) solutions.

4.3.2 Hydrogel characterization

Three series (with a total of seven distinct formulations) of hydrogels with varying precursor concentrations and degrees of methacrylation of Dex-MA-Ly and Gel-MA were fabricated (Table 4.1); these are labeled L-G-1x, L-G-2x and L-G-3x respectively where x is A, B or C depending on the ratio of Dex-MA-Ly to Gel-MA, which was varied from 80:40 to 80:80. At fixed degree of methacrylation of Dex-MA-Ly and Gel-MA, increasing Gel-MA concentration (*e.g.* compare L-G-1A with L-G-1C, Fig. 4.4A and B) somewhat reduces swelling ratio, but dramatically increases storage modulus. Similarly, at fixed Dex-MA-Ly to Gel-MA ratio, increase in DS of either Dex-MA-Ly or Gel-MA (*e.g.* compare L-G-1A with L-G-3A or L-G-1A with L-G-2A) decreases swelling ratio and increases storage modulus (Fig. 4.4A and B). The trend of decreased swelling ratio and increased storage modulus with higher Gel-MA concentration and higher methacrylation is due to increased hydrogel precursor double bond density leading to higher hydrogel crosslinking density. The sol

content increased slightly with increasing Gel-MA concentration (Fig. 4.4C); no statistically significant trend of sol content with DS is discernible due to the relatively large measurement uncertainties.

Fig. 4.4 Properties of hydrogels: (A) swelling ratio, (B) shear storage modulus and (C) sol content. * $P < 0.05$ vs. other hydrogels with Dex-MA-Ly and Gel-MA of the same degrees of substitution; # $P < 0.05$ vs. other hydrogels with the same concentration.

4.3.3 Spreading of SMCs inside hydrogels

Gelatin was incorporated into the hydrogel networks in order to support cell adhesion and promote proteolytic degradation, both of which are needed for cell spreading within a 3D scaffold. It is known that cells are also sensitive to gel mechanics^{19, 21, 37, 281, 282}, which motivates the study of a range of formulations with diverse mechanical properties (Fig. 4.4B). The green fluorescence in Fig. 4.5A shows that the SMCs encapsulated in the hydrogels

are mostly alive at different time points. However, extensive SMC spreading and formation of 3D cellular networks were found only in the softer hydrogels (L-G-1A to L-G-1C and L-G-2A, Fig. 4.5A). The stiffer hydrogels (L-G-2B, L-G-3A and L-G-3B, Fig. 4.5A) exhibited high viability as indicated by high green fluorescent density but inhibited SMC spreading and showed no cellular networks formation after 14 days of culture. On the other hand, SMCs within the softer L-G-1A, L-G-1B, and L-G-1C hydrogels started to acquire elongated morphology after as little as 1 day of culture, and exhibited evidence of 3D cellular network formation after 2 days of culture (Fig. 4.6).

Phalloidin-staining of the cytoskeleton F-actin shows spread out cells with assembled stress fibres (Fig. 4.5Bi). A composite of confocal image sections pseudo-colored as a function of depth indicates extensive 3D spreading of SMCs within the hydrogel (Fig. 4.5Bii).

Fig. 4.5 A (Cont'd)

Fig. 4.5 B

Fig. 4.5 (A) Live/Dead staining of SMCs encapsulated inside hydrogels after 1 day (left column), 7 days (middle column), and 14 days (right column) of culture. Scale bar equals to 200 μm . Cell seeding density: 1×10^6 cells/ml. (B) Cytoskeletal F-actin staining of SMCs in L-G-1C hydrogel after 7 days. Confocal stacks of 300 μm depth were taken. The image sections were projected to one composite image (i, left), and a pseudo-colored composite of image sections of SMCs at varying depths (ii, right) was created using LSM image browser. The scale bar is 100 μm .

Fig. 4.6 Phase contrast microscopic images of smooth muscle cells encapsulated inside L-G-1A (top row), L-G-1B (middle row), and L-G-1C (bottom row) hydrogels after 1 day (left column), 2 days (middle column), and 7 days (right column) of culture. Scale bar is 100 μm . Cell seeding density: 1×10^6 cells/ml.

4.3.4 Proliferation of SMCs inside hydrogels

To quantify the proliferative capacity of SMCs inside the 3D hydrogels, cell viability during 2 weeks of culture was measured using WST-1 reagent in representative hydrogels (*i.e.* L-G-1A, L-G-1B, L-G-2A, and L-G-3A). As shown in Fig.4.7, cells in L-G-1A, L-G-1B and L-G-2A hydrogels proliferated over the two-week study period, as indicated by the increase in WST-1 absorbance with culture time. Within L-G-1A hydrogels, cell viability increased ~ 3 fold during the first two days of culture and less rapidly thereafter. In L-G-1B and L-G-2A hydrogels, cells proliferated rapidly during the first week of culture with viability increasing ~ 4 -5 fold; the viability plateaued or declined somewhat thereafter. In L-G-3A hydrogels, cell viability decreased about 50% during the first two days of culture and then stabilized; this is interpreted to be a result of inhibition of cell spreading.

The four hydrogels were created with identical cell densities but cell proliferation did not begin within the first day of culture (determined by the

absence of BrdU incorporation, discussed below, data not shown), so that the viability at day 1 is proportional to the number of cells encapsulated within the hydrogels (and surviving the first day of culture). The day 1 viability is thus a proxy for the encapsulation efficiency of the hydrogel formulation, *i.e.* the proportion of the cell inoculum incorporated into the gel network. In the context of the present experiments, the encapsulation efficiency depends on the speed with which the precursor solution gels compared with the speed with which cells settle from the precursor solution. The gelation time depends on the concentration of double bonds in the precursor solution while the cell settling time depends on the solution viscosity. The relatively low encapsulation efficiency implied by day 1 viability in the L-G-1A hydrogel is attributable to the low viscosity (quick cell settling) and low double bond concentration (slow gelation) of this hydrogel precursor solution. The L-G-1B precursor solution has higher viscosity due to higher Gel-MA concentration, while the L-G-2A and L-G-3A precursor solutions have higher concentrations of double bonds, with consequent quicker gelation, so that the cell encapsulation efficiencies of these three hydrogels are higher than that of L-G-1A hydrogel.

Cell proliferation was detected using Bromodeoxyuridine (BrdU). BrdU was added to the cell culture medium and the cells were further incubated overnight. During that time, the proliferating cells incorporated BrdU into

newly synthesized DNA. Fluorescent anti-BrdU was used to visualize the incorporated BrdU. Green colored nuclei were seen in L-G-1A and L-G-2A hydrogels after 5 days of culture (Fig. 4.8), which is consistent with the cell viability measurements (Fig. 4.7) showing an increase of cell viability.

Fig. 4.7 Comparison of proliferative capacity, as assessed by WST-1 assay, of SMCs encapsulated within L-G-1A, L-G-1B, L-G-2A, and L-G-3A hydrogels. *: $p < 0.05$ vs. adjacent time point before (same hydrogel).

Fig. 4.8 SMCs showing incorporation of BrdU after encapsulation inside L-G-1A (top row) and L-G-2A (bottom row) hydrogels for 5 days. Scale bar is 50 μm . “Ph” refers to phase contrast image.

4.4 Discussion

Hydrogels have been widely explored as substrates for cell encapsulation, and it is hypothesized that cell functions, including adhesion, spreading, proliferation, and differentiation, can be controlled by manipulating the physical and chemical characteristics of hydrogels^{31, 35, 38}. The aim of this study was to investigate the ability of hydrogels based on natural dextran and gelatin derivatives to support SMC spreading and proliferation and to investigate the effect of mechanical stiffness on 3D SMC spreading and proliferation.

Dextran was functionalized with methacrylate and lysine, and gelatin was modified to introduce methacrylamide groups. By varying the concentration of Gel-MA, as well as the DS of methacrylate, hydrogels with a range of storage moduli (from 898 to 6075 Pa) were produced by UV crosslinking. For cell encapsulation, SMCs were suspended in precursor solution and encapsulated during UV polymerization. Within the hydrogels, SMCs invaded the matrix through spreading, which is characterized by the protrusion of cellular lamellipodia. We have found that SMC spreading and proliferation is sensitive to hydrogel mechanics. The softer hydrogels (with shear storage moduli ranging from 898 Pa to 3124 Pa) promoted fast cell spreading, profound proliferation, and extensive 3D cellular network formation, while in stiffer hydrogels (shear

storage moduli ranging from 4026 Pa to 6075 Pa) SMCs maintained a round morphology and their viability declined by 50% over 2 weeks of culture (Fig. 4.5A and Fig. 4.7). The stiffer hydrogels have correspondingly denser networks that are more obstructive to cell spreading and proliferation. The spreading of SMCs within the hydrogels was clearly accompanied by the assembly of F-actin stress fibers (Fig. 4.5B). This relationship between hydrogel mechanics and SMC spreading agrees with similar studies using PEG-based hydrogels^{19, 34, 37}, although other reported hydrogels differed in the range of mechanical stiffness. For example, in one study using PEG-conjugated fibrinogen hydrogels, a qualitative spindle index was applied to classify the degree of cell spreading³⁴: a spindle index of 4 indicated highly spindled spreading SMCs, while 1 referred to round-shaped SMC with minor lamellipodia. When shear modulus increased from 62 Pa to 497 Pa, the spindle index decreased from 4 to 1 after 24 h in 3D culture. Compared with this study, our hydrogels have higher mechanical stiffness; nevertheless, it is only an approximate comparison, since the parameters regarding characterization of mechanical stiffness of hydrogels differed. However, in our case, even hydrogels with storage modulus as high as 3124 Pa permitted the initiation of cell spreading behavior within 24 h (Fig. 4.5A and Fig. 4.6). In addition to spreading, SMC proliferation and formation of cellular networks in our hydrogels were also extensive, as indicated by the

high-fold increase in cell viability (Fig. 4.7) and densely and extensively organized 3D cellular networks (Fig. 4.5A).

The excellent cellular response (*i.e.* spreading, proliferation and formation of 3D cellular networks) of our dextran and gelatin-based hydrogels may be due to their closer resemblance to native proteoglycan, which may provide a highly bioactive 3D microenvironment. The Gel-MA content confers both cell adhesion and cell-mediated degradation to our hydrogels. Although pristine dextran is non-adhesive, like the bioinert PEG, it is degradable through the action of dextranases which are present in various tissues of the human body, such as liver, spleen, and kidney ²⁶¹. In addition, lysine-modified dextran structurally resembles natural glycosaminoglycans (GAGs), which are an important component of connective tissues, and is also negatively charged like GAGs. Our composite hydrogel based on the copolymerization of gelatin and lysine-modified dextran has a degree of molecular structure-similarity to proteoglycans, which are GAGs covalently linked to a protein. It is well known that proteoglycans can affect the activity and stability of proteins and signaling molecules (*e.g.* growth factors) within ECM matrix, thus influencing cell function ²⁸³. So, we hypothesize that the combination of lysine-modified dextran and gelatin is capable of recapitulating some of the intricacies of natural ECM matrix.

4.5 Conclusion

We have developed novel biocompatible and biodegradable hydrogels based on methacrylate and lysine functionalized dextran and methacrylamide modified gelatin. The mechanical stiffness of our hydrogels can be altered over a relatively wide range of moduli by varying the degree of methacrylation of dextran and gelatin, as well as the concentration of precursor solution. Rapid 3D SMC spreading, profound proliferation and extensive cellular network formation were obtained in a subset of these novel hydrogels which had suitably low mechanical stiffness. Our hydrogels resemble native proteoglycans both structurally and functionally. The excellent cell proliferation and network formation indicate that these hybrid hydrogels based on natural polysaccharides and proteins are promising scaffolds for vascular tissue engineering.

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- ✧ **Liu YX**, Chan-Park MB. A biomimetic hydrogel based on methacrylated dextran-graft-lysine and gelatin for 3D smooth muscle cell culture. *Biomaterials* 31: 1158-1170, 2010.

Chapter 5 – Phenotype and collagen type I synthesis of smooth muscle cells in 3D dextran-based hydrogels

5.1 Introduction

SMC phenotype plays a key role in the performance of vasculature and consequently must be regulated along with spreading and proliferation in vascular tissue engineering ^{6, 7}. Differentiated SMCs in normal blood vessels exhibit a quiescent contractile phenotype with relatively low rate of proliferation and ECM synthesis ^{7, 8, 97}. Under a variety of pathological conditions, such as restenosis and atherosclerosis, SMCs dedifferentiate from their normal quiescent contractile phenotype to a synthetic phenotype characterized by migration, proliferation and abundant ECM production ^{98, 100, 284}. The SMC phenotype transition can be regulated via environmental cues, such as mechanical stimuli, soluble signaling factors and cell-cell and cell-substrate interactions ^{6, 285}. For tissue regeneration, a synthetic SMC phenotype in the early stages of tissue culture is desired to facilitate cellular expansion and deposition of ECM components, such as collagen and elastin, to replace the degrading scaffold but ultimately a transition to contractile phenotype is required for a vasoactive vessel ⁶. This has been achieved in prior

work by our group on 2D culture of SMCs within microstructured scaffolds²⁸⁶.

In this chapter, the phenotype state of SMCs within a particular grade of Dex-MA-Ly and Gel-MA 3D hydrogels (*i.e.* L-G-2A hydrogel in Chapter 4) was monitored during 20 days of culture by the gene expressions of contractile proteins (α -smooth muscle actin (α -SMA) and smooth muscle-myosin heavy chain (SM-MHC)) and nonmuscle-myosin heavy chain B (SMemb), a synthetic SMC marker^{7, 8}. Collagen type I production was also monitored indirectly at gene level and directly using immunostaining.

5.2 Methods

5.2.1 SMC culture and encapsulation within hydrogel

SMCs were cultured and encapsulated in L-G-2A (Table 4.1) hydrogel as described in section 4.2.5. For 2D comparison studies, SMCs were cultured on 24-well TCPS.

5.2.2 Real-time reverse transcription polymerase reaction (real-time RT-PCR)

RNA was collected from cell-laden hydrogels using the combination of Trizol[®] (Invitrogen) and RNeasy[®] Mini Plant Kit (Qiagen) following the procedure of reference²⁸⁷. RNA from 2D TCPS surface was obtained by

RNeasy[®] Mini Plant Kit (Qiagen) following the manufacturer's instructions. The acquired RNA was reverse-transcribed using Sensiscript RT Kit (Qiagen). Real-time PCR analysis was performed in an iQ[™] qPCR system (Bio-Rad) using iQ SYBR[®] Green reagent (Bio-Rad). The relative gene expression values were calculated with the ΔC_T (threshold cycle) methods normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Gene specific primers^{111, 288, 289} are listed in Table 5.1. Statistical analysis using Student's t-test was performed as described in section 4.2.8.

5.2.3 Immunofluorescent staining

After discrete periods of culture, hydrogel constructs were rinsed three times with PBS, fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton X-100, and blocked in 1% BSA/PBS solution. For α -actin and collagen type I staining, monoclonal anti- α -smooth muscle actin antibody (Sigma) and anti-collagen type I antibody (Sigma) were applied at 1:100 dilution at 4°C overnight or for 48 h. After washing in 1% BSA/PBS solution, secondary antibodies (Alexa Fluor[®] 488 goat anti-mouse for α -actin, Alexa Fluor[®] 488 Signal Amplification Kit for collagen type I) were applied at 1:200 dilution at room temperature for 4 h. Images were taken using an LSM 510 confocal microscope (Carl Zeiss, Germany).

Table 5.1 Primers used for real-time RT-PCR.

Gene	Primer sequence (5'-3')	A.T. (°C)	P.S. (bp)	Ref.
GAPDH	F: ATGGGGAAGGTGAAGGTCG R: GGGGTCATTGATGGCAACAATA	58	108	289
Myosin heavy chain11, smooth muscle (SM-MHC or SM1/SM2)	F:TGAACGCACTCAAGAGCAAG R:TCTACGTCCTCCAGACCTTCT	58	77	111
Alpha-actin	F:CATCACCAACTGGGACGA R: GGTGGGATGCTCTTCAGG	58	85	290
Myosin heavy chain10, non smooth muscle (SMemb)	F:CCCATGAAGAGATTCTGTCAATGC R: ACATTCATCCCAAGAAGATGGC	60	151	288
Collagen type I	F: ATGTGGCCATCCAGCTGAC R: TCTTGCAGTGGTAGGTGATGTTCT	58	75	290

A.T., P.S., and Ref. refer to annealing temperature, product size, and references, respectively.

5.3 Results

5.3.1 Differentiation of SMCs inside hydrogels

The expression of mRNA of smooth muscle myosin heavy chain 11 (SM1/SM2), α -actin, non-smooth muscle myosin heavy chain 10 (SMemb), and collagen type I were monitored during 20 days of culture in hydrogels (Fig. 5.1). SM1/SM2 and α -actin are contractile markers ⁷. Collagen type I is an ECM marker whose expression depends somewhat on SMC phenotype. Substantial ECM synthesis is typically associated with proliferative SMC phenotype ²⁹¹. SMemb is profoundly expressed in proliferating SMCs and is considered to be a marker for phenotype change from a contractile to a proliferative type ²⁹².

The gene expression tests were performed on the 3D cultures at three stages, the 4th, 9th and 20th day of culture. Day 9 corresponds to the maximum of cell viability in L-G-2A hydrogel (Fig. 4.7). For comparison with phenotype evolution in 2D culture, mRNA was also extracted from monolayers of SMCs on TCPS at days 1, 4, and 7. The cells in 2D culture reached confluence at day 4. The 2D cultures progressed more rapidly than the 3D cultures and, for the purpose of comparison, the relative state of confluence between cells on TCPS and in L-G-2A was estimated to be comparable (though not at the same absolute state of proliferation) at these time points: 4th day hydrogel vs. 1st day

TCPS, 9th day hydrogel vs. 4th day TCPS, 20th day hydrogel vs. 7th day TCPS.

We first discuss the gene expression pattern in the 2D cultures on TCPS. Expression of the contractile phenotype genes SM1/SM2 and α -actin peaked near confluence and declined in longer culture (Fig. 5.1A and B). The expression pattern of the proliferative phenotype gene SMemb reversed that of the contractile phenotype genes, though the amplitude of variation was much lower (Fig. 5.1C). The expression pattern of the gene for ECM protein collagen type I resembled that of the contractile markers (Fig. 5.1D), which seems paradoxical in view of the known tendency of SMCs in the proliferative state to secrete abundant ECM. The pattern of SM1/SM2 and α -actin expression is consistent with the interpretation of cells in the process of switching from a synthetic/proliferative to a contractile phenotype as they crowd themselves near confluence, a pattern that we have observed in SMC culture in 3D constrained microenvironments²⁸⁶. The decline in expression of SM1/SM2 and α -actin genes at day 7 point is presumably due to cells reaching the completion of their switch from synthetic/proliferative to contractile/quiescent phenotype. Although the expression of SMemb is somewhat higher at day 7 than at day 4, at both days it is lower than at day 1, suggesting an overall trend of decrease in synthetic/proliferative state with passage of time, again consistent with a picture of transition from synthetic/proliferative to quiescent/contractile state. At day 4,

the collagen type I gene expression is also high, like the contractile SM1/SM2 and α -actin gene expression, and it may be that the cells which are reaching, but have not reached, confluence are still producing significant ECM. There is manifestly a dramatic decline in expression of collagen type I gene at day 7, consistent with the picture of the culture assuming a quiescent/contractile state.

Fig. 5.1 Expression of SM1/SM2 (A), α -actin (B), SMemb (C), and collagen type I (D) over 20 days of culture in L-G-2A hydrogels and over 7 days of culture on TCPS. The inset in B enlarges the vertical scale for the α -actin expression in L-G-2A hydrogel. Expression levels shown are relative to GAPDH expression in the same sample. *: $p < 0.05$ vs. TCPS (4th day hydrogel vs. 1st day TCPS; 9th day hydrogel vs. 4th day TCPS, 20th day hydrogel vs. 7th day TCPS); #: $p < 0.05$ vs. same sample prior time point.

In L-G-2A hydrogel, the expression patterns of SM1/SM2, α -actin, SMemb and collagen type I were broadly similar in shape to those on TCPS, but significantly different in absolute level. Here we summarize the details; the implications are discussed in the next section. The initial (4 d) expression level of SM1/SM2 gene is significantly higher than that of day 1 TCPS ($p < 0.015$, Fig. 5.1A). Expression of SM1/SM2 first increased but not significantly ($p > 0.06$, 9th day vs. 4th day), then significantly decreased ($p < 0.015$, 20th day vs. 9th day). The highest expressed SM1/SM2 gene at day 9 was significantly lower than that with TCPS ($p < 0.0003$). The initial (4 d) expressions of the other contractile gene α -actin (Fig. 5.1B) and synthetic marker SMemb (Fig. 5.1C) were significantly lower in L-G-2A hydrogel than on TCPS ($p < 0.015$ and $p < 0.00005$ for α -actin and SMemb respectively). Expression of α -actin increased from 4 days to 9 days of culture ($p > 0.06$, not significantly), and decreased from 9 days to 20 days of culture ($p > 0.3$, not significantly). The α -actin gene expression was

much lower than that of the culture on TCPS at all measured times. Expression of SMemb first significantly decreased ($p < 0.003$, 9th day vs. 4th day) then dramatically increased ($p < 0.0000015$, 20th day vs. 9th day); the final stage measurement of SMemb expression in the hydrogel was much higher than that in the 2D culture. Expression of collagen type I first significantly increased ($p < 0.0006$, 9th day vs. 4th day) and then decreased significantly ($p < 0.025$, 20th day vs. 9th day).

5.3.2 Immunofluorescent staining of α -actin and collagen type I

The expression of smooth muscle specific marker α -actin was confirmed by immunofluorescent staining (Fig 5.2 top row). SMCs in L-G-1A and L-G-2A hydrogels after 9 days of culture stained positive for collagen type I (Fig. 5.2 bottom row), with pericellular localization, indicating that the secreted proteins had not diffused far from the cells.

Fig. 5.2 Immunofluorescent staining of α -actin after 4 days (top row), and collagen type I after 9 days (bottom row) in L-G-1A and L-G-2A hydrogels. Scale bars are 100 μ m (top row) and 50 μ m (bottom row), respectively.

5.4 Discussion

We investigated the evolution of SMC phenotype, which is closely related to SMC function, in our 3D hydrogels. We examined the regulation of phenotype-specific proteins at gene level during 20 days of culture in L-G-2A hydrogel, and compared that with cultures on 2D TCPS. It has been reported

that as SMCs cultured on 2D micropatterned surface approach confluence, their morphology changed toward spindle-like, accompanied by upregulated expression of contractile α -actin, indicating a switch to a more contractile phenotype²⁸⁶. Here, we also observed tendency towards increased contractility through upregulation of contractile genes α -actin and SM1/SM2, and downregulation of synthetic gene SMemb on 2D TCPS near confluence. The switch of SMC phenotype towards more contractile near confluence may be due to the tensional stress imposed on cells by neighbors within a densely packed monolayer^{286, 293, 294}.

In 3D hydrogels, SMCs proliferated during the first week of culture. Confluence was not observed, perhaps due to the low cell seeding density and limited proteolytic degradation of the hydrogels during the culture period. In L-G-2A hydrogels, the two contractile genes measured exhibited a time pattern of expression similar to that of 2D cultures on TCPS (Fig. 5.1), though with substantially lower absolute levels of expression; the α -actin expression is dramatically lower in the 3D culture than in the 2D culture at all time periods. The SM1/SM2 and α -actin marker evidence for phenotype switch is equivocal in view of their relatively low (relative to 2D culture) expression, particularly that of α -actin. The most puzzling feature of the gene expression data, however, is the radical increase in SMemb expression in the 3D culture at day 20. We do

not have independent information on the state of the hydrogel network at this stage of culture, but this large increase in a proliferative marker suggests that the network may have degraded to the point that the cells are more nearly able to freely proliferate.

In the 3D cultures, the cells are from the outset in contact with the hydrogel network, which initially is quite densely crosslinked. This initial constraint may explain the higher early expression of the contractile marker SM1/SM2 and the lower early expression of the synthetic marker SMemb in the 3D culture than in the 2D culture. However, the switch to contractile phenotype at the measured point of maximum proliferation (as measured by the viability study, Fig 4.7) for 3D encapsulated cells is not significant, presumably because the cells have not reached confluence due to the prior restraint on proliferation imposed by the slow degradation of the hydrogel matrix. Instead, with longer culture (20 d) and further matrix degradation, it appears that the SMCs start to change to a synthetic/proliferative state. This is corroborated by the higher ECM gene expression in longer cultures (9 d and 20 d) compared to the initial value. Our results corroborate with the observation that stress environment, mechanical influence such as mechanical stimulation ^{6, 285} and physical constraint ²⁸⁶ can promote contractile phenotype or suppress the synthetic phenotype. The pericellular environment in the 3D culture is very different from

that of the 2D culture at all measured stages, and is dynamic due to proteolytic degradation of the hydrogel network. Cells in the 3D cultures appear to be more synthetic at the latest measured period than in 2D due to their nonconfluent state, coupled with presumed increase in space for spreading due to cell-mediated degradation of the hydrogel matrix.

Although various environmental cues, such as mechanical stress, substrate stiffness, and soluble signal factors (*e.g.* ascorbic acid and TGF- β 1), could be applied to influence SMC phenotype^{6, 250, 285}, regulating SMC phenotype, especially redifferentiating SMCs towards a contractile phenotype, in 3D hydrogels remains challenging compared to 2D culture --- a key question is whether the 3D culture can proliferate to a state resembling 2D confluence with corresponding contractile phenotype. Synchronization of degradation of matrix with active cellular proliferation may be a key factor.

The encapsulated SMCs in our 3D hydrogels produced collagen type I at both transcriptional and protein levels (Fig. 5.1D and Fig. 5.2). Synthesis of *de novo* ECM proteins is beneficial for the remodeling of hydrogel microenvironment; however the diffusion of cell-secreted collagen type I, which may bind to cell surface receptor and influence cell function²²⁹, was limited to the pericellular region (Fig. 5.2). The largely pericellular deposition of collagen type I has also been reported in PEG-protein or peptide hybrid

hydrogels with encapsulated SMCs^{19, 34, 250}. This may be due to the limited degradation of the hydrogels, which may inhibit the diffusion of ECM molecules. Regulating the distribution of *de novo* ECM proteins within 3D hydrogels remains another challenge for cell encapsulation for tissue engineering or tissue regeneration.

5.5 Conclusion

SMCs cultured in our 3D hydrogels showed evidence of dynamic phenotype modulation by the gel. The cells initially exhibited a mild degree of contractile gene expression, attributed to the tight mechanical constraint of the highly crosslinked gel. After prolonged culture, synthetic/proliferative gene markers increased, which may be due to decreased mechanical constraint as the hydrogel began to proteolytically degrade. Cells in these 3D hydrogels are generally more synthetic than in 2D. *De novo* ECM component collagen type I was detected within hydrogels at both transcription and translation levels. Although promising for vascular tissue engineering, further study and perhaps additional modalities for regulation of SMC phenotype control in 3D hydrogels are needed.

This chapter has been reproduced from the following paper published by the PhD student.

- ✧ **Liu YX**, Chan-Park MB. A biomimetic hydrogel based on methacrylated dextran-graft-lysine and gelatin for 3D smooth muscle cell culture. *Biomaterials* 31: 1158-1170, 2010.

Chapter 6 – Impact of endothelial cells on smooth muscle cells in 3D culture

6.1 Introduction

In blood vessels, smooth muscle cells (SMCs) and endothelial cells (ECs) are the two important cellular components for vessel function. Communication between SMCs and ECs through direct physical contact^{295, 296} or synthesis and diffusion of specific mediators^{91, 297, 298} regulates the behaviors of both ECs and SMCs, and the structural and functional maintenance of vessels is dependent on such communication²⁹⁹⁻³⁰¹.

ECs have marked impact on SMC behaviors. The EC-produced vasoactive molecules, including endothelin, platelet-derived growth factor (PDGF), transforming growth factor (TGF)- β , prostacyclin *etc*, influence SMC behaviors such as spreading, proliferation, migration, differentiation, and secretion of ECM proteins^{91, 296, 302-305}. Much work has been done to determine the regulatory effects of ECs on SMCs and vice versa. These investigations were performed in various ways: direct co-culture in which ECs were seeded directly on top of SMCs⁹¹, bilayer co-culture in which ECs and SMCs were seeded on opposite sides of a porous membrane^{296, 302, 303}, and SMC culture using

EC-conditioned medium²⁹⁶. Although *in vitro* EC-SMC interactions have been shown to lead to more biomimetic environment, the 2D culture of SMCs reported^{91, 296, 302, 303}, which is unlike the native situation, may induce behaviors different from that of *in vivo*.

Several co-culture models using 3D cultured SMCs have also been investigated thus far³⁰⁵⁻³⁰⁹. A co-culture model with SMCs sandwiched between two layers of collagen gel and ECs seeded on the top surface has been used to explore the effects of SMCs on ECs³⁰⁶. Although approximating the vessel wall architecture, this co-culture model has the limitation that SMCs were essentially presented in a monolayer. Bilayered poly(ethylene glycol) diacrylate (PEGDA) hydrogel constructs composed of SMCs and ECs encapsulated in two different layers were developed as a 3D co-culture model³⁰⁵. However, the spherical morphology of SMCs within PEGDA hydrogels and the 3D encapsulated rather than the 2D monolayer arrangement of ECs are limitations of the system in mimicking the native condition. In another co-culture model, SMCs were embedded in collagen gel, and ECs were cultured directly on the surface of the cell-laden gel³⁰⁷⁻³⁰⁹. ECs did not show significant proliferation on collagen gel, not to mention the formation of monolayer³⁰⁹. Also the growth of SMCs was suppressed in 3D collagen gels compared to 2D culture¹³⁸.

In previous chapters, we have reported biomimetic hydrogels which

promoted 3D SMC spreading and proliferation. The excellent SMC proliferation in these hydrogels offers the possibility to study the effect of ECs on 3D-cultured SMCs. In this chapter, we reported an EC/SMC co-culture system using SMCs encapsulated inside the L-G-2A hydrogel developed in Chapter 4. Our initial results showed that it was difficult to maintain an integrated monolayer of ECs on the surface of hydrogels itself, and during longer time cultures, the ECs detached from the surface, which may be due to hydrogel compaction. So in this co-culture system, ECs were cultured on surface of tissue culture polystyrene (TCPS) and allowed to form a sub-confluent monolayer prior to co-culture with SMCs which were encapsulated in hydrogel (Fig. 6.1).

Fig. 6.1 EC/SMC co-culture model. SMCs were encapsulated in hydrogels and exposed to a monolayer of ECs.

Hydrogels with encapsulated SMCs and cultured in the presence of ECs

are denoted “EC⁺ constructs”. Cell/hydrogel constructs were also cultured without the EC monolayer as the control group; these are referred to hereafter as “EC⁻ constructs”. Using this co-culture model, the effect of ECs on SMC spreading and proliferation in a 3D environment was investigated. The transcriptional expressions of SMC-specific smooth muscle (SM) α -actin, calponin, smooth muscle-myosin heavy chain (SM-MHC), smoothelin, and nonmuscle-myosin heavy chain B (SMemb) were measured in both EC⁺ and EC⁻ constructs to investigate the impact of ECs on SMC differentiation. The ECM production of SMCs was also measured at transcriptional level.

6.2 Experimental details

6.2.1 Co-culture of ECs and SMCs

Human umbilical artery endothelial cells (HUAECs) were cultured on the surface of 24-well TCPS using Endothelial Basal Medium supplemented with 2.5% fetal bovine serum (FBS), Bovine Brain Extract, hydrocortisone, human epidermal growth factor (hEGF), and gentamicin/amphotericin-B (GA)), as described in section 3.2.1. Depending on the seeding density, ECs were cultured for 2-3 days to sub-confluence and then subjected to co-culture with SMCs as described below.

SMCs were encapsulated in L-G-2A (Table 4.1) hydrogel as follows:

SMCs were suspended in hydrogel precursor solution; unless otherwise indicated, 80 μ l of cell-laden precursor solution was deposited onto the wells of 48-well nontreated TCPS (BD Falcon), and subjected to low intensity UV illumination (365 nm, 20 mW/cm²) for 5 min under argon atmosphere. After UV irradiation, the cell/hydrogel constructs were washed with PBS and supplied with DMEM containing 10% FBS. One day after cell encapsulation, the constructs were transferred to the wells of 24-well TCPS which has a monolayer of ECs and supplied with DMEM containing 10% FBS (Fig. 6.1). The medium was refreshed every two days. In order to avoid problems associated with long time culture of ECs, such as detachment, the SMC-laden hydrogel constructs were transferred to other freshly cultured EC monolayers every 3-4 days.

6.2.2 Characterization of SMC spreading and proliferation

SMC spreading in the hydrogels was monitored after seeding using an Axiovert 200 Motorized Inverted Microscope System and recorded with a digital CCD camera. SMC proliferation was tested via quantification of cell viability using Cell Proliferation Reagent WST-1. Following incubation for various periods, EC⁺ and EC⁻ constructs (fabricated from 40 μ l of precursor solution) were transferred to the wells of a new 24-well TCPS and supplied

with 400 μ l of fresh DMEM without phenol red or FBS. 40 μ l of WST-1 reagent was added to each well. After incubation for an additional 4 h, the absorbance of the formazan dye solution in culture medium at 440 nm was recorded. 6 parallels were averaged for each hydrogel sample, and data are expressed as mean $\pm$ standard deviation values.

6.2.3 Gene expression analyse

RNA was extracted from cell/hydrogel constructs using the RNeasy[®] Mini Plant Kit (Qiagen) following the manufacturer's protocols. cDNAs were generated from 200 ng of total extracted RNA using Omniscript[®] Reverse Transcription Kit (Qiagen) with oligo-dT primer. Gene expression levels were determined via real-time PCR analysis using iQ SYBR[®] Green reagent on an iQTM qPCR system (Bio-rad). Melt curve analysis was performed to ensure a single amplification for each gene. The ΔC_T method as normalized to GAPDH was applied to calculate the relative gene expression. Gene specific primers for α -actin, collagen type I, SMem and GAPDH were the same as those listed in Table 5.1. Primers for smoothelin, calponin, elastin, and SM-MHC (also referred to as SM1/SM2 in Chapter 5)^{289, 290} are listed in Table 6.1.

Table 6.1. Primers used for real-time RT-PCR.

Gene	Primer sequence (5'-3')	A.T. (°C)	P.S. (bp)	Ref.
Smoothelin	F: CCCTGGCATCCAAGCGTTT R: CTCCACATCGTTCATGGACTC	62	137	289
Calponin1, basic	F: GAGTCAACCCAAAATTGGCAC R: GGACTGCACCTGTGTATGGT	58	138	289
Elastin	F: GAGCTTTTGCTGGAATCCCA R: GGCAGTTTCCCTGTGGTGTAG	62	130	290
Myosin heavy chain11, smooth muscle (SM-MHC)	F: CTGCAGCTTGGAATATCGT R: GAGTGAGGATGGATCTGGTG	52	136	289

A.T., P.S., and Ref. refer to annealing temperature, product size, and references, respectively.

6.2.4 Degradation of hydrogel constructs

Following culture for certain periods, cell/hydrogel constructs were washed with PBS and freeze-dried. The dry weight of the hydrogel constructs was measured to investigate the degradation of hydrogels during cell culture.

6.3 Results

6.3.1 Impact of ECs on SMC spreading and proliferation

ECs were cultured on TCPS for 2-3 days (Fig. 6.2A) before the beginning

of co-culture with SMCs. The ECs were sub-confluent at the beginning of co-culture. SMCs were encapsulated in L-G-2A hydrogels. Initially, SMCs had a round morphology (Fig. 6.2B). After 1 day of culture, some of the cells started to spread out (Fig. 6.2C). After 1 day of culture, the SMC/hydrogel constructs were transferred to TCPS with a monolayer of ECs.

Fig. 6.2 Phase contrast microscopic images of (A) EC monolayer on TCPS after 2-3 days of culture, (B) SMCs just after encapsulation and (C) cultured for 1 day within L-G-2A hydrogel. Scale bar is 100 μm . SMC seeding density in hydrogel: 1.8×10^6 cells/ml.

To examine the effect of EC monolayer on SMC spreading, phase contrast images of SMCs within EC⁺ and EC⁻ constructs were taken during 7 days of culture (corresponding to 6 days of exposure of SMCs to EC monolayer in the EC⁺ group). After 4 days of culture, more spreading SMCs and denser cellular network were present in EC⁺ constructs than in EC⁻ constructs (Fig. 6.3). This phenomenon became more obvious after 7 days of culture.

Fig. 6.3 Morphology of SMCs encapsulated within L-G-2A hydrogels and cultured for 2 days (left column), 4 days (middle column), and 7 days (right column) with ECs (top row) and without ECs (bottom row). Scale bar is 100 μm . SMC seeding density: 1.8×10^6 cells/ml.

The SMC proliferation in EC^+ and EC^- constructs during 2 weeks of culture was quantified via analyzing the number of viable cells using WST-1 reagent. As shown in Fig. 6.4, the general trend of cell proliferation with culture time in EC^+ and EC^- constructs were similar: the number of viable cells initially increased, but decreased with longer cultures. In EC^+ constructs, SMC proliferation was stimulated by the EC co-culture initially (at 4th day and shorter period of culture), as indicated by the significantly higher WST-1 absorbance compared with EC^- constructs. However, this stimulatory effect disappeared with longer cultures: the numbers of viable cells in EC^+ and EC^- constructs were not significantly different on and after 7 days of culture.

Fig. 6.4 Measurement of cell proliferation, using WST-1 assay, of SMCs encapsulated within L-G-2A hydrogels with and without the presence of ECs (EC⁺ and EC⁻ constructs). *: $p < 0.05$, EC⁺ vs. EC⁻ constructs at the same time point; #: $p < 0.05$, vs. prior time point (comparison was performed within EC⁺ and EC⁻ groups respectively, except that the 2 d result of EC⁺ constructs was compared with the 1 d result of EC⁻ constructs).

The comparable cell viability in EC⁺ and EC⁻ constructs with longer (day 7 and beyond) culture periods seems not to agree with higher density of cellular networks in EC⁺ constructs (Fig. 6.3). However, significantly higher degree of compaction was observed in EC⁺ constructs compared with EC⁻ constructs during culture (data not shown), which may be due to the promoted SMC proliferation in the early culture periods in the presence of ECs. We propose that

the reduced gel volume resulted in denser cellular networks in EC⁺ constructs.

6.3.2 Impact of ECs on SMC differentiation

It has been well demonstrated that SMC differentiation occurs during normal development, under pathophysiological conditions, as well as during *in vitro* culture^{7, 8, 98, 310-312}. SMC differentiation is ultimately modulated at transcriptional level³¹⁰⁻³¹². In order to investigate the effect of ECs on SMC differentiation, the expressions of a number of genes were analyzed during 2 weeks of culture. These genes include SMC contractile marker genes (specifically α -actin, calponin, SM-MHC, and smoothelin), synthetic marker gene (SMemb), and ECM protein genes (collagen type I and elastin).

α -actin, calponin, SM-MHC, and smoothelin are contractile apparatus proteins. They participate in the contraction function of SMCs. In EC⁻ constructs, the trends of transcriptional expressions of these contractile proteins, except smoothelin, showed a similar pattern with culture time (Fig. 6.5A, B, and C): there were significant increases during initial culture; maximum expressions were reached at 4th day; the expression levels declined thereafter. For smoothelin, in EC⁻ constructs (Fig. 6.5D), the expression had a maximum level at the 2nd day. Also, the 1.2-fold maximum increase (by comparing expression on the 2nd day with that on the 1st day) of smoothelin was

considerably less than the maximum increase of other contractile marker genes (7.8 fold for α -actin, 14.5 fold for calponin, and 2.6 fold for SM-MHC obtained by comparing day 4 expressions with day 1 expressions).

In EC⁺ constructs, the general trend of expressions of α -actin and calponin with culture time resembled the corresponding ones in EC⁻ constructs (Fig. 6.5A and B): the gene expression increased initially, and then declined, though the maximum expressions were achieved at 10th day instead. There was no significant difference between the maximum expressions of α -actin, as well as calponin, of SMCs in EC⁺ and EC⁻ constructs ($p=0.44$ for α -actin; $p=0.27$ for calponin). The α -actin and calponin curves for the EC⁺ constructs seem to be merely shifted to the later time compared to the corresponding ones for the EC⁻ constructs.

Once exposed to ECs, SM-MHC expression of SMCs significantly increased ($p=0.04$, EC⁺ constructs 2nd day vs. EC⁻ constructs 1st day) (Fig. 6.5C). Thereafter, the expression decreased significantly and remained at a low level until the 10th day, after which a sharp increment led to the maximum expression. The 14th day maximum expression level was slightly higher than the 2nd day expression in EC⁺ construct, and was comparable to the maximum in EC⁻ constructs.

Smoothelin expression in both EC⁺ and EC⁻ constructs had a similar

dynamic trend (Fig. 6.5D). There seems to be an oscillation of some kind. However, in general, the expression level was higher at the beginning and lower with longer culture periods. The EC⁺ constructs had a consistently higher expression level than EC⁻ constructs, except at the 4th day.

Fig. 6.5 (Cont'd)

Fig. 6.5 Expression of (A) α -actin, (B) calponin, (C) SM-MHC, (D) smoothelin, and (E) SMemb over 14 days of culture in EC⁺ and EC⁻ constructs. Expression levels shown are relative to GAPDH expression in the same sample. *: significant difference ($p < 0.05$) between EC⁺ and EC⁻ constructs at the same time point; #: significant difference ($p < 0.05$) vs. prior time point (within the same group of constructs except that the 2 d result of EC⁺ constructs was compared with the 1 d result of EC⁻ constructs).

The EC⁺ and EC⁻ constructs also had similar dynamic expression patterns of SMemb (Fig. 6.5E), which was lower at the beginning (on day 7 and before) but increased thereafter. Initially, the expression levels in EC⁺ constructs were lower than in EC⁻ constructs. It was after the 7th day that this trend was reversed with EC⁺ having higher level of SMemb expression. There is a hint of an oscillation about the trend in these curves.

6.3.3 Impact of ECs on ECM production of SMCs

In vascular tissue engineering, ECM synthesis is required to replace the degrading scaffold. Synthesis of ECM is also indicative of SMC phenotype state. It is generally considered that SMCs in a more synthetic phenotype increase their synthesis of ECM components⁸. We have investigated the synthesis of collagen type I and elastin at transcriptional level. As shown in Fig. 6.6A, EC⁺ and EC⁻ constructs had similar expression dynamics of collagen type I: the levels increased with time. The expression of collagen type I in EC⁺ constructs was lower than in EC⁻ constructs at all the evaluated time points except on the 10th day. However, the difference between these two groups was not significant at most of the measured points.

There was significant difference in the expression patterns of elastin in EC⁺ and EC⁻ constructs (Fig. 6.6B). In EC⁻ constructs, SMCs had considerably lower and stable elastin transcriptional expressions during 2 weeks of culture. EC⁺ constructs had significantly higher levels of elastin expression than EC⁻ constructs at the evaluated time points except on the 4th day. However, there was a large oscillation in elastin synthesis of SMCs in EC⁺ constructs.

Fig. 6.6 Gene expressions of ECM proteins: (A) collagen type I and (B) elastin, over 14 days of culture in EC⁺ and EC⁻ constructs. Expression levels shown are relative to GAPDH expression in the same sample. *: significant difference ($p < 0.05$) between EC⁺ and EC⁻ constructs at the same time point; #: significant difference ($p < 0.05$) vs. prior time point (within the same group of constructs except that the 2 d result of EC⁺ constructs was compared with the 1 d result of EC⁻ constructs).

6.3.4 Impact of ECs on hydrogel degradation

The degradation of hydrogels with encapsulated SMCs was analyzed via measuring the dry weight of constructs during 2 weeks of *in vitro* culture. In Fig. 6.7, it is shown that the dry weight of both EC⁺ and EC⁻ constructs decreased with time. With the presence of ECs, the EC⁺ constructs seemed to degrade faster than EC⁻ constructs. At all the evaluated time points, the dry weights of EC⁺ constructs were lower than that of EC⁻ constructs. However, the differences were not significant except at the 2nd day. Due to the incorporation of natural gelatin, the hydrogels based on dextran and gelatin would be degraded by cell-secreted enzymes, such as matrix metalloproteinases (MMPs). The presence of ECs could enhance the degradation by secretion or activation of MMPs such as MMP-1 and MMP-2³¹³.

Fig. 6.7 Dry weight of cell-laden hydrogel constructs (EC⁺ and EC⁻) during 14 days of culture. *: significant difference ($p < 0.05$) between EC⁺ and EC⁻ constructs at the same time point. (n=6)

6.4 Discussion

An EC/SMC co-culture model is presented here in which SMCs encapsulated in 3D hydrogels were exposed to a monolayer of ECs. This co-culture model is superior in that the SMCs were kept in a 3D microenvironment mimicking the native condition. Although no direct EC-SMC physical contact was present, the exposure of SMC/hydrogel constructs directly to EC monolayer is expected to be superior to the model using EC-conditioned medium²⁹⁶, in which the effect of EC-secreted regulators

on SMCs may be neglected due to their short life times. Additionally, SMCs may also influence EC secretion of regulators. This bidirectional communication is absent in cultures with EC-conditioned medium.

Upon exposure to ECs, the initial (before 7 d) SMC proliferation was promoted within 3D hydrogels (Fig. 6.4). The EC regulatory, either inhibitory or stimulatory, effect on SMCs is known to be dependent on the culture state of ECs. For example, sub-confluent ECs stimulated SMC proliferation whereas no such stimulatory effect was evident with confluent ECs³¹⁴. In our co-culture model, a stimulatory effect on SMC growth was detected. The monolayer of ECs in our model was actually sub-confluent, as shown in Fig. 6.2A: gaps existed between cells, and formation of tight cell-cell junctions was limited. So the result is consistent with the reported results³¹⁴. The initial (2 d) faster hydrogel degradation of EC⁺ constructs may also be responsible for the enhanced SMC proliferation (Fig. 6.7). The creation of space with hydrogel degradation would promote SMC proliferation. This is consistent with the reported observation that in 3D hydrogels, the “steric hindrance” effect of the dense matrix would suppress cell growth, since there were not sufficient space for cells to divide¹³⁸.

Higher compaction of SMC-laden hydrogels was also observed with EC⁺ constructs (data not shown), which may be as a result of higher rate of SMC

proliferation in the early culture periods. The compaction led to the formation of much denser cellular networks in EC⁺ constructs (Fig. 6.3). The stimulation of SMC growth and formation of denser cellular networks favor the use of hydrogels for vascular tissue engineering, since high cell density and deposition of ECM are required to replace and remodel the degradable hydrogel scaffold.

In vascular tissue engineering, suitably timed SMC phenotype modulation is believed to be the key to success⁶: a synthetic phenotype is required initially for vessel remodeling whereas a contractile phenotype is required ultimately for vasoactivity. In chapter 5, we have shown that SMCs in 3D hydrogels without co-culture with ECs were generally in a more synthetic phenotype. Others have reported that ECs promoted the contractile phenotype of SMCs in 2D cultures³¹⁵. In this chapter, the impact of ECs on SMC phenotype modulation in 3D hydrogels was investigated. SMC phenotype in EC⁻ constructs was investigated as control. Although EC⁻ constructs were investigated in chapter 5, here we directly compared their behavior with EC⁺ constructs, included more genes (calponin and smoothelin) and also extended data points to initial cultures (1st day and 2nd day). We shall first discuss the impact of our hydrogels on the SMC phenotype in the absence of ECs and then in the presence of ECs.

SMC contractile markers including α -actin, calponin, SM-MHC, and smoothelin are often used to define the phenotype of SMCs. The expressions of

these markers are downregulated when SMC phenotype is switched from contractile to synthetic ^{7, 8, 98}. In EC⁺ constructs, the expression of contractile genes α -actin, calponin, and SM-MHC were found to have similar dynamics (Fig. 6.5A, B, and C): they first increased and peaked at 4th day, and declined thereafter. The initial (4 d and before) increase of gene expression levels of these contractile proteins may be due to the establishment of increased cell-cell contact (Fig. 6.3) which increases the tensional force imposed on cells ^{293, 294} as SMCs spread and form 3D cellular networks. The same phenomenon has been shown in our previous work in 2D SMC cultures (Chapter 5). The general trends of α -actin and SM-MHC for the EC⁺ constructs (Fig. 6.5A and C) are consistent with those in Chapter 5 (Fig. 5.1A and B). (Calponin was not investigated in Chapter 5.) However, the increase here was much more significant, which may be due to the higher seeding density here (1.8×10^6 cells/ml) compared to that in Chapter 5 (1.0×10^6 cells/ml).

J.P. Stegemann has also shown that the expression of α -actin protein in 2D-cultured SMCs initially increased and then dropped ¹³⁸. However, in the same study, no such an increase was observed when SMCs were 3D cultured inside collagen gel. Instead, the expression of α -actin protein was downregulated significantly and rapidly during 3D culture. This may be caused by the suppression of SMC growth and hence the establishment of cell-cell

contact in collagen gel. Compared with collagen gel, our dextran and gelatin based gels promoted SMC growth and formation of 3D cellular networks. As a result, upregulation in the expressions of contractile genes resembling that in 2D cultures was observed.

Distinct from α -actin, calponin, and SM-MHC, smoothelin transcription level in EC⁻ constructs only marginally increased during the initial 2 days of culture. A significant decline was observed thereafter. It seems that the establishment of cell-cell contact has less influence on smoothelin transcription than on α -actin, calponin, and SM-MHC transcription. In contrast to α -actin, calponin, and SM-MHC, it is known that smoothelin is a more sensitive and advanced SMC contractile marker¹⁰⁴. Its expression is downregulated rapidly when SMCs are switched to a more synthetic phenotype⁸. Once its expression ceases, it is thought to be impossible to trigger its re-expression *in vitro*¹⁰⁴. In our hydrogels, although the increased cellular tensional force has induced the upregulation of contractile markers α -actin, calponin, and SM-MHC, it was not sufficient to induce the expression of smoothelin. Much denser cellular networks may be needed for the complete differentiation of SMCs in hydrogels.

Comparing the EC⁺ and EC⁻ constructs (Fig.6.5A, B, and C), the plots of α -actin, calponin, and SM-MHC for the co-culture seem to be similar in shape to the corresponding ones for EC⁻ except that the EC⁺ ones were shifted to the

right *i.e.* delayed. However, the presence of ECs had no influence on the maximum transcription levels of any of these genes. It seems that ECs delayed the onset of maximum transcription of α -actin, calponin, and SM-MHC, although the cellular networks in EC⁺ constructs were denser and SMC proliferation during the initial (before 7 days) culture in EC⁺ constructs was higher. Fig. 6.5A, B, and C indicate that in EC⁺ constructs, the transition towards a more contractile phenotype during the formation of cellular networks was delayed.

However, for the more advanced contractile smoothelin, there was no such a delayed expression. Although the transcription levels were slightly higher in EC⁺ constructs, the general dynamics of smoothelin transcription was not significantly influenced by ECs. This difference associated with impacts of ECs on transcription of smoothelin, α -actin, calponin, and SM-MHC could be attributed to different regulatory mechanisms at transcriptional level^{316, 317}. We postulate that though the cellular network leads to increased expression of the contractile genes (Fig. 6.5A, B & C), ECs may themselves secrete or stimulate SMCs to secrete certain growth factor(s) that can modulate contractile protein expression at transcriptional levels in EC⁺ constructs. Further, this regulatory effect may function only transiently (*i.e.*, during the initial period) due to adjustable secretion levels of the growth factors. We hypothesize that upon

exposure of SMCs to ECs, growth factors secretion was stimulated with a higher level; but with longer cultures, due to certain feedback mechanisms, this stimulation effect vanished and resulted in the similar levels of growth factors in both EC⁻ and EC⁺ constructs.

It is generally believed that α -actin, calponin, and SM-MHC genes have multiple CArG elements (*i.e.*, a CC(AT-rich)(6)GG motif) in their promoter-enhancer regions^{310, 311, 318}. Serum response factor (SRF) binds to the CArG elements and regulates the transcription of these genes^{316, 317, 319}. Although smoothelin (which refers to smoothelin-B in this work) has 2 CArG-like elements in the promoter, its expression is believed to be CArG-independent³²⁰. Myocardin has been identified as a potent SRF coactivator, which activates the transcription of these CArG-containing SMC marker genes^{310, 311, 316, 317, 319}. Overexpression of myocardin has been shown to induce expression of α -actin, calponin, and SM-MHC genes but not smoothelin gene in SMC precursor cells³¹⁷.

The expression of myocardin is downregulated by PDGF-BB, and PDGF-BB also inhibits myocardin-SRF interaction as well as SRF/myocardin association with CArG elements in SMC marker genes^{312, 321}. Through combinatorial mechanisms, PDGF-BB induces the repression of numerous CArG-containing SMC marker genes including α -actin, SM-MHC, and

calponin^{312, 322}. ECs have been shown to increase the expression of PDGF-BB gene of co-cultured SMCs⁹¹. Cultured ECs also release PDGF-BB⁹¹. It is possible that the release pattern of PDGF-BB in our co-culture model is responsible for the initial delayed onset of maximum transcription of α -actin, calponin, and SM-MHC. Since smoothelin does not have a myocardin-SRF-dependent regulatory pathway, its expression was largely uninfluenced by the presence of ECs.

SMemb is reported to be a dedifferentiated SMC marker^{7, 106, 199}. It is expressed in embryonic SMCs and the expression declines in adult SMCs¹⁰⁶. However, in proliferating SMCs, the expression of SMemb is quickly and markedly upregulated⁸. The upregulated expression of SMemb (after day 4) collaborates with the observation of downregulated expression of smoothelin in both constructs (after day 2), and indicates a tendency towards synthetic phenotype. Considering all the five gene expressions in Fig. 6.5, a fully differentiated SMC phenotype was not induced even in the presence of ECs although there was increase in some contractile gene expressions.

It is noteworthy that initially upon exposure to ECs (at 2nd day), there was an upregulation in the transcriptions of all the examined contractile genes (*i.e.* α -actin, SM-MHC, calponin, and smoothelin), and a downregulation in SMemb. There must be biochemical factors other than PDGF-BB in the co-culture

model that induced such a phenotype switch towards more contractile phenotype. It may be TGF- β , which unlike PDGF-BB, can increase the expression levels of α -actin, SM-MHC, and calponin^{57, 323} and decrease the expression of SMemb²⁸⁸, so as to induce the contractile phenotype of SMCs⁸. Co-culture with ECs has been demonstrated to increase the expression of TGF- β gene in SMCs⁹¹. We postulate that in EC⁺ construct, TGF- β effect is more significant on day 2 but PDGF-BB is more significant on day 4. The different significance could be attributed to their secreted levels. However, the effects of both growth factors may return to the same relative level as those in EC⁻ construct on day 6 and beyond, so that the curves for the various gene transcription levels in Fig 6.5 for both EC⁺ and EC⁻ constructs are similar but the EC⁺ curve is delayed.

We hypothesize that the coordination of multiple biochemical factors, such as TGF- β and PDGF-BB, are responsible for the phenotype modulation of SMCs in co-culture with ECs. Additional data associated with the release dynamics of these factors in this co-culture model would be helpful to prove such a hypothesis.

It appears that the presence of ECs initially (day 2) promoted a more contractile phenotype of SMCs in 3D hydrogels compared to SMCs in EC⁻ constructs, possibly due to TGF- β secretion. However, shortly after the initial

period, this effect was reversed: the phenotype of SMCs in EC⁺ was more synthetic than that in EC⁻, possibly because PDGF-BB was secreted. The effect of ECs on SMC phenotype is known to be dependent on the EC state of proliferation; quiescent ECs inhibit SMC proliferation, whereas proliferating ECs promote SMC proliferation^{301, 324}. Dependent on the culture state of ECs, different impacts may be imposed on the co-cultured SMCs. In our co-culture model, ECs were still probably in the proliferative state. Primary ECs were cultured in medium supplied with growth factors to sub-confluence prior to co-culture. During co-culture, the medium was changed to DMEM without growth factors. This resulted in the detachment of some ECs from the TCPS making the ECs still rather proliferative during the initial part of the co-culture. *In vivo*, damage to the endothelium of vessels often induces the proliferation and migration of SMCs³²⁵. So we hypothesize that the dynamic modulation of SMC phenotype by co-cultured ECs is strongly dependent on the culture state of ECs, which is associated to the release pattern of signal molecules including PDGF and TGF- β by both co-cultured ECs and SMCs.

SMCs encapsulated with hydrogels synthesized ECM proteins collagen type I and elastin (Fig. 6.6). The synthesis of collagen type I was not greatly influenced by the presence of ECs. In both EC⁺ and EC⁻ constructs, the transcription of collagen type I increased with culture. The presence of ECs

significantly increased elastin transcription, in line with other reported co-culture work³⁰⁵. In native blood vessels, elastin is not only an ECM protein which confers elasticity, but also a regulator of SMC proliferation, migration, and differentiation^{88, 326, 327}. In fact, many of the current tissue-engineered blood vessels have failed due to the lack of elastin synthesis^{6, 88}. The synthesis of elastin in the 3D encapsulated SMCs is quite promising for the purpose of vascular tissue engineering.

6.5 Conclusion

The impact of ECs on SMCs in 3D hydrogels was investigated using a co-culture model in which SMCs were encapsulated in hydrogels and exposed to a monolayer of ECs. In the presence of ECs, SMC growth was enhanced initially and the formation of dense 3D cellular networks was promoted. Our hydrogel favors extensive network formation either with or without EC co-culture and this led to increased cell-cell contact and cellular tensional force and the upregulation of contractile α -actin, calponin, and SM-MHC genes. Comparing the SMC cultures with and without ECs, the general trends and maximum transcriptions of α -actin, calponin, and SM-MHC were similar in both cases, but the time point to reach these maximum transcriptions was delayed in the presence of ECs. The encapsulated SMCs showed some

contractility which is not so usual, but full differentiation of SMCs was not obtained either with or without EC co-culture, indicated by the lower expression of smoothelin and higher expression of SMemb. The dynamic modulation of SMC phenotype is hypothesized to be caused by the culture state of ECs. ECs also promoted the synthesis of elastin at transcriptional level. In order to obtain a functional tissue-engineered blood vessel, co-culture with ECs is one promising way to promote SMC-remodeling of 3D hydrogels; however, modulation of SMC phenotype towards full differentiation is still a challenge.

Chapter 7 – Conclusions and recommendations

Two series of dextran- and gelatin-based hydrogels were developed in this study. We showed that dextran, a polysaccharide, and gelatin, a protein, can be suitably modified to produce hydrogels which supported 3D SMC encapsulation. Most interestingly, we found a softer grade of dextran-*graft*-Lysine methacrylate (Dex-MA-Ly) mixed with methacrylamide modified gelatin (Gel-MA) showed desirable characteristics. This hybrid network chemically resembles proteoglycan, which is an important component of native ECM. It supported extensive SMC proliferation and 3D cellular network formation, and induced a generally more synthetic SMC phenotype than 2D TCPS. These are desirable for the positive remodeling of the hydrogel scaffolds. Culture with higher cell seeding density (1.8×10^6 cells/ml) in Dex-MA-Ly/Gel-MA hydrogel resulted in the formation of denser cellular networks, with the consequence of increased cellular tensional forces and SMC phenotype switch toward a more contractile state. Until now, few hydrogels have been reported to be able to viably encapsulate the SMCs^{19, 21, 28, 37}, not to mention supporting the formation of dense cellular networks to trigger SMC phenotypic switch from synthetic towards more contractile¹³⁸. Co-cultured ECs promoted SMCs proliferation and the formation of a much denser cellular

network. ECs also influenced SMC phenotype by delaying the transition towards a more contractile. The detailed conclusions are given below.

7.1 Hydrogels based on Dex-MA-AD and gelatin for 3D SMC and 2D EC cultures (D-G series)

A methacrylate- and aldehyde-bifunctionalized dextran (Dex-MA-AD) was synthesized by first modifying dextran with glycidyl methacrylate followed by oxidation using sodium periodate. A low degree of oxidation was chosen (DS of aldehyde of 13.9 ± 1.3) in order to obtain Dex-MA-AD with high water solubility. Hydrogels based on interpenetrating networks of Dex-MA-AD and gelatin were formed through UV-crosslinking in the presence of a photoinitiator. The methacrylate and aldehyde groups on Dex-MA-AD enabled the formation of polymethacrylate kinetic chains and Schiff bases respectively. Both the polymerization and the Schiff base reaction contributed to the covalent crosslinking in this system. Increasing gelatin concentration introduced more Schiff base but also led to increased viscosity of the precursor solution, which caused a steric hindrance of methacrylate polymerization. The mechanical properties of hydrogels were influenced by these two effects.

ECs attached and spread well on the surface of these hydrogels. SMCs

were successfully encapsulated within the hydrogels by UV irradiation without significant loss of cell viability. Spreading and proliferation of encapsulated SMCs within the hydrogels were observed. In these 3D hydrogels, the incorporation of gelatin serves the dual purpose of providing cell adhesion sequences and enzymatic degradability. The latter makes space available for cells to spread.

The time course of spreading and proliferation was related to gelatin concentration and matrix stiffness. The hydrogel with highest gelatin content (D-G-6) promoted the quickest spreading and proliferation. This was followed by D-G-2, which contains the least gelatin but is the least rigid, and then D-G-4. It was observed that high ligand density and soft matrix promoted cell spreading. However, SMC spreading was rather limited in these D-G series.

7.2 Hydrogels based on Dex-MA-Ly and Gel-MA for 3D SMC culture (L-G series)

By grafting lysine to Dex-MA-AD, dextran was functionalized with both the methacrylate and lysine groups (Dex-MA-Ly). Gelatin methacrylamide (Gel-MA) was prepared by the reaction of gelatin with methacrylic anhydride. The introduction of lysine in Dex-MA-Ly increased its compatibility with Gel-MA. In addition, Dex-MA-Ly more closely resembles the structure of

glycosaminoglycans (GAGs) than does Dex-MA-AD.

Hydrogels based on Dex-MA-Ly and Gel-MA were formed through UV-crosslinking. The formation of this second series of hydrogels through polymerization only was more controllable than that of the D-G series in which Schiff base reaction occurred between gelatin and Dex-MA-AD. The swelling ratio and mechanical stiffness of these hydrogels could be controlled by varying the concentration of Gel-MA, as well as the degree of substitution of methacrylate of both Dex-MA-Ly and Gel-MA.

SMCs were encapsulated within L-G hydrogels with different mechanical stiffness. Within the softer hydrogels (with shear storage moduli ranging from 898 Pa to 3124 Pa), fast cell spreading and profound cell proliferation were noted. The viability as measured using WST-1 assay in these hydrogel increased about 3 folds during 2 weeks of culture. Extensive 3D cellular network formation was also observed within the softer hydrogels. In the stiffer hydrogels (shear storage moduli ranging from 4026 Pa to 6075 Pa) the SMCs maintained a round morphology and their viability declined by 50% over 2 weeks of culture. It is thought that the denser networks of stiffer hydrogels are more obstructive to cell spreading and proliferation.

Compared with the D-G series, the spreading and proliferation of SMCs in the optimum compositions (for example L-G-2A) of these L-G series were

significantly improved. As they resemble native proteoglycans structurally and functionally, our Dex-MA-Ly/Gel-MA hybrid hydrogels provide a highly bioactive 3D microenvironment. They are promising scaffolds for vascular tissue engineering.

7.3 SMC phenotypic modulation inside 3D dextran-based hydrogels

The SMC phenotype within the L-G-2A hydrogel was examined by investigating the transcriptional expression of phenotype-specific proteins during 20 days of culture. The dynamic phenotype modulation of SMCs inside the gel due to proliferation and gel degradation was observed. Initially the SMCs exhibited a mild degree of contractile gene expression, which we attribute to the tight mechanical constraints of the highly crosslinked gel. After prolonged culture, in the course of which the cells experienced decreased mechanical constraint as the hydrogel began to proteolytically degrade, the synthetic/proliferative gene markers were upregulated. Cells in these 3D hydrogels were generally more synthetic than on 2D TCPS. The synthetic SMC phenotype facilitates cellular proliferation and ECM production, which are desirable during the early stages of construction of a tissue-engineered vascular graft. However, the ultimate transition to a contractile phenotype, needed for

vasoactivity, was not observed in L-G-2A hydrogel during the 20 days of culture. This may be due to the low cell seeding density and limited proteolytic degradation.

7.4 Impact of ECs on SMCs in 3D hydrogels

An EC/SMC co-culture model was developed with the SMCs encapsulated in 3D hydrogels and exposed to a monolayer of ECs. This model is advantageous in that the SMCs were in a 3D microenvironment which mimics native conditions, and bidirectional regulatory effects between EC and SMC could occur.

Without EC co-culture, SMCs encapsulated in L-G-2A hydrogels with a higher seeding density (1.8×10^6 cells/ml in Chapter 6 vs. 1.0×10^6 cells/ml in Chapter 5) showed upregulation of transcriptional expressions of several contractile genes (specifically α -actin, calponin, and smooth muscle-myosin heavy chain (SM-MHC)), a phenomena that was not usually observed when SMCs were encapsulated. This upregulation may be due to increased cell-cell contact and with the consequence of increased cellular tensional forces during the formation of cellular networks.

In the presence of ECs, SMC growth was promoted and the formation of much denser cellular networks was enhanced. ECs dynamically modulated

SMC phenotype. ECs promoted a more contractile SMC phenotype initially (on the 2nd day). The general expression trends of SMC α -actin, calponin, and SM-MHC in the co-culture model were similar to these of SMCs without EC co-culture: there was an upregulation of these contractile gene expressions during culture. The maximum expression levels of these genes were similar for SMCs with or without EC co-culture; however, the time point to reach these maximum transcriptions was delayed in the presence of ECs. Though the SMCs showed a degree of contractility, their full differentiation to the contractile phenotype was not obtained in both cases (with or without ECs). ECs also promoted SMC synthesis of elastin at transcriptional level.

Although the full differentiation of SMCs was not realized, the formation of denser cellular networks and synthesis of elastin suggest that co-culture with ECs may be one potential approach to stimulate the formation of functional vascular grafts *in vitro*.

7.5 Recommendations for future work

7.5.1 Degradation of hydrogels

We have developed bioactive hydrogels based on natural dextran and gelatin which were able to promote SMC spreading and proliferation in 3D environments. The spreading of SMCs within these hydrogels is due to the

incorporation of gelatin which introduces both cell adhesion sequences and proteolytic degradation sites to the hydrogels. More detailed research into the degradation properties of hydrogels with encapsulated SMCs is recommended.

The stiffer hydrogels inhibited SMC spreading and proliferation. This is thought to be due to the obstructive characteristic of denser networks. However, if an initially stiff hydrogel could degrade fast enough to generate adequate space, we speculate that encapsulated SMCs will spread in this type of stiff 3D hydrogel. It seems likely that in at least some tissue-engineered blood vessel applications, particularly in instances of emergency interventions, it will be necessary to produce functional grafts on timescales shorter than those needed for *ex vivo* maturation of the graft vessel structure, so that the initial mechanical strength of the graft will need to be provided mostly by the tissue scaffold rather than the tissue itself. This suggests the need to precisely control the degradation of the scaffold so that it permits the vessel tissue to rapidly regenerate and mature but also retains adequate strength as this happens.

The degradation of our dextran-based hydrogels can be tuned by various methods. Dextranase, an enzyme which catalyzes the degradation of dextran, can be added exogenously or endogenously. However, the influence of dextranase on SMCs is unclear at present. It could be tested through the exogenous supply of dextranase to 2D cultured SMCs. The degradation

properties of hydrogels can also be tailored by incorporating hydrolytically degradable components, such as β -malic acid-containing poly(ethylene glycol) dimethacrylate. This polymer hydrolyses rapidly and is biocompatible, as previously reported by our group³²⁸.

7.5.2 Regulation of SMC phenotype

In 3D cultures within our hydrogels, the SMCs possessed a generally more synthetic phenotype than on 2D culture. This is beneficial for eventual remodeling of the hydrogel constructs. However, what is ultimately desired for a functional tissue-engineered blood vessel is the transition towards a fully differentiated contractile phenotype before implantation. This is required to inhibit intimal hyperplasia and for normal vasoactivity in the grafts. Encapsulation of SMCs at a higher density or co-culture of SMCs with ECs has resulted in a degree of contractility but not the full differentiation of SMCs. The stimulation of SMCs towards a more contractile phenotype at the right stage (*i.e.* start when cells have already secreted enough ECM to replace the artificial matrix) and the ultimate full differentiation of SMCs are challenges in vascular tissue engineering, and many aspects of SMC phenotype regulation still remain to be understood.

The timely transition of SMC phenotype may be accomplished through the

controlled sequential delivery of suitable growth factors, including platelet-derived growth factor (PDGF) and transforming growth factor (TGF)- β . PDGF is a potent chemoattractive factor for SMCs, and stimulates SMC proliferation and migration^{329, 330}, but downregulates the expression of smooth muscle (SM)-specific proteins³³¹⁻³³³. TGF- β , on the other hand, is essential for the induction of contractile SMC phenotype. For example, TGF- β 1 has been shown to increase α -actin, SM-MHC and SM-calponin levels in cultured SMCs³²³, and TGF- β 2 inhibits proliferation and increases α -actin and desmin levels in cultured porcine SMCs³³⁴. By first stimulating SMCs with PDGF, and later with TGF- β , a switch from synthetic to contractile phenotype may be feasible.

Alternatively, 3D micropatterns may be employed to control the phenotype of SMCs. We have shown in our previous work that a 3D micropattern allowed SMC proliferation at low cell density. When the cells approached confluence, unidirectional orientation was induced and a shift from synthetic to a more contractile phenotype was promoted²⁸⁶. The shift of phenotype is believed to be due to the physical constraint imposed by the micropatterns. In another study, skeletal muscle tissue networks with aligned and differentiated muscle fibers were created by casting cell/hydrogel mixture onto a 3D micropattern with staggered elongated posts³³⁵.

Thus, we propose that micropatterns may be applied to engineer vascular

tissue with oriented SMCs (Fig. 7.1). By trapping cell encapsulating hydrogel in a 3D micropattern with guiding posts, it is postulated that SMCs may initially proliferate and secrete ECM. However, nearing confluence, the physical constraint of the micropattern as well as cell-cell contact may cause the SMCs to align and switch to contractile phenotype. The micropatterns may inhibit the swelling of hydrogel and thus decrease extensive cell spreading. This effect should be taken into consideration in formulating the hydrogel precursor solution. In addition, the dense networks of SMCs required for alignment have so far been difficult to obtain in our current hydrogels. Modulating the degradation of the hydrogels may help towards this goal.

Fig. 7.1 Proposed induction of SMC alignment within hydrogels via 3D micropattern. Polydimethylsiloxane (PDMS) molds with an array of posts will be fabricated. Hydrogel precursor solution with cells will be injected into the PDMS mold. Upon UV irradiation, SMCs will be encapsulated in the hydrogel which is trapped by the posts. The physical constraint of the pattern may induce the alignment of SMCs.

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Appendix: Publication

- ✧ **Liu YX**, Chan-Park MB. A biomimetic hydrogel based on methacrylated dextran-graft-lysine and gelatin for 3D smooth muscle cell culture. *Biomaterials* 31: 1158-1170, 2010.
- ✧ **Liu YX**, Chan-Park MB. Hydrogel based on interpenetrating polymer networks of dextran and gelatin for vascular tissue engineering. *Biomaterials* 30: 196-207, 2009.
- ✧ Chan-Park MB, Shen JY, Cao Y, Xiong Y, **Liu YX**, Rayatpisheh S, Kang GCW, Greisler HP. Biomimetic control of vascular smooth muscle cell morphology and phenotype for functional tissue-engineered small-diameter blood vessels. *Journal of biomedical materials research* 88A: 1104-1121, 2009.